การโคลนและลักษณะสมบัติของไคโตไบเอสจาก *Aeromonas caviae* D6

นางสาวศรีสุดา ตระกูลน่าเลื่อมใส

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาชีวเคมี

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2550

CLONING AND CHARACTERIZATION OF CHITOBIASE FROM *Aeromonas caviae* D6

Miss .Sirsuda Trakunaleamsai

A Thesis Submitted in Partial Fulfillment of the Requirement
for the Degree of Master of Science Program in Biochemistry
Faculty of Science
Chulalongkorn University
Academic 2007

Thesis Title	CLONING AND CHARACTERIZATION OF CHITOBIASE FROM *Aeromonas caviae* D6
By	Miss .Sirsuda Trakunaleamsai
Field of Study	Biochemistry
Thesis Advisor	Assistant Professor Rath Pichyangkura, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

S. Hannongbua

..... Dean of the Faculty of Science

(Professor Supot Hannongbua, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Professor Aran Incharoensakdi, Ph.D.)

..... Thesis Advisor

(Assistant Professor Rath Pichyangkura, Ph.D.)

..... Member

(Associate Professor Siriporn Sittipraneed. Ph.D.)

..... Member

(Associate Professor Vichien Rimphanitchayakit, Ph.D.)

ศรีสุดา ตระกูลน่าเลื่อมใส: การโคลนและลักษณะสมบัติของไคโทไบเอสจาก *Aeromonas caviae* (CLONING AND CHARACTERIZATION OF CHITOBIASE FROM *Aeromonas caviae* D6)
อ.ที่ปรึกษา: ผศ.ดร.รัฐ พิชญางกูร, 140 หน้า.

น้ำตาลเอ็น-อะซิลทิล-ดี-กลูโคซามีน (GlcNAc) มีบทบาทและความสำคัญต่อการรักษาผู้ป่วยที่เป็นโรคข้อเสื่อมเป็นอย่างสูง ยังผลให้เกิดการศึกษาและพัฒนากระบวนการผลิตของสารดังกล่าว ในการศึกษานี้ได้ทำการโคลนยีนไคทิเนส (*chiA*) จากแบคทีเรียสายพันธุ์ *Bacillus licheniformis* SK-1 และยีนเอ็นอะซิลทิลกลูโคซามินิเดรส (*agd97*) จากแบคทีเรียสายพันธุ์ *Aeromonas caviae* D6 และทำการแสดงออกร่วมกันในเชื้ออีโคไลสายพันธุ์ BL21 (DE3) และ XL-1 blue โดยใช้โปรโมเตอร์ T7 (pET-17b) และโปรโมเตอร์ที่ติดมากับยีนเพื่อการแสดงออกตามลำดับ จากผลการแสดงออกของยีนในเชื้ออีโคไลทั้งสองสายพันธุ์ พบว่าเอนไซม์เอ็นอะซิทิลกลูโคซามินิเดรสจากอีโคไลสายพันธุ์ BL21 (DE3) ที่มี pETAgd97-ChiA มีค่าแอคติวิตี 15.66 U/ml ซึ่งสูงกว่าจากอีโคไลสายพันธุ์ XL-1 blue/pBSK60-Agd97 ซึ่งมีค่า 0.207 U/ml เมื่อพยายามนำยีน *chiA* มาต่อร่วมกับยีน *agd97* พบว่าไม่สามารถได้การจัดเรียงตัวที่ต้องการ อย่างไรก็ดี เราพบว่าเมื่อมียีน *chiA* แทรกอยู่หน้ายีน *agd97* เอนไซม์เอ็นอะซิลทิลกลูโคซามินิเดรสจากอีโคไลสายพันธุ์ BL21(DE3) มีค่าแอคติวิตีสูงกว่า 76 เท่า เมื่อใช้โปรโมเตอร์ T7 จากเชื้ออีโคไลสายพันธุ์ XL-1 blue ดังนั้นจึงได้ทำเอนไซม์เอ็นอะซิลทิลกลูโคซามินิเดรสจากอีโคไลสายพันธุ์ BL21(DE3)ให้บริสุทธิ์ ด้วย DEAE-cellulose และ sephadex G-100 พบว่าเอ็นไซม์ได้สเปซิฟิกแอคติวิตีเพิ่มขึ้น 2.4 เท่า โดยมีค่าแอคติวิตีคงเหลือ 1.4 เปอร์เซนต์ และเอนไซม์บริสุทธิ์มีค่า pH และอุณหภูมิที่เหมาะสมต่อการทำงานของเอนไซม์คือ 6 และ 45 องศาเซลเซียส ตามลำดับ ส่วนช่วงค่า pH และอุณหภูมิที่เอนไซม์ยังคงความเสถียรอยู่คือ 6 ถึง 10 และต่ำกว่า 40 องศาเซลเซียส ตามลำดับ

ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต .....
สาขาวิชา.....ชีวเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา .....
ปีการศึกษา.....2551

4772492723 : MAJOR BIOCHEMISTRY
KEY WORD : *N*-ACETYLGLUCOSAMINIDASE / CHITINASE / *Aeromonas caviae* / *Bacillus licheniformis* SK-1 / *N*-acetylglucosamine
SRISUDA TRAKUNALEAMSAI : CLONING AND CHARACTERIZATION OF CHITOBIASE FROM *Aeromonas caviae* D6.
THESIS ADVISOR: ASST.PROF. RATH PICHYANGKURA, Ph.D. 140 pp.

N-acetyl-D-glucosamine (GlcNAc) has an important role for the treatment of osteoarthritis. Consequently, GlcNAc production has been studied and developed. In this research, Chitinase (*chiA*) and *N-acetylglucosaminidase* (*agd97*) genes from *Bacillus licheniformis* SK-1 and *Aeromonas caviae* D6 respectively were cloned and gene cassettes containing both genes were constructed. Attempts were made to co-express both genes in *E. coli* BL21 (DE3) and XL-1 blue by using pET-17b and chi60 promoter from *Serratia sp.* TU09 in the pBSSK$^-$ vector. The expression of *agd97* gene from pETAgd97-ChiA in *E. coli* BL21 (DE3) gave the activity of 15.656 U/ml, higher than expression of pBSK60-Agd97 by chi60 promoter in *E. coli* XL-1 blue, which gave the activity of 0.207 U/ml. Our attempts to construct gene cassettes were unsuccessful. However, when *chiA* gene was placed in a reverse orientation in front of *agd97* gene, the activity of agd97 increased 76 fold compared to the activity observed from chi60 promoter in *E. coli* XL-1 blue. Hence, the expressed *E. coli* BL21 (DE3) /pETAgd97-ChiA was purified using DEAE-cellulose and sephadex G-100 column chromatography. Agd97 from *E. coli* BL21 (DE3) was purified 2.4 fold with a 1.4% yield. The optimum pH and temperature of the purified enzyme was 6 and 45°C, respectively. The enzyme has the highest stability over the pH rang from 6 to 10 and at temperature below 40°C.

Department.....Biochemistry..... Student's signature .....
Field of study.....Biochemistry.....Advisor's signature .....
Academic year.....2007.....

ACKNOWLEDGEMENTS

Fist of all, I would like to express my respect and sincerely thank to my advisor, Assist. Prof. Dr. Rath Pichyangkura. Him supports and valuable suggestions in various aspects. Without him kindness and understanding, this work could not be accomplished.

In addition, I also would like to express my deeply indebted and special appreciation to Prof. Dr. Aran Incharoensakdi, Assoc. Prof. Dr. Siriporn Sittipraneed and Assoc. Prof. Dr. Vichien Rimphanitchayakit, serving as the members of the committees, for their helpful suggestions and valuable comments.

My appreciation is also expressed to Dr Somchai Pongpattanakitshote Nation Center for Genetic Engineering and Biotechnology. Him proof my thesis.

There are so many friends to mention from so little space to fill. I would like to give a deep thank to my closely friends (nong Aun, Tip) and friends in the Department of Biochemistry and Program in Biotechnology for their friendship, kind assistance and support.

Finally, I would like to express my deep gratitude to Assist. Prof. Dr Savit Trakulnaleamsai (my sister) and my family for their love, warmth, understanding, helping, supporting and encourangement with care which have enabled me to carry out this study successfully whereas I have spent rarely whole my time with this thesis rather than with them.

CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

ABBREVIATIONS

A	Absorbance, 2′-deoxyadenosine (in a DNA sequence)
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
C	2′-deoxycytidine (in a DNA sequence)
°C	Degree Celsius
CCMM	Colloidal chitin minimum medium
Da	Dalton (s)
DEAE	diethylaminoethyl
DNA	Deoxyribonucleic acid
dNTP	2′-deoxynucleoside 5′- triphosphate
EDTA	Ethylenediaminetrichloroacetic acid
et al.	Et. Alii (latin), and others
etc.	Et cetera (latin), other things
G	2′-deoxyguanosine (in a DNA sequence)
GlcNAc, NAG	*N*-acetyl-D-glucosamine
i.e.	Id est (latin), that is
IPTG	isopropyl-thiogalactoside
kb	kilobase pairs in duplex nucleic acid
	kilobase pairs in single-standed nucleic acid
kDa	kiloDalton (s)
L	Liter
LB	Luria-Bertani
M	Mole per liter (Molar)
mg	Miligram
mg/mL	Miligram per mililitre
min	Minute
mL	Mililitre

mM	Millimolar
MW	Molecular weight
ng	Nanogram
μg	Microgram
μL	Microliter
μM	Micromolar
NaCl	Sodium chloride
OD	optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PNAC	Partially *N*-acetylated chitin
pNP-β-GlcNAc	para-Nitrophenyl N-Acetyl-β-D- Glucosamine
RNase	ribonuclease
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
sp.	Species
T	2′-deoxythymidine (in a DNA sequence)
TE	Tris-EDTA buffer
TEMED	*N, N, N′, N′*- tetramethyl ethylene diamine
TLC	Thin layer chromatography
v/v	Volume by volume
w/w	Weight by weight

CHAPTER I

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

1.1 Chitin

Chitin, a linear β-1,4-*N*-acetyl D-glucosamine (NAG, GlcNAc) polysaccharide (Cabib, 1987), is the most abundant renewable natural resource in the world next to cellulose (Deshpande, 1986). It is a major structural component of fungal cell walls, the exoskeletons of invertebrates including insects, crustaceans and extracellular polymer of some bacteria (Nicol, 1991). Approximately 75% of the total weight of shellfish, such as shrimp, crab and krill, is considered waste, and chitin comprises 20 to 58% of the dry weight of the said waste (Wang and Chang, 1997). In nature, chitin has been estimated an annual production between 10^{10} and 10^{11} tons. The highest amount of chitin with respect to total dry weight is found in Crustaceans. Thus crustacean shells were used as a main source of chitin by most chemical industries. Chitin has a broad range of applications in biochemical, food, and various chemical industries. It has antimicrobial, anticholesterol and antitumor activities (Patil *et al*., 2000; Gooday, 1999). Chitin and its related materials are also used in wastewater treatment (Flach *et al*., 1992), drug delivery (Kadowaki *et al*., 1997), wound healing, and dietary fiber (Dixon, 1995; Muzzarelli, 1977; Muzzarelli *et al*., 1999).

1.2 The structure of chitin

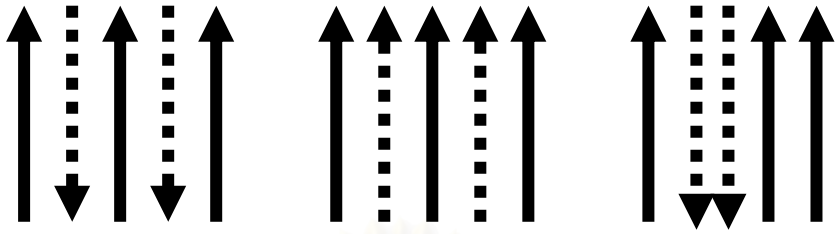
The chemical structure of chitin, similar to that of cellulose, is a straight chain homopolymer of β-1,4-*N*-acetyl D-glucosamine (NAG, GlcNAc). This structure resemble cellulose with the C-2 hydroxyl group replaced by an acetamido residue. Chitin can be processed into many derivatives, such as chitosan, chitin oligosaccharide and chitosan oligosaccharide.

The structure of chitin determined by polarized light and electron microscope indicates that the chains of chitin usually orientate in high degree of order (Kramer and Koga, 1986). The X-ray diffraction studies reveal that chitin occurs in three

polymeric forms, α, β and γ-form. The polymer chains in alpha-form, which is arranged in an antiparallel, are tightly boned. Different to parallel arrangement, β-form, and γ forms were mixture of antiparallel and parallel strands, and has less crystallinity than the alpha chitin, they are easily dispersed in water, and are more easily degradable by lysozyme and chitinase than alpha and beta forms colloidal chitin as shown in Figure 1.1. The α-form, the most abundance in nature is more stable than the other.

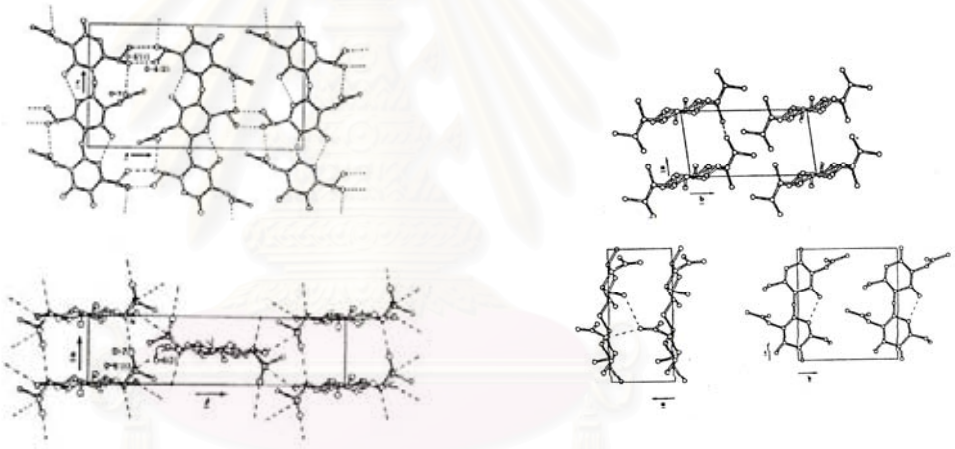
Several reports are available regarding the presence of chitin in fungal cell walls (Table 1.1). In filamentous fungi and basidiomycetes, it comprises 16% of the dry weight of the organism. Cell walls of Mucoraceae are known to have chitosan in addition to chitin. In yeast, the amount of chitin in the cell wall is much lower, but bud scars have been shown to be largely composed of chitin (Kuranda and Robbins, 1991).

(A)



α-Chitin β-Chitin γ-Chitin

(B)



α-chitin **β-chitin**

Figure 1.1. Model of α-chitin, β-chitin and γ-chitin.

The poly N-acetylglucosamine chains [N-acetylglucosamine]$_n$ were represented by arrows.(A) Structure of α -and β -chitin and hydrogen bond linkages between the C=O…NH groups (B) (Minke and Blackwell, 1978).

Table 1.1 Chitin content of selected crustaceans, insects, molluscan organs and fungi (Tharanathan and Kittur, 2003)**.**

Organism	Chitin content (%)	Organism	Chitin content (%)
Crustacean		**Insects**	
Cancer (crab)	72.1[c]	*Periplaneta* (cockroach)	2.0[d]
Carcinus (crab)	64.2[b]	*Blatella* (cockroach)	18.4[c]
Paralithodes (king crab)	35.0[b]	*Colcoptera* (beetle)	27-35[c]
Callinectes (blue crab)	14.0[a]	*Diptera* (true fly)	54.8[c]
Crangon (shrimp)	69.1[c]	*Pieris* (sulphur butterfly)	64.0[c]
Alasakan shrimp	28.0[d]	*Bombyx* (silk worm)	44.2[c]
Nephrops (lobster)	69.8[c]	*Calleria* (wax worm)	33.7[c]
Homarus (lobster)	60-75[c]	**Fungi**	
Lepas (barnacles)	58.3[c]	*Aspergillus niger*	
Molluscan organs		*Penicillium notatum*	42.0[e]
Clamshell	6.1	*Penicillium chrysogenum*	18.5[e]
Oyster shell	3.6	*Saccharomyces cereviseae*	20.1[e]
Squid, skeleton pen	41.0	*Mucor rouxii*	2.9[e]
Krill, deproteinized shell	40.2	*Lactarius vaiiereus*	44.5
		(mushroom)	19.0

[a]Wet body weight

[b]Dry body weight

[c]Organic weight of cuticle

[d]Total weight of cuticle

[e]Dry weight of the cell wall

1.3 The applications of chitin and its derivatives

Chitin and chitosan have strong anti-bacterial, anti-fungal and anti-viral properties that make it extremely useful in medical applications such as bandages, wound dressings, surgical sutures, periodontal treatments, and cataract surgery. Extensive research has shown the chitin and its derivatives chitosan to be non-toxic and non-allergenic. Chitin is fully biodegradable and therefore environmentally friendly.

Chitin and chitosan have been extensively examined and tested by researchers world-wide in a wide range of medical applications (H.K. and Meyers, 1995), food and nutrition uses, cosmetics, beauty aids and other new discoveries. Today, mainly in the U.S. and Japan, more than two million people take chitin and chitosan as dietary supplements.

Chitooligosaccharides used as neutricutial agents, anti cancer and carbohydrate precursor, are prepared by partial hydrolysis of chitin with hydrochloric acid or enzyme by degradation and transglycosylation. Acid hydrolysis gives products that have low degree of polymerization, varying from monomer to trimer and gives acid wastes. Differ from acid hydrolysis, enzymatic hydrolysis gives high degree of polymerization, specific products ranging from monomer to heptamer that can be used for different applications (Aiba, 1994).

N-acetylglucosamine (GlcNAc) is used as a dietary supplement and pharmacological agent. GlcNAc is produced by acid hydrolysis of chitin or enzymatic degradation.

1.4 *N*-acetylglucosamine (GlcNAc, NAG)

N-acetylglucosamine (GlcNAc, NAG) is a simple amino sugar, a monosaccharide with an amino group as part of its structure. There are two sources of acetylglucosamine; it comes from the exoskeletons of marine animals, and is also produced synthetically. GlcNAc is made in the body of all animals from glucose, also called blood sugar. Complex (polymeric) carbohydrates containing *N*-acetyl-D-glucosamine , referred to as glycosaminoglycans, become associated with or attached to proteins to form a class of compounds generally refered to as proteoglycans. GlcNAc is part of the makeup of body tissues and blood vessels, and the protective converings over the digestive, respiratory and genitourinary organs. In this capacity, it is involved in the regulation of what enters and leaves the body and the movement of substances into and between cells of the body. Amino sugars make up over half of the glycosaminoglycans of interstitial tissue that fills the spaces between cells and forms the cellular “glue” that binds cells together. This material is a gel-like matrix of collagen protein and glycoaminoglycans, holding cells in place and regulating those substances which pass between cells.

In humans, GlcNAc is precursors of the disaccharide unit in glycosaminoglycans (such as hyaluronic acid, chondroitinsulfate and keratin sulfate), which is necessary to repair and maintain health cartilage and joint function. GlcNAc by stimulating the metabolism of chondrocytes in the articular cartilage cells is beneficial in treatment of osteoarthritis and by stopping or slowing down disorders like osteoarthritis. Hence, GlcNAc is a valuable pharmacological agent in the treatment of a wide variety of aliments. However, GlcNAc has not been widely commercialized mainly due to the lack of an economical process for its production that is acceptable for food and medicine.

The production of GlcNAc can also achived by enzymatic reaction (Aiba, 1994). The enzyme hydrolysis of chitin gives *N*-acetylglucosamine performed by a chitinolytic system, The action of which is known to be synergistic and consecutive (Deshpande, 1986). However, there are very few reports on the commercial production of GlcNAc and glucosamine by enzymatic hydrolysis of their respective

polymers, since the progress of the reaction depends on the synergistic and concecutive action of various chitinolytic enzymes present in the complex. To make the process commercially viable, (Sakai *et al* (1991)

1.5 Chitinolytic enzyme

1.5.1 Chitinase

Based on amino acid sequence similarity, chitinolytic enzymes are grouped into 3 families, 18, 19, and 20 of glycosyl hydrolases (Henrissat and Bairoch, 1993). Family 18 is diverse in evolutionary terms and contains chitinases from bacteria, fungi, viruses, animals, and some plant chitinases. Family 19 consists of plant chitinases (classes I, II, and IV) and some *Streptomyces* chitinases (Hart *et al*., 1995). The chitinases of the two families, that is, 18 and 19, do not share amino acid sequence similarity. The amino acid residues found in the active site and includes glutamic acid (E) residue which played a crucial role in catalytic mechanism. Two glutamic residues are important in the mechanism of the action of Family 19 (Henrissat *et al*., 1991). Family 18 and 19 chitnase have completely different 3-D structures and molecular mechanisms and are therefore likely to have evolved from different ancestors (Suzuki *et al*., 1999). Family 20 includes the β-*N*-acetylhexosaminidases from bacteria, *Streptomycetes*, and humans. Bacterial chitinases are clearly separated into three major subfamilies, A, B, and C (Figure 1.2), based on the amino acid sequence of individual catalytic domains (Watanabe *et al*., 1993). Subfamily A chitinases have the presence of a third domain corresponding to the insertion of an α+β fold region between the seventh and eighth $(\alpha/\beta)_8$ barrel. On the other hand, none of the chitinases in subfamilies A and B have this insertion. Several chitinolytic bacteria that possess chitinases belonging to different subfamilies like *Serratia marcescens* (Suzuki *et al*., 1999), *Bacillus circulans* WL-12 (Alam *et al*., 1995), and *Streptomyces coelicolor* A3 (2) (Saito *et al*., 1999) are reported.

1.5.1.1 Proposed catalytic mechanism of chitinases

Family 19 chitinase

Family 19 chitinase from barley seeds has a three-dimensional structure similar to that of hen egg white lysozyme, especially in the substrate binding and catalytic core composed of a three stranded β-sheet and two α- helices (Monzingo *et al*., 1996). From this finding, it can be speculated that barley chitinase has a catalytic mechanism similar to that of hen egg white lysozyme. Contrary to speculation, hydrolytic products from barley chitinase reaction were found to be in α-form as determined by ^{1}H-NMR spectroscopy, indicating that the chitinase inverts the anomeric form through its catalytic reaction (Hollis *et al*., 1997). Regardless of the structural similarity, the catalytic mechanism of family 19 chitinase is different from that of hen egg white lysozyme. Some structural difference in the catalytic center between the barley chitinase and hen egg white lysozyme would result in the different catalytic mechanisms. As reported by Withers and his co-workers, the distance between the two catalytic residues is closely related to the catalytic mechanism (Wang *et al*., 1994). In the case of retaining enzymes, the average distance between the two catalytic residues is about 4-5 Å, while the distance is about 10-11 Å in inverting enzymes. In fact, the distance between Glu35 and Asp52 in hen egg white lysozyme is 4.6 Å. In the site-directed mutagenesis study of barley chitinase, the mutation of Glu67 to Gln completely eliminated its activity, and that of Glu89 impaired the activity to 0.25 % of that of the wild type. Glu67 and Glu89 are most likely to be a proton donor and a second catalytic residue like Asp52 in the lysozyme, respectively (Andersen *et al*., 1997). In the crystal structure, the distance between the two catalytic residues is 9.3 Å. Obviously, the difference in catalytic mechanism between hen egg white lysozyme and barley chitinase is ascribed to the distance between the two catalytic residues. The longer separation between the catalytic residues seems to be a structural feature characteristic of family 19 chitinase. The reaction of inverting glycosyl hydrolases which have two largely separated catalytic residues is often explained by a single displacement mechanism (Kuroki *et al*., 1995). The mechanism is shown in Figure 1.3, scheme I. At first, the general acid, Glu67, protonates the β-1,4- glycosidic oxygen atom, forming an oxocarbonium ion intermediate, and then the water molecule activated by the general base, Glu89, attacks the C1 atom of the

intermediate state from the a-side to complete the reaction. The separated location of the two catalytic residues might permit the water molecule to be located in-between the anomeric C1 atom and the carboxyl oxygen of the general base (Glu89). This location of the water molecule would result in the anomeric inversion of the reaction products. From the molecular dynamics simulations (Brameld and Goddard, 1998), however, Glu89 was found not only to activate the nucleophilicity of the water molecule but to act as a stabilizer of the carbonium ion intermediate. In addition, the simulation study indicated that the $(GlcNAc)_6$ substrate binds to barley chitinase with all sugar residues in a chair conformation; that is, no sugar residue distortion was found in family 19 chitinase complexed with the substrate. Chitinase from yam (*Dioscorea opposita*) was reported to produce a-form of the product, indicating that the chitinase is an inverter (Fukamizo *et al*., 1995). Chitinase from another plant was reported to be an inverter as well (Dahlquist *et al*., 1969 and Iseli *et al*., 1996). All of these inverting chitinases from plant should have a similar catalytic mechanism.

Family 18 Chitinases

Family 18 chitinases have not been studies as extensively as those from family 19. They were reported to yield hydrolysis products which retain the anomeric configuration at C1′ (Brameld *et al*., 1998; Brameld and Goddard, 1998 and Yannis *et al*., 2001) and two proposed catalytic mechanism.

The earlier proposed catalytic mechanism (shown in figure 1.3, Scheme II) invoked a substrate assistance mechanism (Brameld *et al*., 1998). That is, the *N*-acetyl group at position 2 for the scissile sugar may itself facilitate the reaction via formation of a transient oxazolinium intermediate (Terwisscha *et al*., 1995). Unlike the enzymes described thus far, family 18 chitinases have a catalytic $(\alpha/\beta)_8$-barrel domain. The catalytic residues of this enzyme family were first reported by Watanabe and his co-worker for chitinase A1 from *Bacillus circulans* WL-12 (Watanabe *et al*., 1993). Site-directed mutagenesis of Glu204 completely eliminated its activity, and the residue was considered to be a proton donor in its catalysis. From the sequence comparison, the glutamic acid residue was found to be conserved in all chitinases in family 18. In *Serratia marcescence* chitinase A, the catalytic carboxylate corresponding to Glu204 of *B. circulans* chitinase A1 is Glu315. Like hen egg white lysozyme, *B. circulans* chitinase A1 produce β-anomer (Armand *et al*., 1994), hence

is a retaining enzyme. As described above, in retaining enzymes, the location of the second carboxylate is close to that of the proton donor carboxylate (< 5 Å). In the consensus region of the catalytic domain of family 18 chitinases, there are several conserved carboxylic amino acid residues, for example, Asp200 and Asp202 in chitinase A1 from *B. circulans*, Asp311 and Asp313 in chitinase A from *S. marcescens*. Site-directed mutagenesis of Asp200 and Asp202 in *B. circulans* chitinase A1 impaired the enzymatic activity, but did not completely eliminate the activity (Watanabe et al., 1993 and Watanabe et al., 1994). The location of these residues does not correspond to that of the second carboxylate in lysozyme (Asp52) or in family 19 barley chitinase (Glu89). Thus, the second carboxylate cannot be identified in any family 18 chitinase. The family 18 chitinases should have a different mechanism of catalysis. Recent studies on the family 18 chitinases indicate that the catalytic reaction of the enzymes takes place through a substrate-assisted mechanism. A putative oxocarbonium ion intermediate is stabilized by an anchimeric assistance of the sugar *N*-acetyl group after donation of a proton from the catalytic carboxylate to the leaving group. Such stabilization might occur either through a charge interaction between the C1 carbon and the carbonyl oxygen of the *N*-acetyl group or via an oxazoline intermediate with a covalent bond between C1 carbon and the carbonyl oxygen. The mechanism does not require the second carboxylate and can rationalize the anomer retaining reaction of the enzymes without the second carboxylate. This mechanism was first proposed for the spontaneous acid-catalyzed hydrolysis of 2-acetamido-substituted polysaccharides in solution, and applied to the lysozyme mechanism (Lowe *et al*., 1967). Experimental evidence of the substrate assistance in family 18 chitinase has been first provided by the crystal structure of the inhibitor allosamidin bound to chitinase from *Hevea brasiliensis* (Terwisscha *et al*., 1995). Recent studies by quantum mechanical calculation supported the substrate-assisted mechanism in family 18 chitinase (Brameld *et al*., 1998).

The last proposed catalytic mechanism in chitinase A from *Serratia marcescens* suggest that residues Asp313 and Try390 along with Glu315 play a central role in the catalysis (Yannis *et al*., 2001). Yannis *et al*. proposed that after the protonation of the substrate glycosidic bond, Asp313 that interacts with Asp311 flips to its alternative position where it interacts with Glu315 thus forcing the substrate acetamido group of -1 sugar to rotate around the C2-N2 bond. As a result of these structural changes, the water molecule that the hydrogen-bonded to Try390 and the

NH of the acetamido group is displaced to a position that allows the completion of hydrolysis (Scheme III). In this mechanism, we will not observe an oxazoline ring

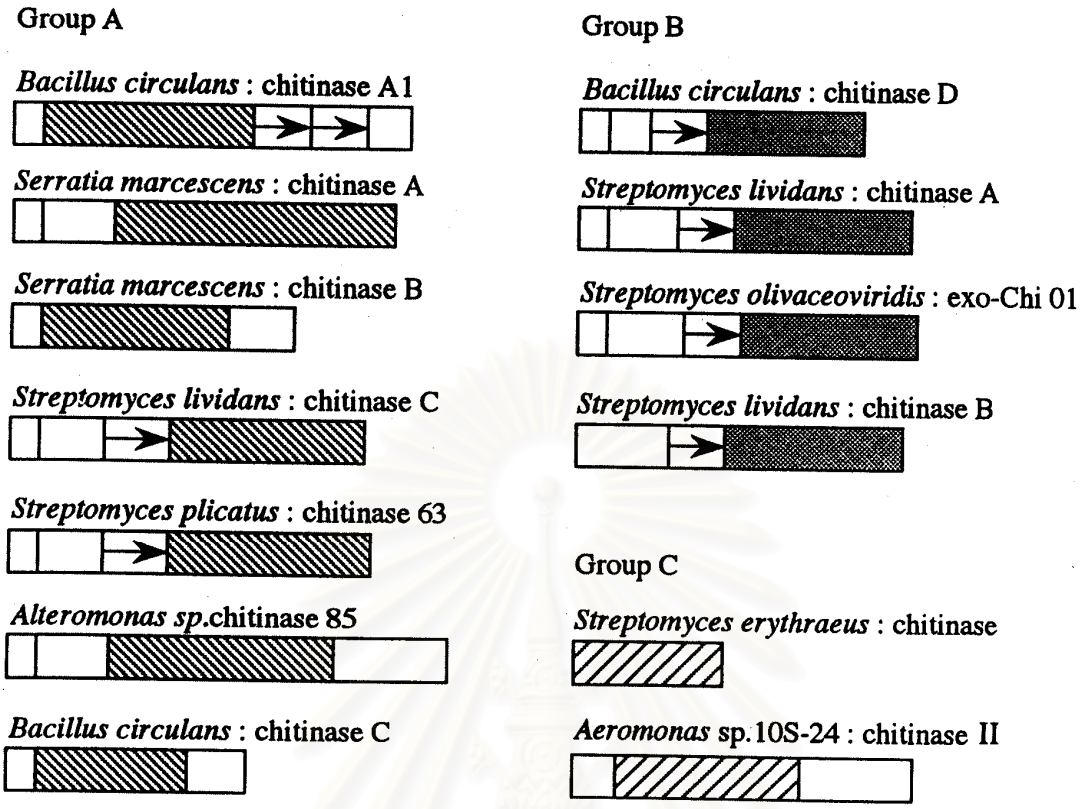
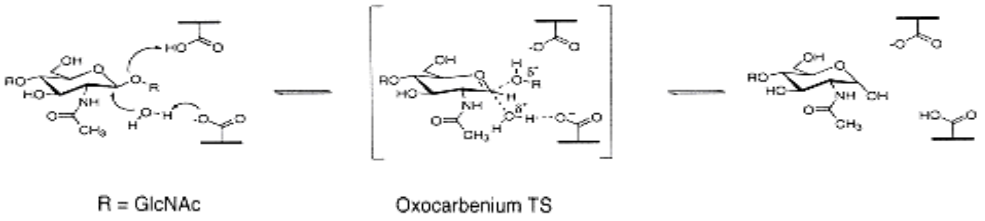
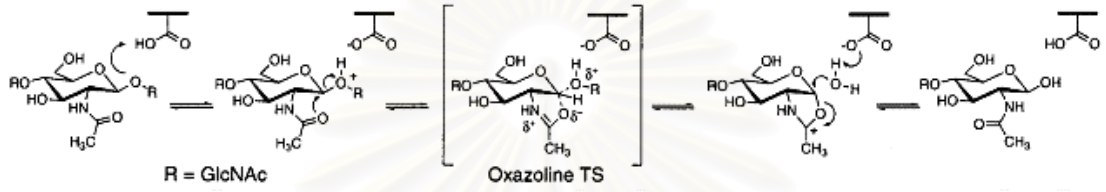


Figure 1.2 Classification of the bacterial chitinases based on the homology of similarity amino acid sequence of individual catalytic domains. Shadowed boxes indicated the homologous regions of individual chitinase to the catalytic domain of *Bacillus circulans* chitinase A1; Group A, *B. circulans* chitinase D; Group B, or *Streptomyces erythraeus* chitinase; Group C. Arrows indicate fibronectin type III like domain (From Hart *et al*., 1995). Amino acid similarity within the catalytic domain of *B. circulans* chitinase was used to classify other bacterial chitinases into 3 groups. Group A is similar with chitinase A1, Group B is similar with chitinase D, and Group C with no similarity with chitinase from *B. circulans*.

Scheme I



Scheme II



Scheme III

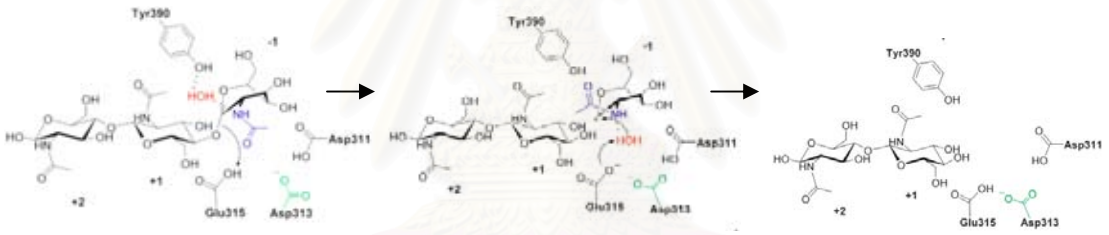


Figure 1.3 **Chitinase mechanisms**. The single-displacement hydrolysis mechanism proposed for family 19 chitinases. Two acidic residues are required in the active site, and the hydrolysis product shows inversion of the anomeric configuration (scheme II). The double-displacement hydrolysis mechanism proposed for family 18 chitinases. Protonation of a GlcNAc residue in a boat conformation leads to an oxazoline intermediate, which may be hydrolyzed to form a product with retention of the anomeric configuration (scheme II, III).

1.5.1.2 Reaction mechanism of chitinase.

Makino *et al*., 2006 proposed the postulated reaction mechanisms of chitinase in hydrolysis of chitin and polymerization of GlcN β-(1,4) GlcNAc monomer 1. In the hydrolysis, the glycosidic oxygen of the saccharide chain placed between the donor site and the acceptor site of chitinase is protonated by the carboxylic acid in the active center of the enzyme immediately after recognition as illustrated in stage 1. Then, the acetamido oxygen at the C2 position of the saccharide unit at the donor site attacks the neighboring C1 carbon to form the corresponding oxazolinium ion stabilized by another carboxylate in the active center, leading to scission of the glycosidic linkage (stage 2) (Herissat, 1991; Henrissat *et al*., 1996; Sakamoto *et al*., 2001 and Tews *et al*., 1997; htttp://afmb.cnrs-mrs.fr/CAZY/index.html.). Nucleophilic attack by a water molecule from the β-side opens the ring of the oxazolinium to accomplish the hydrolysis reaction, giving rise to the hydrolyzate having a β-configuration (stage 3). In the polymerization, the oxazoline monomer is effectively recognized at the donor site of chitinase as a TSAS (Kobayashi *et al*., 2006). The nitrogen atom in the oxazoline ring is immediately protonated by the carboxylic acidafter the recognition to form the corresponding oxazolinium ion (stage1′), which is stabilized there. Then, the hydroxy group at the C4 of another monomer or the growing chain end attacks the C1 of the oxazolinium from the β-side (stage 2′), resulting in the formation of β(1,4) glycosidic linkage (stage 3′). Repetition of these reactions is a ring-opening polyaddition, leading to the formation of a chitin-chitosan hybrid polysaccharide under total control of regioselectivity and stereochemistry. The key-point is the structure of the transition state (or the intermediate), which is commonly involved in both stages 2 and 2′ as a protonated oxazolinium moiety. Monomer 1 is very close to the moiety, showing the importance of the concept of a transition-state analogue substrate (TSAS) monomer in Figure 1.4. (Kobayashi *et al*., 1995; Kobayashi, 1997; Kobayashi 1999; Kobayashi *et al*., 2001a,b,c; Kobayashi, 2005; and Kobayashi *et al*., 2006)

1.5.1.3 Synergistic action of multiple forms of chitinases

Most of the chitinolytic organisms produce multiple isomeric forms of chitinases, which may result from posttranslational processing of a single-gene product or, more often, the products of multiple genes. The heterogeneity of chitinases was attributed to posttranslational modifications such as differential glycosylation and/or proteolysis.

Multiple chitinolytic enzymes have been reported in several microorganisms such as *S. marcescens* (Suzuki *et al*., 2002), *Aeromonas* sp. No. 10S-24 (Ueda, *et al*.1995), *Pseudomonas aeruginosa* K-187 (Wang and Chang, 1997), *B. circulans* WL-12 (Mitsutomi *et al*., 1998), *Bacillus licheniformis* X-7u (Takayanagi *et al*., 1991), *Streptomyces* sp. J. 13-3 (Okazaki *et al*., 1995), and *Streptomyces griseus* HUT 6037 (Itoh *et al*., 2002).

Suzuki *et al*., (2002) reported the synergistic action of chitinases Chi A, Chi B, and Chi C1 of *S. marcescens* 2170 on chitin degradation. They proposed that despite having similar catalytic domains, Chi A and Chi B were considered to digest chitin chains in the opposite direction. Chi A was proposed to degrade the chitin chain from the reducing end, whereas Chi B, from the nonreducing end. Addition of Chi A after treatment of powdered chitin with Chi B and vice versa was generally improved chitin degradation efficiency.

A thermophilic bacterium, *B. licheniformis* X-7u, possesses four chitinases, I, II, III, and IV. Chitinases II, III, and IV produced $(GlcNAc)_2$ and GlcNAc, whereas chitinase I predominantly produced $(GlcNAc)_2$. Chitinases II, III, and IV also

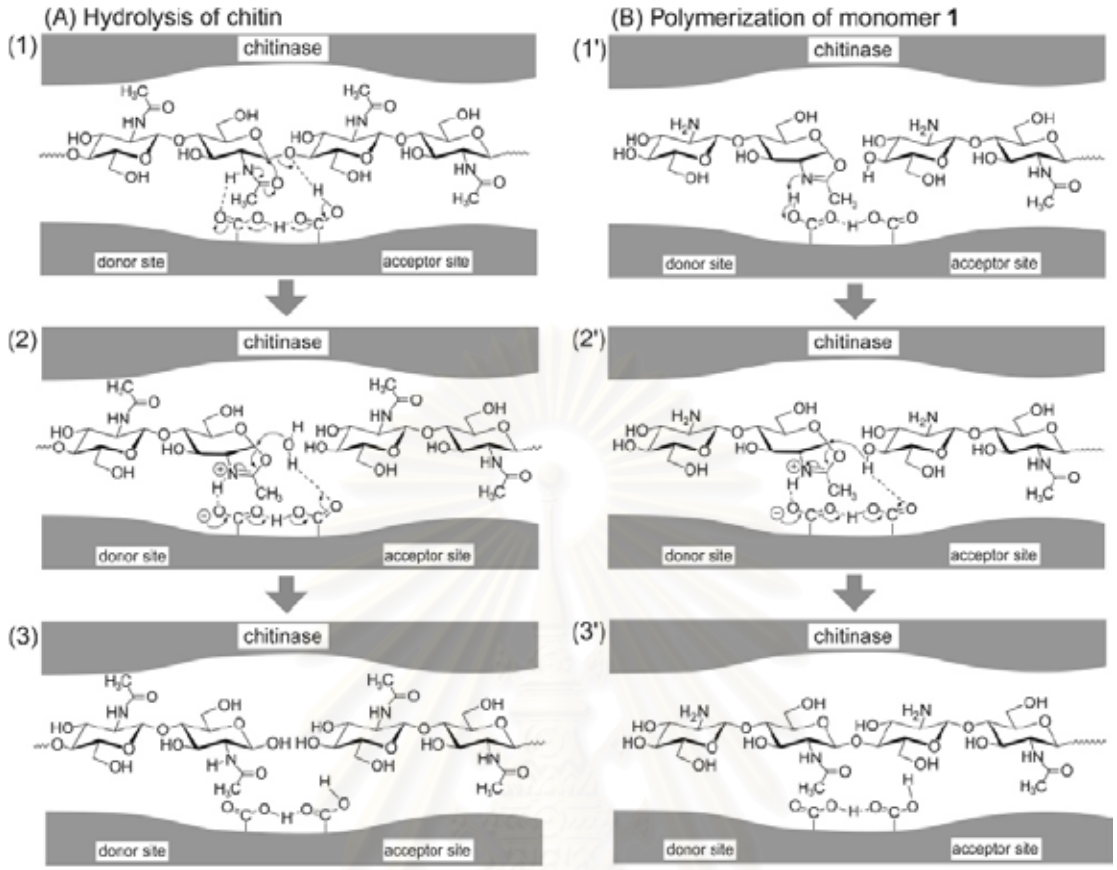


Figure 1.4 Postulated reaction mechanisms of chitinase catalysis (Makino *et al*., 2006)**.**

1.5.2 *β-N*-acetylglucosaminidase

β-*N*-acetylglucosaminidase (sometimes termed chitobiase; EC 2.1.30) are widely distributed in animal tissues, higher plants and microorganisms and are known to be useful enzymes for structural studies of the carbohydrate moieties of glycoproteins. β-*N*-acetylglucosaminidase act preferentially on a dimmer, diacetylchitobiose, some enzymes cleave GlcNAc units from the non-reducing ends of chitin chains (Gooday, 1990). The enzyme has been classified under the family 20 glycosyl hydrolases (Henrissat, 1996), which also comprises of the human hexosaminidase. Deficiency of which cause gangliosidoses, like Tay-Sach's and Sandhoff's diseases (Neufeild, 1989). A unigue feature proposed for the family 20 glycosyl hydrolases is the probable anchimeric assistance of the C2 acetamido group of the substrate in catalysis. *β-N*-acetylglucosaminidase along with certain other chitinolytic enzymes are proposed to follow an acid-base reaction mechanism, with a single protein carboxylate functioning as the catalytic acid, while the nucleophile is the polar acetamido group of the substrate GlcNAc (Davies, 1995 and White, 1997). Except for the human hexosaminidase and the chitobiase from *Serratia marcescens*, little information is available on the active site nature of other *N*-acetylglucosaminidase. Site directed mutagenesis of the human hexosaminidase indicated the involvement of βArg^{211} and βGlu^{355} in the catalytic function (Brown, 1991 and Pennybacker *et al*., 1997). However , the human hexosaminidase hydrolyzes broth terminally linked GlcNAc as well as GalNAc residues from the non-reducing end of glycoconjugates. Based on the X-ray structure of *S. marcescens* chitobiase complexed with the substrate (chitobiose), Vorgaris et al. (Tews., *et al*, 1996) gave evidence for the involvement of a single carboxylic acid in the catalytic mechanism.

Xiqian *et al*., 2004 proposed cloning *β-N*-acetylglucosaminidase gene from *A.hydrophila* strain SUWA-9. The ORF identified is 2,661 nucleotides long. The amino acid sequence deduced showed a high similarity to those of bacterial *β-N*-acetylhexosaminidase classified in family 20 of glycosyl hydrolases and the purified enzyme hydrolyzed *N*-acetylchitooligomers from dimmer to pentamer and produced GlcNAc as a final product.

For the complete hydrolysis of chitin, most chitinolytic microorganisms have an enzyme system consisting of two hydrolases: chitinase and β-*N*-acetylglucosaminidase (chitobiase) or β-*N*-acetylhexosaminidase. Chitin is the frist attacked by chitinase, releasing β-*N*-acetylchitooligosaccharides. These oligosaccharides are converted into *N*-acetylglucosamine, and the monosaccharide is then metabolized by variety of organisms.

1.6 *Bacillus licheniformis*

Typically, the cells are motile by peritrichous flagella and are aerobic. These latter feature of the microorganism has been commercially exploited for over a decade. *Bacillus licheniformis* is a uniquitous, saprophytic, soil bacterium which is thought to contribute to nutrient cycling due to its ability to produce a wide variety of enzymes. *B. licheniformis* has been used for industrial production of proteases, amylases and antibiotics (Claus and Berkeley, 1986).

Although the genus *Bacillus* is rather heterogenous based on a wide rang of DNA base ratios (32 to 69 mol% G+C) (Claus and Berkeley, 1986), these species are rather homogeneous based on DNA-DNA hybridization studies. Historically, *B. licheniformis* and two closely related species, *B. subtilis,* and *B. pumilus,* were grouped taxonomically into what was known as the subtilis-group (Priest *et al*., 1988). However, recently methods have been developed that allow *B. licheniformis* to be differentiated from these other species (Gordon, 1973). Peitsch

B. licheniformis is not a frank human pathogen, but has on several occasions been isolated from human infections. Diseases attributed to *B. licheniformis* included bacteremia, opthalmitis following trauma, and their reports of food poisoning based on circumstantial evidence (Tabbasa and Tarabay, 1979).

The used of *B. licheniformis* for industrial production of enzymes should not caused environmental hazards. First, the number of microorganisms released from the fermentation facility is low. In addition, *B. licheniformis* is ubiquitous in the environment, and the released expected from fermentation facilities operating under the conditions of this exemption will not significantly increase the frequency of this occurrence, even if a scenario for high exposure to *B. licheniformis* released from the fermentation facility to livestock could be envisioned.

When *Bacillus circulans* was grown in chitin, six distinct chitinase molecules were detected in the culture supernatant. These chitinases (A1, A2, B1, B2, C, and D) showed the following distinct sizes and isoelectric points: Mr 74,000, p*I* 4.7 (A1); Mr 69,000, p*I* 4.5 (A2); Mr 38,000, p*I* 6.6 (B1); Mr 38,000, p*I* 5.9 (B2); Mr 39,000, p*I* 8.5 (C); and Mr 52,000, pI 5.2 (D). Among these chitinases, A1 and A2 had the highest colloidal-chitin-hydrolyzing activities. Chitinase A1 showed a strong affinity to insoluble substrate chitin. Purified chitinase A1 released predominantly chitobiose [$(GlcNAc)_2$] and a trace amount of *N*-acetylglucosamine (GlcNAc) from colloidal chitin. *N*-terminal amino acid sequence analysis of chitinases A1 and A2 indicated that chitinase A2 was generated from chitinase A1, presumably by proteolytic removal of a *C*-terminal portion of chitinase A1. Since chitinase A2 did not have the ability to bind to chitin, the importance of the *C*-terminal region of chitinase A1 to the strong affinity of chitinase A1 to substrate chitin was suggested. Strong affinity of the chitinase seemed to be required for complete degradation of insoluble substrate chitin. Chitinase A1 is the key enzyme in the chitinase system of this bacterium.

In this study, the chitinase from bacteria was observed because chitinase activity has been found in a wide variety, easy cultivation, large quantity of enzyme production and most of them secrete enzyme out of the cell which simplify the purification. We study in *B. licheniformis* SK-1 because of this bacteria is an active producer of chitinase in our lab. In addition, the study of Sanya, 2006 showed that it can produce *N*-acetylchitobiose. For cloning, there are few reports about chitinase from *B. licheniformis* available. Recently, polymerase chain reaction (PCR) technique for cloning chitinase genes is the main interest for my study.

1.7 *Aeromonas caviae*

A. caviae is facultatively anaerobic gram-nagative rods which are oxidase positive, rode which are oxidase positive, motile by polar flagellation. Although phylogennetically more closely related to the *Enterobacteriaceae*, aeromonads are grouped in the family *Vobrionaceae*.

A hallmark characteristic of species of *Aeromonas* is their ability to secrete a wide variety of enzymes associated with pathogenenicity and environmental

adaptability. Among the most intensively studied are lipases, protease, chitinases and amylases.

A. sp. produced several distinct extracellular chitinases. These can be grouped into the A, B, and C homology groups proposed by Watanabe and co-workers (Watanabe *et al*., 1993). ChiA of *A. caviae* (Sitrit *et al*., 1995) belongs to group A. *A. sp*. 10S-24 produces five extracellular chitinases, several of which have been cloned and further studied (Shiro *et al*., 1996). Chi III and the enzyme produced from ORF 3 belong to group B, while Chi II belongs to group C. Four *chitinase* genes (ORFs 1-4) have been found in a cluster on the chromosome of *A. sp*. 10S-24, but no evidence for the products of enzymes from ORFs1 or 2 was found in culture supernatant. Only the N-terminal region of ORF 4 was included on the DNA fragment cloned.

Inbar and Chet (1991) isolated from soil a stain of *A. caviae* which showed a high level of chitinolytic activity. The chitinolytic enzyme found from *A. caviae* has two types, endo-chitinase and exo-chitinase (β-*N*-acetylhexosaminidase), (Xiqian, 2004).

In conclusion, the used of *A. caviae* isolated β-*N*-acetylglucosaminidase gene. In this study, the β-*N*-acetylglucosaminidase from bacteria was observed because this bacteria *A. caviae* D6 is active high produce β-*N*-acetylglucosaminidase in our lab. Therefore, β-*N*-acetylglucosaminidase gene from D6 was cloned and expressed with chitinase from SK-1 for produced GlcNAc.

1.8 Molecular cloning

A number of attempts have been made to clone and express genes from several organisms such as *B. circulans* WL-12 (Mitsutomi et al. 1998), *Enterobacter agglomerans* (Chernin *et al*., 1997), and *S. marcescens* 2170 (Suzuki *et al*., 1998) into *E. coli*.

These proteins were expressed in *E. coli* to study the degradation of chitin by chitinases ChiA, ChiB, and ChiC from *S. marcescens* (Suzuki *et al*., 2002). The chitinase gene from *S. marcescens* was also expressed in *E. coli* and *Pseudomonas fluorescens* 701E1, and it was reported that the gene was expressed more efficiently in *E. coli*, as compared with *Pseudomonas* (Fuchs *et al*., 1986). The chitinase gene from *Streptomyces lividans* was cloned in *E. coli* to study induction pattern (Miyashita *et*

al., 1991). They suggested the role of a 12-bp direct repeat in the induction of chitinase by chitin and its repression by glucose.

Recently, chitinase A from *Enterobacter* sp. G-1 and chitosanase A from *Matsuebacter chitosanotabidus* were cloned in the yeast *Schizosaccharomyces pombe* to study the functional expression of these enzymes and their effect on morphogenesis in *S. pombe*. In this host, chitinase was expressed inside the cells, whereas chitosanase was expressed as a secretion product (Shimono *et al*., 2002).

A number of reports are available on the cloning of chitinases either to increase biocontrol efficiency of *Bacillus thuringiensis* to prepare highly active chitinase preparation or to produce transgenic plants for increased resistance against insects. Sampson and Gooday, (1998) reported chitinolytic activities of two strains of *B. thuringiensis*. They reported enhanced virulence of *B. thuringiensis* with increased chitinase production. Two chitinases, Chi35 and Chi25, from *Streptomyces thermophilus* OPC 520 were cloned in *E. coli*, and it was reported that the polysaccharide binding domain of Chi 35 is involved in the hydrolysis of insoluble chitin and antifungal activity (Tsujibo *et al*., 2001). A family 18 *chitinase* gene, *chiA*, from thermophile *Rhodothermus marinus* was cloned and expressed in *E. coli*. It was reported to be the most thermostable chitinase isolated from bacteria (Hobel *et al*. 2005). In another report, two chitinase genes encoding ChiCH and ChiCW of *Bacillus cereus* 28-9 were cloned in pGEX-6P-1 and expressed in *E. coli* cells as soluble glutathione S-transferase-chitinase fusion proteins (Huang and Chen, 2005).

Such studies are essential for designing a more efficient chitinase producer and production of transgenic plants that can be used for the control of fungal and insect pathogens. Furthermore, biochemical and molecular studies could lead to a better understanding of the chitinase secretory process and the development of cloning strategies suitable for secretion of desired products.

β-*N*-acetylglucosaminidase from various source were used for isolated β-*N*-acetylglucosaminidase gene, cloning, expression in *E. coli*. (Tanaka *et al*., 2003, Kubota *et al*., 2004, and Okada *et al*., 2007).

1.9 Production of chitooligosaccharides, glucosamine, and GlcNAc

Chitooligosaccharides, glucosamines, and NAcGlc have an immense pharmaceutical potential. Chitooligosaccharides are potentially useful in human medicines. For example, chitohexaose and chitoheptaose showed antitumor activity. A chitinase from *Vibrio alginolyticus* was used to prepare chitopentaose and chitotriose from colloidal chitin (Murao *et al*., 1992). A chitinase preparation from *S. griseus* was used for the enzymatic hydrolysis of colloidal chitin. The chitobiose produced was subjected to chemical modifications to give novel disaccharide derivatives of 2-acetamido 2-deoxy D-allopyranose moieties that are potential intermediates for the synthesis of an enzyme inhibitor, that is, *N, N′*-diacetyl-β-chitobiosyl allosamizoline (Terayama *et al*., 1993).

Specific combinations of chitinolytic enzymes would be necessary to obtain the desired chain length of the oligomer. For example, the production of chitooligosaccharides requires high levels of endochitinase and low levels of *N*-acetylglucosaminidase and exochitinase, whereas the production of GlcNAc requires higher proportion of exochitinase and *N*-acetylglucosaminidase (Aloise *et al*., 1996). Alternatively, transglycosylation activity of a variety of endochitinases and *N*-acetylglucosaminidases will also be useful to generate desired chitooligomers, oligomers with changed glycosidic linkages and glycopeptides.

Nanjo *et al*., (1989) observed the accumulation of hexamer when tetramer or pentamer was incubated with *Nocardia orientalis* chitinase. A chitinase from *T. reesei* also exhibited a similar type of efficient transglycosylation reaction. They reported the accumulation of hexamer and dimer as the major product when the enzyme was reacted with tetramer (Usui *et al*., 1990). They also observed a chain elongation from dimer to hexamer and heptamer using lysozyme catalysis in the presence of 30% ammonium sulfate in a buffered medium. Chi-26 from *Streptomyces kurssanovii* showed the accumulation of hexamer in the reaction mixture containing tetramer and pentamer (Stoyachenko *et al*., 1994).

The transglycosylation reaction of *Mucor hiemalis* endo-β-*N*-acetyl glucosaminidase was used for the preparation of sugar derivatives modified at C-1 or C-2 for the synthesis of glycopeptides (Yamanoi *et al*., 2004).

A chitinolytic enzyme preparation from *N. orientalis* IFO12806 was used for the preparation of GlcNAc from chitooligosaccharides (Sakai *et al*. 1991). Crude

bacterial chitinases from *Burkholderia cepacia* TU09 and *B.* licheniformis SK-1 were used for the hydrolysis of α-chitin (from crab shells) and β-chitin (from squid pens) to produce NAG (Pichyangkura *et al*., 2002). Sashiwa *et al*., (2002) produced NAG from α-chitin using crude chitinolytic enzymes from *Aeromonas hydrophila* H-2330.

Objectives of this study

1. Cloning of β-*N-acetylglucosaminidase* (*agd97*) gene from *A. caviae* D6 and Chitinase (*chiA*) gene from *B. licheniformis* SK-1.
2 Expression of *agd97* and *chiA* genes.
3 Characterization of recombinant Agd97 protein.

Scope of study

1 To clone *agd97* and *chiA* from *A. caviae* D6 and *B. licheniformis* SK-1, respectively.
2 To express *agd97* and *chiA* in *E. coli* BL21 (DE3) and XL-1 blue.
3 To optimize the expression condition of *agd97* and *chiA* genes in *E. coli* BL21 (DE3) and XL-1 blue.
4 To purify recombinant Agd97 protein.
5 To characterize purified Agd97

Expected benefits

The *agd97* and *chiA* heterologous genes will be expressed in *E. coli*. This result will be useful information for industrial application in GlcNAc production.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model H-88LL, Kokusan Ensinki Co., Ltd., Japan

Autopipette: Pipetman, Gilson, France

Centrifuge, refrigerated: Model J2-21, Beckman Instrument Inc., U.S.A.

Centrifuge, microcentrifuge: Model MC-15A, Tomy Seiko Co., Ltd., Japan

Electrophoresis unit: 2050 MIDGET, LKB, Sweden and Mini protein, Bio-Rad, U.S.A.; Submarine Agrarose Gel Electrophoresis unit

Gene Pulser^{R}/*E.coli* Pulser.^{TM} Cuvettes: Bio-Rad, U.S.A.

GeneAmp PCR System 2400, PERKIN-ELMER, U.S.A.

Gel Doc : BioDoc-It^{Tm} Imaging system,Model M20, Cambridge, UK

Gel Document: SYNGENE, England

Incubator: Model 1H-100, Gallenkamp, England

Incubator shaker: Model G-76, New Brunswicks Scientific Co., Inc., U.S.A.

Incubator, water bath: Model M20S, Lauda, Germany

Lamina flow: HT123, ISSCO, U.S.A.

Light box: 2859 SHANDON, Shandon Scientific Co., Ltd., England

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, U.S.A.

Magnetic sterrer and heater: Model IKAMA®GRH, JANKE&KUNKEL GMBH&CO.KG, Japan

Membrane filter, cellulose nitrate, pore size 0.45 μm : Whatman, Japan

Microcentrifuge tubes 0.5 and 1.5 ml, Axygen Hayward, U.S.A.

Microwave oven: KOR-6C27, Daewoo International Co., Ltd., Korea

pH meter: Model PHM95, Radiometer Copenhegen, Denmark

Power supply: Model POWER PAC 300, Bio-Rad, U.S.A.

Sonicator: Model W375, Heat systems-ultrasonics, U.S.A.

Spectrophotometer: Spectronic 2000, Bausch&Lomp, U.S.A.

Spectrophotometer UV-240, Shimadzu, apan, and DU Series 650, Beckman, U.S.A.

Thin-wall microcentrifuge tubes 0.2 mL, Axygen Hayward, U.S.A.
Thin layer chromatography (TLC): DC-Plastikfolien cellulose, Merck, Germany
UV transluminator: Model 2011 Macrovue, SanGabriel California, U.S.A.
Vortex: Model K-550-GE, Scientific Industries, Inc, U.S.A.
Water bath Buchi 461: Switzerland

2.2 Chemicals

Acetonitrile: (Methy cyanide) Sigma, U.S.A.
Acetone: Mallinekrodit, U.S.A.
Acrylamide: Merk, U.S.A.
Agarose: GIBCOBRL, U.S.A.
Aqua sorb: Fluka, Switzerland
[α-^{35}S]-dATP: Amersham, U.S.A.
Ammonium persulphate: Sigma, U.S.A.
Ammonium sulphate: Sigma, U.S.A.
Ampicillin: Sigma, U.S.A.
Bacto-Agar: DIFCO, U.S.A.
β-mercaptoethanol: Fluka, Switerland
Bovine serum albumin: Sigma, U.S.A.
5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal): Sigma, USA
Bromphenol blue: Merck, Germany
Chloramphenicol: Nacalai tesque, Inc., Japan
Chloroform: BDH, England
Coomassie brilliant blue R-250: Sigma, U.S.A.
[γ-^{32}P]dATP: Amersham, U.S.A.
DEAE-cellulose resin: Sigma, U.S.A.
Dialysis tubing: Sigma, U.S.A.
di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy
di-Sodium ethylenediaminetetra acetate: M&B, England
DNA marker: Lamda(λ)DNA digest with *Hin*dIII, BioLabs, Inc., USA 100 base pair DNA ladder, Promega Co., USA

Ethidium bromide: Sigma, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

Ethylene diamine tetraacetic acid (EDTA): Merck, Germany

Ethylene glycol chitin: Seikaguku Corporation, Japan

Ficoll type 400: Sigma, U.S.A.

Flaked chitin: Sigma, U.S.A.

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycerol: Merck, Germany

Glycine: Sigma, U.S.A.

Glucose: BDH, England.

Hydrochloric acid: Carlo Erba Reagenti, Italy

Isoamyl alcohol: Merck, Germany

Isopropyl-1-thio-β-D-galactopyranoside (IPTG): Sigma, U.S.A.

Low molecular weight calibration kit for SDS electrophoresis: Amersham, U.S.A.

100 bp marker: GIBCOBRL, U.S.A.

Magnesium sulphate 7-hydrate: BDH, England

Methanol: Merck, Germany

N, *N′*-methylene-bis-acrylamide: Sigma, U.S.A.

NNN′N′-tetramethyl-1,2-diaminoethane(TEMED): Carlo Erbo Reagenti, I taly

Peptone from casein pancreatically digested: Merck, Germany

Phenol: BDH, England

*p*NP-β-GlcNAc: para-Nitrophenyl N-Acetyl-β-D- Glucosamine

Polyvinylpyrrolidone: Sigma, U.S.A.85% Phosphoric acid: Mallinckrodt, U.S.A.

Potassium acetate: Merck, Germany

Potassium chloride: Merck, Germany

Potassium dihydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy

Potassium ferricyanide: BDH, England

Potassium hydroxide: Carlo Erba Reagenti, Italy

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

QIAquick Gel Extraction Kit: QIAGEN, Germany

Sodium acetate: Merck, Germany

Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy

Sodium citrate: Carlo Erba Reagenti, Italy

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate: Boehringer Mannheim Gmbtt, Germany

Sodium hydroxide: Carlo Erba Reagenti, Italy

Standard molecular weight marker protein: New England BioLabs, Inc, U.S.A.

Tris(hydroxymethyl)-aminomethane: Carlo Erba Reagenti, Italy

Tryptone: Scharlau, Microbiology, England.

2,7-Diamino,-10-ethyl-9-phenyl-phenanthridinium bromide: Sigma, U.S.A.

Xylene cyanole FF: Sigma, U.S.A.

Yeast extract: DIFCO, U.S.A and Scharlau, Microbiology, England

2.3 Enzyme and Restriction enzymes

DNA polymerase I (Klenow): New England BioLabs, Inc., U.S.A.

Lysozyme: Sigma, U.S.A

Pfu DNA polymerase: Fermentas, Inc., U.S.A.

Proteinase K: Sigma, U.S.A

Restriction enzyme: GIBCOBRL, U.S.A. and New England BioLabs, Inc., U.S.A.

RNase: Sigma, U.S.A

Taq polymerase: Pacific science, France

T_4DNAligase: New England BioLabs, Inc., U.S.A

Vent polymerase: New England BioLabs, Inc., U.S.A.

2.4 Primers

All oligonucleotide primers used in this research were synthesized form Bioservice Unit(BSU) of NSTDA, Pacific Science, Thailand and Ward Medic, Thailand.

2.5 Bacterial strains

Aeromonas caviae strain D6, used as a source of β-*N*-Acetylglucosaminidase (*agd97*) gene was isolated from soil in Nakhon pathom of Thailand,

Bacillus licheniformis strain SK-1, was used as a ource of chitinase (*chi A*) gene was isolated from the central of Thailand

.

2.6 Host cells

E. coli BL 21(DE3), genotype: F^- *omp*T *hsd*S_B *(r_B^- m_B^-) gal dcm* (DE3), was used as a host for expression.

E. .coli BL 21(DE3) pLysS, genotype: F^- *omp*T *gal dcm* lon *hsd*S_B *(r_B^- m_B^-)* λ(DE3) pLysS(cm^R), was used as a host for expression.

E. coli DH5α, genotype : *F΄, Φ*80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F) U169 *end*A1, RecA1, *hsd*R17(r_K-m_{K+}), *deo*R, *thi*-1, *sup*E44, λ-*gyr*A96, *rel*A1 (Liss, L.R., 1987) was purchased from GIBCOBRL, U.S.A. was used as a host for cloning.

E. coli Rosetta(DE3) pLysS, genotype: F^- *omp*T *hsd*S_B *(r_B^- m_B^-) gal dcm* λ(DE3 [lacI lacUV5-T7 gene l ind l sam 7 nin5]) pLysSRARE (Cam^R), was used as a host for expression.

E. coli TOP10, genotype: F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endAl λ^- was used as a host for cloning.

E. coli XL-1-Blue, genotype: recA1, relA1, endA1, gyrA96, thi-1, hsdR17, supE44, lac[*F΄*, proAB, lac/^{9}ZΔM15Tn10(Tet^r)] (Dower, 1990) was purchased from GIBCOBRL, U. S.A. was used as a host for cloning.

2.7 Vectors

Plasmid pBluescriptSK(-) (Stratagene) had promoter *chi60* was used as an alternative vector for cloning and expression of chitinase (*chiA*) and β-*N*-Acetylglucosaminidase (*agd97*) gene into *E. coli.*

pET-17b was used as an expression vector for cloning of β-*N*-Acetylglucosaminidase (*agd 97*) gene and chitinase (*chi A*) gene.

pGEM®-Teasy (QIAGEN) was used as an alternative vector for PCR cloning and subcloning of β-*N*-Acetylglucosaminidase (*agd97*) gene into *E. coli.*

2.8 Bacterial culture media

2.8.1 Luria-Bertani broth (LB medium)

The following medium was used as LB medium (Sambrook *et.al.*, 1989) containing 1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5% NaCl which was prepared and adjusted pH to 7.5 with NaOH. For solid medium, the medium was supplemented with 2.0% (w/v) agar. Medium was steriled for 20 minutes at 121^{o}C. If needed, selective antibiotic drug was then supplemented.

2.8.2 Colloidal chitin minimum medium (CCMM)

Colloidal chitin minimum medium was used for enzyme production. The medium containing 0.02% (w/v, dry weight) colloidal chitin, 0.05%(w/v) yeast extract, 0.1%(w/v) $(NH_4)_2SO_4$, 0.03%(w/v) $MgSO_4.7H_2O$, 0.6%(w/v) KH_2PO_4 and 1%(w/v) K_2HPO_4 with pH 7.5. For solid medium, the medium was supplemented with 2.0% (w/v) agar. All media were adjusted pH to 7.5 with KOH or KCl and sterilized at 121 $^{\circ}$C for 15 min by autoclaving

2.9 Determination of Chitinolytic activity

2.9.1 Chitinase

2.9.1.1 Colloidal chitin agar plate for chitinolytic screening

Chitinase producing strains were screened by conventional plate assay using colloidal chitin as a substrate. The principal is based on the capability of chitinase in hydrolyzing insoluble and opaque chitin substrate resulting in clear zone formation around the bacterial colony.

For this study, chitinase activities were determined by the turbidity reduction of a colloidal chitin suspension during chitinolysis and colorimetric reducing sugar producing activity assay, modified Schale's method (Imoto, 1971). Chitinase

activity was assayed in 1.5 ml of a reaction mixture containing 0.1% colloidal chitin in 0.1 M phosphate buffer pH 6.0 and 0.1 ml of enzyme solution.

2.9.1.2 Colorimetric method

Chitinolytic activity was measured quantitatively by detecting the amount of reducing sugar, a product of enzymatic hydrolysis, based on the Schales' s method.

The enzyme assay was performed as described in the following. A 100 µl of appropriate diluted enzyme solution was added to 75 µl of 2% colloidal chitin (final 1 mg/ml), 150 µl of 1 M phosphate buffer pH 6.0 (final 0.1 M) and adjust volume to 1.5 ml with distilled water. After incubation at 60 °C for 10 minutes, the reaction was stopped by boiling. Two milliliters of color reagent, 0.5 g of potassium ferricyanide in 1 litre of 0.5 M Na_2CO_3 was added. The mixture was heated in boiling water for 15 minutes. After cooling at room temperature, small particles were removed from the mixture by centrifugation at 10,000 rpm for 10 minutes.

The absorbance of the supernatant (A_1) was measured at 420 nm by a spectrophotometer versus distill water. A blank value (A_0) was obtained when denatured enzyme (heating in boiling water for 20-30 minutes) was used instead of the enzyme in the reaction. The difference between A_0 and A_1 was used to determine the reducing property equivalent to amount of *N*-acetylglucosamine from standard curve.

One unit (U) of enzyme activity was defined as the amount of enzyme able to produce reducing sugar property equivalent to 1 µmol of *N*-acetylglucosamine per min. Specific activity was defined as units per mg protein of an enzyme sample.

2.9.2 β-*N*-acetylglucosaminidase

β *N*-acetylglucosaminidase or chitobiase activity was measured quantitatively by detecting the amount of *p*-nitrophenol, a product of enzymatic hydrolysis when *p*-nitrophenol-*N*-acetylglucosamine was used as substrate.

The enzyme assay was performed as described in the following. A 100 µl of appropriate diluted enzyme solution was added to 100 µl of 2.5 mM *p*-nitrophenol-*N*-acetylglucosamine, 150 µl of 1 M phosphate buffer pH 6.0 and adjust

volume to 0.5 ml with distilled water. After incubation at 37 °C for 30 minutes, one milliliter of Na_2CO_3 was added, stand for 5 minutes at room temperature then measured at 420 nm. The standard curve for *p*-nitrophenol was showed (See Appendix A).

One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of pNP per min under condition. Specific activity was defined as units per mg protein of an enzyme sample.

2.10 Protein determination

The protein concentration was determine by the method of Bradford *et al*., (1976) The reaction mixture 1.1 ml containing 2.5-20 μg of protein, 1 ml of Bradford working buffer and 100 μl of protein solution were vigorously mixed and incubated at room temp for 2-60 minutes. After that, the protein concentration was monitored by measuring the absorbance at 595 nm and the amount of protein was calculate from the standard curve of protein standard (BSA). The protein standard protein curve was show in Appendix B

.

2.11 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Bollag *et al*., 1996. The slab gel system consisted of 0.1 % SDS (W/V) in 10% separating gel and 5% stacking gel. Tris-glycine (25 mM Tris, 192 mM glycine and 0.1% SDS), pH 8.3 was used as electrode buffer. in The proteins were mixed with 5x sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue and 14.4 mM β-mercaptoethanol) by a ratio of 5:1 and boiled for 10 minutes before loading to the gel. The electrophoresis was run from cathode towards anode at a constant current (30 mA) at room temperature. The standard molecular weight markers were phosphorylase B (MW 97,000), bovine serum albumin (MW 66,000), ovalbumin (MW 45,00), carbonic anhydrase (MW 31,00), trypsin inhibitor (M 20,100) and lactabumin (MW 14,400). After electrophoresis, protein in the gel were visualized by coomassie blue staining.

2.11.1 Gel staining

2.11.1.1 Coomassie blue staining

The gel was transferred to a small box containing Coomassie staining solution (1% (w/v) Coomassie Blue R-250, 45% (v/v) methanol, and 10% (v/v) glacial acetic acid). After agitating for 30 minutes on the shaker, the stain solution was poured out and the Coomassie destaining solution (10% (v/v) methanol and 10% (v/v) glacial acetic acid) was added. The gel was deatained several times until the gel background was clear.

2.12 General techniques in genetic engineering

2.12.1 Plasmid extraction

Plasmids from *E. coli* were extracted by rapid alkaline lysis method (Sambrook *et al*., 2001) or the QIAprep Miniprep Kit (Qiagen) was used for plasmids. For the alkaline lysis method, after collecting the cells grown in 5 ml LB medium, 100 µl Solution I (50 mM Tris-HCl pH 7.5, 10 mM EDTA and 100 µg/ml RNase A) was added. After resusupension, 200 µl Solution II (0.2 N NaOH and 1% (w/v) SDS) was then added and mixed by inversion. Then, 150 µl Solution III (3 M solution acetate, pH 4.8) was added and mixed gently by inversion and centrifuged at 12,000 rpm for 15 minutes. The supernatant was then collected and transferred to new microtube. The DNA was extracted with an addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 V/V), centrifugation at 12,000 rpm for 20 minutes. The plasmid DNA was precipitated by the addition of 2 volume of absolute ethanol of the aqueous phase and then kept at -70^{o}C for 2 hours. The DNA pellet was collected by centrifugation at 12,000 rpm for 10 minutes and washed with 70% ethanol. After drying, the pellet was dissolved in an appropriate volume of TE buffer, pH 8.0 containing 20 µg/ml DNase-free pancreatic RNase.

2.12.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate, identify and purify fragment of DNA using 0.8% or 1% agarose depending on DNA fragment size in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) (Ausubel *et al*., 2002). DNA samples in 1X loading buffer were loaded into the wells. The gel was run at 100 volts until bromophenol blue reached the bottom of the gel. After electrophoresis, the gel was stained by immersion in H_2O containing ethidium bromide (0.5 μg/ml) for 15-30 minutes. DNA fragment on agarose gel were visualized under a long wavelength UV light. The concentration and molecular weight of DNA sample was estimated from the intensity and relative mobility of the standard DNA marker.

2.12.3 Preparation of *E. coli* competent cells for electroporation method

A single colony of *E. coli* cells was inculated into 1,000 ml LB medium with 1% inoculum size. Cells were grown at 37^{o}C with vigorous shaking until OD_{600} was about 0.6. The culture was chilled on ice for 10 minutes and then centrifuged at 5,000 rpm for 10 minutes at 4^{o}C. The cells were washed with 1 liter of cold distilled water, of spun down and washed again with 0.5 liter of cold distilled water. After centrifugation, the cells were resuspended in approximately 20 ml of 10% glycerol in distilled water and centrifuged at 5,000 rpm for 10 minutes at 4^{o}C. Finallly, the cell pellets were resuspended to a final volumn of 2 to 3 ml in 10% glycerol. This suspension was divided into 40 μl aliquots and stored at 80^{o}C until used (Sambrook *et al*., 2001).

2.12.4 Transformation into host cell *E. coli* by electroporation

The recombinant plasmids were transformed into completent cells of *E. coli* DH5α, TOP10, JM109, XL-1 blue, BL21 (DE3), BL21 (pLys), and Rosetta. In the electroporation step, cuvette and sliding cuvette holder were chilled on ice. The Gene Pluser apparatus was set to the 25 μF capacitor, 2,5 kV, and the pluse controller unit was set to 200 Ω. Competent cells were gently thawed on ice. One microliter of

recombinant plasmid was mixed with 40 µl of the competent cells and then placed on ice for 1 minute. This mixture was transferred to a cold cuvette and the cuvette was applied with one pluse of electric shock at the above setting. Subsequently, LB medium was added immediately to the cuvette. The cells were quickly resuspended with a pasteur pipette. Then the cell suspension was transferred to new tube and incubated at 37^{o}C. for 1 hour with shaking. Finally, this suspension was spreaded onto the LB agar plates containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-gal and incubated at 37^{o}C for 10-12 hours.

2.13 Identification of *Aeromonase caviae* (D6)

2.13.1 Morphological and biochemical properties

The *Aeromonase caviae* bacteria was identified by Department of Medical Sciences, according to its morphological and biochemical properties such as gram staining, fermentative production of acids from various carbon sources and the activity of various enzyme.

2.13.2 Molecular genetic properties

2.13.2.1 Chromosomal DNA extraction

Chromosomal DNA of *Aeromonase caviae* D6. was prepared by the method of Maniatis *et al*., (2001). A single colony was incubated into 10 ml of LB medium (1.0% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.5) and incubated at 37^{O}C for 16 hours with shaking. Then each 1.5 ml of culture was centrifuged in microcentrifuge tube at 12,000 rpm for 2 minutes. The pellet was resuspended in 350 µl SET buffer. Thirty microlitres of RNase (10 mg/ml) and 20 mg lysozyme were added and incubated for 1 hour at 37^{o}C. After incubation, 1/10 volumn of 10% SDS and 10 mg proteinase K were added incubated at 50^{o}C overnight until solution became clear. After incubation, 50 µl 5 M Sodium acetate was added. The DNA was extracted with an addition of an equal volume of phenol-chloroform-isoamylalcohol (25:24:1 V/V)

mixed gently, and centrifuged at 12,000 rpm for 10 minutes. A viscous fluid formed at the aqueous layers was carefully transferred to a new microcentrifuge tube, to ensure the complete extraction of DNA. DNA was precipitated by the addition of 2.5 volume of 95% ethanol to the aqueous phase and collected by centrifugation at 10,000xg for 10 minutes. The DNA was washed with 70% ethanol. After drying, the pellet was dissolved in an appropriated volume of TE buffer. Finally, DNA concentration was estimated by submerged agarose gel electrophoresis in comparison with known amount of λ/*Hin*dIII maker.

2.13.2.2 Agarose gel electrophoresis

Electrophoresis through agarose is the standard method used to separate, identify, and purify DNA fragments. The 0.4 g of agarose was added to 50 ml electrophoresis buffer 1X TAE buffer in Erlenmeyer flask and heated until complete solubilization. The agarose solution was left at room temperature to 50^{o}C before pouring into an electrophoresis mould. When the gel was completely set, the DNA samples were mixed with gel loading buffer and loaded into agarose gel. Electrophoresis was performed at constant voltage of 10 volt/cm until the bromophenol blue migrated to appropriately distance through the gel. The gel was stained with 2.5 μg/ml ethidium bromide solution for 5 minutes and destained to remove unbound ethidium bromide with distilled water for 10 minutes. DNA fragments on agarose gel were visualized under a long wavelength UV light. The concentration and molecular weight of DNA sample was compared with the intensity and relative mobility of the standard DNA marker (λ/*Hin*dIII)

2.13.2.3 16S rRNA gene amplification

Chromosomal DNA from *Aeromonase caviae* D6 was used as a template for the 16S rRNA sequence amplification. The amplification procedure followed the method described by Edwards *et al.* (1989). The sense pB and antisense pG′ primer sequences were 5′-TAACACATGCAAGTCGAACG-3′ and 5′-GTACACACCGCCCGT-3′, respectively. PCR was performed with *pfu* DNA polymerase following this procedure, predenaturation at 94^{o}C for 5 minutes following

by 30 cycles of denaturation at 94^{o}C for 1 minutes, annealing at 55^{o}C for 2 minutes and extension at 72^{o}C for 3 minutes. Then, 1 cycle of final extension at 72^{o}C for 5 minutes was added. After final extension, the PCR products were kept at 4^{o}C. The nucleotide sequence of the amplified DNA fragment (~1400 bp). The amplified PCR product was purified by Qiagen quick Gel Extraction Kit (Qiagen, Germany) and sequences by the dideoxy-chain termination method with fluorescent primer (Edwards *et al*., 1989)

2.13.2.4 Computer search for sequence similarities

Nucleotide sequence obtained from 2.13.2.3 was compared with other 16S rRNA bacterial sequence previously published nucleotide sequence from the EMBL-GenBank-DDBJ database. Multiple sequences were aligned to find the position of nucleotide sequences by using the CLUSTAL W program.

A. caviae D6 were grown overnight at 37^{o}C in 5 ml of LB medium. After that, 1.0% of the cell culture was inoculated into 100 ml CCMM medium containing 2% flake chitin and was cultured at 37^{o}C with shaking at 250 rpm.

2.14 Cloning of the β-*N*-acetylglucosaminidase (*agd 97*) gene from *A. caviae* D6 using the PCR technique

2.14.1 Preparation of template

Chromosomal DNA of *A. caviae* D6 was prepared by the method of Sambrook *et al*., (2001) described in 2.13.2.1. The DNA solution was used as template in each PCR mixture.

2.14.2 Primer design

The primer pairs used for full length amplification of *agd 97* gene of *A. caviae* D6 were designed from 5′ and 3′ of *adg 97* gene of *A. puncta* AJ833914 from the nucleotide sequence database of GenBank. The primer pairs were designed with two difference restriction sites for directional cloning into pGEM®-T easy with the same

restriction sites. The sequence of forward primer (Agd_pGF**)** contained *Nde*I site (underline) whereas that of reverse primer (Agd_pGR) contained *Xho*I site (underline)

2.14.3 PCR amplification of *agd 97* gene fragments

The full length *agd 97* gene was amplified using PCR method. Twenty five Microliters reaction mixture contained 0.2 mM dNTPS, 1X reaction buffer, 50 ng DNA template and 10 pmole of each primer. The thermocycle consisted of predenaturation at 94^{o}C for 4 minutes, and 30 cycles of denaturation at 94^{o}C for 1 minute, annealing at 55^{o}C for 2 minutes, extension at 72^{o}C for 3 minutes following by final extension at 72^{o}C for 5 minutes. The PCR products were electrophoresed through agarose gel. Finally, the putative full length *agd97* gene fragment was recovered from agarose gel by QIA Quick gel extraction kit.

2.15 Recombinant DNA preparation

2.15.1 Construction of the *agd 97* gene into pGEM®- T easy

2.15.1.1 Ligation of the PCR product with pGEM®- T easy vector and transformation

The PCR product from 2.14.3 was ligated to sites of *Nde*I and *Xho*I pGEM®-T easy vector (Appendix C) at molar ratio of 1: 3 (DNA vector: inserted DNA) The ligation mixture of 10 µl contrained 25 ng of vector DNA, 75 ng of the gene fragment, 1X ligation buffer and 2 units of T_4 DNA ligase. The mixture was incubated overnight at 16^{o}C and then transformed into *E. coli* TOP10 by electroporation. After incubation on LB_Amp-IPTG-X-gal agar plate for 16 hours. The white colonies were selected. The present of insert in the recombinant plasmids were confirmed by sequencing.

2.15.1.2 Selection of positive recombinant

After incubation at 37^{o}C 16 hours, the white colonies were selected. The present of insert in the recombinant plasmids were confirmed by sequencing.

2.15.1.3 Recombinant plasmid characterization

The recombinant *E. coli* TOP 10 clones were grown in LB medium containing 100 µg/ml ampicillin at 37^{o}C for 16 hours with shaking. The cells cultures were collected in each 1.5 ml microcentrifuge tube by centrifugation at 12,000 rpm for 2 minutes. Then 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH 8.0) was added and the cell pellet was resuspended by repeated pipetting. After that, the 200 µl of freshly prepared solution II (0.2 N NaOH and 1% SDS) was added and gently mixed by inverting the tube and placed on ice for 5 minutes. Then, 150 µl of cooled solution III (3 M solution acetate, pH 4.8) was added and the tube was placed on ice for 5 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes and the supernatant was transferred to a new microcentrifuge tube. Then, DNA solution was extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1 V/V). The plasmid DNA was precipitated by the addition of 2 volume of absolute ethanol of the aqueous phase and then kept at -70^{o}C for 2 hours. The DNA pellet was collected by centrifugation at 12,000 rpm for 10 minutes and washed with 70% ethanol. After drying, the pellet was dissolved in an appropriate volume of TE buffer, pH 8.0 containing 20 µg/ml DNase-free pancreatic RNase (modified from Miniprep Sambrook *et al*., 2001). After that, the plasmids DNA were completely digested with *Nde*I-*Xho*I, *Pst*I, and *EcoR*I. The size of recombinant plasmids and the inserted DNA were estimated by submerged agarose gel electrophoresis compared with the λ/*Hin*dIII Finally, the inserted DNA fragments in the recombinant plasmids were confirmed to be *agd97* gene by sequencing.

2.15.2 Construction of the *agd97* gene into pET-17b

2.15.2.1 Vector DNA preparation (modified from Miniprep in Sambrook *et al*., 2001)

The *Escherichia coli* BL21 (DE3), which harboured pET-17b (Appendix D) plasmid was grow in 5 ml LB medium (1% tryptone, 1% NaCl and 0.5% yeast extract, pH 7.2) containing 100 µl ampicillin at 37^{o}C for 16 hours with shaking. The cell culture was collected in each 1.5 microcentrifuge tube by centrifugation at 12,000 rpm for 2 minute. Then 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH 8.0) was added and the cell pellet was resuspended by repeated pipetting. After that, the 200 µl of freshly prepared solution II (0.2 N NaOH and 1% SDS) was added and gently mixed by inverting the tube and placed on ice for 5 minutes. Then, 150 µl of cooled solution III (3 M solution acetate, pH 4.8) was added and the tube was placed on ice for 5 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes and the supernatant was transferred to a new microcentrifuge tube. Then, DNA solution was extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1 V/V). The plasmid DNA was precipitated by the addition of 2 volume of absolute ethanol of the aqueous phase and then kept at -70^{o}C for 2 hours. The DNA pellet was collected by centrifugation at 12,000 rpm for 10 minutes and washed with 70% ethanol. After drying, the pellet was dissolved in an appropriate volume of TE buffer, pH 8.0 containing 20 µg/ml DNase-free pancreatic RNase.

The expression vector pET-17b was linerized with *Nde*I and *Xho*I. The linear-formed pET-17b was recovered from agarose gel by QIA Quick gel extraction kit.

2.15.2.2 The *agd97* gene fragment preparation

The recombinant plasmid from 2.15.1 was digested with *Nde*I and *Xho*I. The DNA fragment of *agd97* gene was harvested from agarose gel by QIA Quick gel extraction kit.

2.15.2.3 Ligation of the *agd97* gene fragment with pET-17b vector and transformation

The *Nde*I and *Xho*I digested *agd97* gene fragment was ligated to the *Nde*I and *Xho*I digested pET-17b vector by the method described in 2.15.2.1 and then transformed into *E. coli* DH5α by electroporation. After incubation on LB Amp agar plate for 16 hours.

2.15.2.4 Selection of positive recombinant

After incubation at 37^{o}C 16 hours, the white colonies were selected.

2.15.2.5 Recombinant plasmid characterization

The recombinant *E. coli* DH5α clones were grown in LB medium containing 100 μg/ml ampicillin at 37^{o}C for 16 hours with shaking. The cells cultures were collected in each 1.5 ml microcentrifuge tube by centrifugation at 12,000 rpm for 2 minutes. Then the plasmid from individual clone was extracted as described in 2.12.1. After that, the plasmids DNA were completely digested with *Nde*I-*Xho*I, *Xho*I and *Pst*I. The size of recombinant plasmids and the inserted DNA were estimated by submerged agarose gel electrophoresis compared with the λ/*Hin*dIII. Finally, the inserted DNA fragments in the recombinant plasmids were confirmed to be *agd97* gene by sequencing.

2.15.2.6 Retransform recombinant plasmid into *E. coli* of expression hosts

The recombinant plasmid from 2.15.2.5 were transformed into the competent cells of *E. coli* BL21 (DE3), BL21 (pLysS), and Rosetta by electroporation as described in 2.12.4. After incubation at 37^{o}C 16 hours on LB-Amp agar (BL21 (DE3), BL21 (pLysS) and LB-chloramphenicol agar (Rosetta), the white colonies were selected.

2.15.2.7 Recombinant plasmid characterization

The recombinant plasmid from 2.15.2.6 were characterized by method described in 2.15.2.5

2.15.2.8 Retransform recombinant plasmid into *E. coli* of expression hosts again

The recombinant plasmid from 2.15.2.5 were transformed into the competent cells of *E .coli* BL21 (DE3), BL21 (pLysS), and Rosetta by electroporation as described in 2.12.4. After incubation at 37^{o}C 16 hours on LB-Amp containing 0.5%, 1% glucose agar (BL21 (DE3), BL21 (pLysS) and LB-chloramphenicol 0.5%, 1% glucose agar (Rosetta), the white colonies were selected.

2.15.2.9 Recombinant plasmid characterization

The recombinant *E. coli* BL21 (DE3), BL21 (pLysS) clones were grown in LB-Amp containing 0.5% and 1% glucose medium, and *E. coli* Rosetta clones were grown in LB-chloramphenical containing 0.5% and 1% glucose medium at 37^{o}C for 16 hours with shaking. Then the plasmid from individual clone was extracted as described in 2.13.1. After that, the plasmids DNA were completely digested with *NdeI-XhoL*. The size of recombinan plasmids and the inserted DNA were estimated by submerged agarose gel electrophoresis compared with the λ/*Hind*III.

2.15.3 Construction of *agd97 gene* into pBSSK(-) chi60 promoter Vector

2.15.3.1 Vector DNA preparation (modified from Miniprep in Sambrook *et al.*, 2001)

The *Escherichia coli* XL-1 blue, which harboured pBSK60 plasmid was grow in 5 ml LB medium (1% tryptone, 1% NaCl and 0.5% yeast extract, pH 7.2)

containing 100 µl ampicillin at 37°C for 16 hours with shaking. The cell culture was collected in each 1.5 microcentrifuge tube by centrifugation at 12,000 rpm for 2 minute. Then 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA, pH 8.0) was added and the cell pellet was resuspended by repeated pipetting. After that, the 200 µl of freshly prepared solution II (0.2 N NaOH and 1% SDS) was added and gently mixed by inverting the tube and placed on ice for 5 minutes. Then, 150 µl of cooled solution III (3 M solution acetate, pH 4.8) was added and the tube was placed on ice for 5 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes and the supernatant was transferred to a new microcentrifuge tube. Then, DNA solution was extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1 V/V). The plasmid DNA was precipitated by the addition of 2 volume of absolute ethanol of the aqueous phase and then kept at -70°C for 2 hours. The DNA pellet was collected by centrifugation at 12,000 rpm for 10 minutes and washed with 70% ethanol. After drying, the pellet was dissolved in an appropriate volume of TE buffer, pH 8.0 containing 20 µg/ml DNase-free pancreatic RNase.

The expression vector pBSK60 was linerized with *Nco*I and *Xho*I and made to blunt end of *Nco*I by Klenow. The linear-formed pBSK60 vector was recovered from agarose gel by QIA Quick gel extraction kit.

2.15.3.2 The *agd97* gene fragment preparation

Subclone of the recombinant plasmid from 2.15.1.1 by digested with *Nde*I and *Xho*I and made to blunt end of *Nde*I by Klenow.The DNA fragment was harvested from agarose gel by QIA Quick gel extraction kit.

2.15.3.3 Ligation of the *agd97* gene fragment with pBSK60 Vector and transformation

The *NdeI* and *XhoI* digested *agd97* gene fragment was ligated to the *Nco*I and *Xho*I digested pBSK60 vector by the method described in 2.15.3.1 and then transformed into *E. coli* DH5α by electroporation. After incubation on LB-Amp agar plate for 16 hours.

2.15.3.4 Selection of positive recombinant

After incubation at 37°C 16 hours, the white colonies were selected.

2.15.3.5 Recombinant plasmid characterization

The recombinant *E. coli* DH5α clones were grown in LB medium containing 100 μg/ml ampicillin at 37°C for 16 hours with shaking. The cells cultures were collected in each 1.5 ml microcentrifuge tube by centrifugation at 12,000 rpm for 2 minutes. Then the plasmid from individual clone was extracted as described in 2.12.1. After that, the plasmids DNA were completely digested with *Xho*I**,** *Pst* I, *BamH*I, and *Pst*I-*Xba*I. The size of recombinant plasmids and the inserted DNA were estimated by submerged agarose gel electrophoresis compared with the λ/*Hin*dIII and 10 kb DNA Ladder marker. Finally, the inserted DNA fragments in the recombinant plasmids were confirmed to be *agd97* gene by sequencing.

2.16 Expression of the *agd97* gene in *E. coli* DH5α

2.16.1 Enzyme production

The *E .coli* XL-1 blue transfomants were grown overnight at 37°C in 5 ml of LB containing 100 μg/ml ampicillin. After that, 1.0% of the cell culture was inoculated into 50 ml of the same medium and was cultured at 37°C with shaking and cultivation was continued at 37°C for 24 hours. The supernatant were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C.

2.16.2 Agd97 activity

The activity of pBSK60-Agd 97 in the recombinant clones was determined by the method described in 2.9.2.

2.16.3 Protein determination

Protein concentration of pBSK60-Agd 97 from the recombinant clones was determined by Bradford method (Bradford *et al*., 1976) as described in 2.10 with bovine serum albumin as the standard protein. The protein standard curve was show in Appendix B.

2.16.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins from 2.16.1 was analyzed by SDS-PAGE to determine the denature protein as described in 2.11.

2.17 Optimization for the *agd97* gene expression

2.17.1 Effect of *E. coli* on Adg97 activity

The transformants of *E .coli* DH5α, TOP10, JM109, and XL-1 blue were grown overnight at 37^{o}C in LB medium containing 100 μg/ml ampicillin. After that, 1.0% of cell culture was inoculated into 50 ml of the same medium and was cultured at 37^{o}C with shaking at 250 rpm. The supernatant was collected by centrifugation at 10,000 rpm for 10 minutes at 4^{o}C. The activity of Agd97 was measured by method described in 2.9.2.

2.17.2 Effect of medium volume on Adg97 activity

The transformants of *E. coli* goodness from 2.17.1 were grown overnight at 37^{o}C in LB medium containing 100 μg/ml ampicillin. After that, 1.0% of cell culture was inoculated into 25, 50, 75, 100 and 125 ml of the same medium and was cultured at 37^{o}C with shaking at 250 rpm. The supernatant was collected by centrifugation at 10,000 rpm for 10 minutes at 4^{o}C. The activity of Agd97 was measured by method described in 2.9.2.

2.17.3 Effect of production timing on Adg97 activity

The transformants of *E. coli* from 2.17.1 were grown overnight at 37^{o}C in LB medium containing 100 μg/ml ampicillin. After that, 1.0% of cell culture was inoculated into optimum volume from 2.17.2 of the same medium and was cultured at 37^{o}C with shaking at 250 rpm for 7 days. The supernatant was collected by centrifugation at 10,000 rpm for 10 minutes at 4^{o}C. The activity of Agd97 was assayed by method described in 2.9.2.

2.17.4 Protein patterns of supernatant

One milliliter of supernatant of transformant cell cultured were harvested at various times (1, 2, 3, 4, 5, 6, and 7 days production) by centrifugation. The supernatant was used for the protein pattern determination by SDS-PAGE as described in 2.11.

2.18 Product hydrolytic produced by pBSK60-Agd 97 enzyme from Recombinant clone *E. coli* XL-1 blue

2.18.1 Production of hydrolysis by pBSK60-Agd97 enzyme

The production of hydrolytic by co-hydrolysis of crude enzyme pBSK60-Agd 97 with crude enzyme Chi60 (from *Serratia sp*.) was determined by incubating the crude enzyme in 100 mM phosphate buffers, pH 6, and β-chitin at different temperatures hydrolytic 37^{o}C overnight.

2.18.2 Detection of hydrolytic product

Qualitative detection of *N*-acetylglucosamine was performed by using Thin Layer Chromatography (TLC). Aliquots (10 μl) of the reactions mixtures were spotted onto silica gel plate (Merck), *N*-acetyl chitooligosaccharide were used as standards. TLC was developed with isopropanol : ethanol : water (5:2:1 [v/v/v]) solvent system. After drying the TLC plate, aniline-diphenylamine reagent (4 ml of aniline, 4 g of

diphenylamine, 200 ml of acetone and 30 ml of 85% phosphoric acid) was sprayed entirely to the plate and then dried in hood. *N*-acetylglucosamine will appear as a gray spot after reaction was catalyzed at 130^{o}C. for 3 minutes (Tanaka *et al*., 1999)

2.19 Cloning of the *chiA* and *agd97* heterologus genes from *Bacillus licheniformis* (SK-1) and *A. caviae* (D6)

2.19.1 Preparation of the *chiA* gene template

Chromosomal DNA of *B. licheniformis* SK-1 culture with shaking at 50^{o}Cwas prepared by the method of Sambrook *et al*., (2001) as described in 2.12.1. DNA solution was used as template in each PCR mixture.

2.19.2 Primer design

The primer pairs used for full length amplification of *chiA* gene of *B. licheniformis* SK-1 were designed from the nucleotide sequence of *B. licheniformis* SK-1. The primer pairs were designed for indirectional cloning into pBSK60 and pET-17b vector. The first pairs the sequence of forward primer contained promoter of *chiA* (OChiA_proF), and the other pairs the sequence of forward primer contained ribosome binding sites of pET-17b (ChiA_RbsF) (underline) whereas that of reverse primer (ChiA_R, ChiA_R) , respectively.

2.19.3 PCR amplification of *chiA* gene fragments

The full length *chiA* had promoter of *chiA* gene was amplified using PCR method. Twenty five microliters reaction mixture contained 0.2 mM dNTPS, 1X reaction buffer, 50 ng DNA template and 10 pmole of each primer. The thermocycle consisted of predenaturation at 95^{o}C for 4 minutes, and 30 cycles of denaturation at 95^{o}C for 30 sec, annealing at 61^{o}C for 30 sec, extension at 72^{o}C for 4 minutes following by final extension at 72^{o}C for 5 minutes. The PCR products were

electrophoresed through agarose gel. Finally, the putative full length *chiA* gene fragment was recovered from agarose gel by QIA Quick gel extraction kit.

The full length *chiA* had ribosome binding site of pET 17b gene was amplified using PCR method**.** Twenty five microliters reaction mixture contained 0.2 mM dNTPS, 1X reaction buffer, 50 ng DNA template and 10 pmole of each primer. The thermocycle consisted of predenaturation at 95^{o}C for 4 minutes, and 30 cycles of denaturation at 95^{o}C for 30 sec, annealing at 65^{o}C for 30 sec, extension at 72^{o}C for 4 minutes following by final extension at 72^{o}C for 5 minutes. The PCR products were electrophoresed through agarose gel. Finally, the putative full length *chiA* gene fragment was recovered from agarose gel by QIA Quick gel extraction kit.

2.20 Construction of the *chiA* and *adg97* heterologus genes into pBSK60

2.20.1 Vector DNA preparation (modified from Miniprep in Sambrook *et al*., 2001)

The plasmid was extracted from *E. coli* XL-1 blue, which harboured *pBSK60-Agd97* plasmid by method described in 2.13.1

The expression vector pBSK60-Agd97 was linerized with *Xba*I and made to blunt end. The linear-formed pBSK60-Agd97 was recovered from agarose gel by QIA Quick gel extraction kit.

2.20.2 Ligation of the PCR product with pBSK60-Agd97 vector and transformation

The PCR product from 2.19.3 (had promoter of chiA) was ligated to sites of *Xba*I pBSK60-Agd 97 vector by method described in 2.15.1.1 and then transformed into *E. coli* XL-1 blue by electroporation. After incubation on LB-Amp agar plate for 16 hours.

2.20.3 Selection of positive recombinant

After incubation at 37^{o}C 16 hours, the white colonies were selected.

2.20.4 Recombinant plasmid characterization

The recombinant *E. coli* XL-1 blue clones were extracted plasmid as described in 2.12.1. After that, the plasmids DNA were completely digested with *Eco*RI, *Xho*I, and *Pst*I. The size of recombinant plasmids and the inserted DNA were estimated by submerged agarose gel electrophoresis compared with the λ/*Hin*d*III* and 10 kb DNA Ladder marker. marker.

2.21 Expression of the *agd97* and *chiA* gene heterologus in *E. coli* XL-1 blue

2.21.1 Chitinase (*chiA*) activity

2.21.1.1 Detection of phenotype *chiA* gene

Transformants harboring *chiA* gene were detected by the formation of clearing zone around the colonies on screening medium which consisted of 0.25% yeast extract, 0.02% colloidal chitin, and 100 μg/ml ampicillin.

2.21.1.2 Enzyme activity assay

The activity of ChiA in recombinant clones was determined by the method described in 2.9.1

2.21.2 Agd97 activity

2.21.2.1 Enzyme activity assay

The activity of pBSK60-97 in recombinant clones was determined by the method described in 2.9.2

2.21.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins from 2.21.1 was analyzed by SDS-PAGE to determine the denature protein as described in 2.11

2.22 Construction of the *chiA* and *agd97* heterologus genes into pET17b

2.22.1 Vector DNA preparation (modified from Miniprep in Sambrook *et al*., 2001)

The *Escherichia coli* TOP10, which harboured *pET-Agd97* plasmid was extracted plasmid by method described in 2.12.1

The expression vector pET-Agd97 was linerized with *Xba*I, made to blunt end and dephosphorylated. The linear-formed pET-Agd97 was recovered from agarose gel by QIA Quick gel extraction kit.

2.22.2 Ligation of the PCR product with pET-Agd 97 vector and transformation

The PCR product from 2.19.3 (had RBS of pET-17b) was ligated to sites of *Xba*I pET-Agd97 vector by method described in 2.15.1.1 and then transformed into *E. coli* TOP 10 by electroporation. After incubation on LB-Amp agar plate for 16 hours.

2.22.3 Selection of positive recombinant

After incubation at 37^{o}C 16 hours, the white colonies were selected.

2.22.4 Recombinant plasmid characterization

The recombinant *E. coli* TOP 10 clones were extracted plasmid as described in 2.12.1. After that, the plasmids DNA were completely digested with *Xho*I**,** *EcoR*I and *EcoR*I-*Sal*I. The size of recombinant plasmids and the inserted DNA were estimated

by submerged agarose gel electrophoresis compared with the λ/*Hin*dIII and 10 kb DNA Ladder marker.

2.22.5 Retransformation recombianant into *E. coli* of expression host

The recombinant plasmid from 2.22.4 were transformed into the competent cells of *E. coli* BL21 (DE3) by electroporation. After that flowing steps method 2.22.1.3, 2.22.1.4 respectively.

2.23 Expression of the *agd97* gene from pETAgd97-ChiA in *E. coli* BL21 (DE3)

The *E. coli* BL21 (DE3) transformants were grown overnight at 37^{o}C in LB-amplicillin. After that, 1.0% of the cell culture was inoculate into the same medium and cultured the same condition. When the turbidity at 600 nm had reached 0.5, IPTG was added to the final concentration of 0.2 mM to induce pET-Agd97 gene expression, for 24 hours. The supernatant was collected for enzyme activity assay and the activity of pET-Agd 97 in the recombinant clones was determined by the method described in 2.9.2.

2.24 Optimization of expression condition for Agd97 from pETAgd 97-ChiA activity

2.24.1 Effect of IPTG concentration

The transformant of *E. coli* BL21 (DE3) were grown the same in 2.24. When the turbidity at 600 nm had reached 0.5, the transformant was induced by IPTG at the final concentration of 0, 0.1, 0.2, 0.4, 0.6 and 1 mM. The supernatant was collected by centrifugation and assayed activity by the method described in 2.9.2.

2.24.2 Effect of production timing

The transformant of *E. coli* BL21 (DE3) were grown the same in 2.24, and the transformant was induced by optimum final concentration of IPTG from 2.24.1. At various times after induction (0, 3, 6, 9, 12, 15, 18, 21, and 24 hours). The supernatant was collected by centrifugation and assayed activity by the method described in 2.9.2.

2.24.3 Protein patterns of supernatant pET-Agd97

One milliliter of supernatnat culture were harvested at various time (0, 3, 6, 9, 12, 15, 18, 21, and 24 hours after induction) by centrifugation. The protein pattern determination by SDS-PAGE as described in 2.11

2.25 Purification of Agd 97 from recombinant clone

2.25.1 Recombinant clone culture

Starter inoculum was prepared by inoculation 20 ml of recombinant clone from the starter into 2 L LB medium added amplicillin, and cultivated for 3 hours with 250 rpm shaking at 37^{o}C. The 2 L of cell culture was induced by IPTG and cultivated for 6 hours at the same condition as described previously.

2.25.2 Supernatant collection and preparation

Cultivated supernatant was harvested by centrifugation at 10,000 rpm for 20 minutes, 4^{o}C, and then dissolved in 10 mM Tris-HCl (pH 7.0) buffer containing 10% glycerol and 1% 2-mercaptoethanol, and dialyzed the same buffer before determination of enzyme activity and protein concentration as described in 2.9.2 and 2.10 respectively.

2.25.3 Enzyme purification steps

The supernatant from 2.25.2 was purified by the following steps. All operations were carried out at 4^{o}C. The buffer used in DEAE cellulose chromatrography step was 10 mM Tris-HCl (pH7.0) buffer containing 10% glycerol and 1% 2-mercaptoethanol and G100 step was 10 mM Tris-HCl (pH7.0).

2.25.3.1 DEAE-cellulose column chromatography

DEAE- cellulose was activated by washing with 0.5 N NaOH twice before rewashing with deionized water until pH was 7.0. The activated DEAE-cellulose was resuspended in 10 mM Tris-HCl buffer and packed into column followed by washing with the same buffer for 10 column volume at a flow rate of 1 ml/min. The 10 ml dialyzed protein solution (10.6989 mg proteins) from 2.25.2 was applied into the column followed by equilibrating with the same buffer for 5-10 column volume at a flow rate of 1 ml/min. After the unbound proteins had been eluted, the column was continuously washed until the absorbance at 280 nm of eluent decreased to baseline value. After that, the bounded proteins were eluted gradient from the column with 0.01M Tris-HCl contraining 1 M NaCl. The fractions of 3 ml were collected by fraction collector. The protein elution profile was monitored by measuring the absorbance at 280 nm and the enzyme activity was detected as described in 3.9. The NaCl concentration was investigated by measuring the conductivity. The active fractions were pooled. The pooled protein solution was dialyzed against 0.01 M Tris-HCl and concentration protein solution was 1 ml before determination of the enzyme activity

2.25.3.2 Sephadex G-100 column chromatography

Sephadex G-100 had been wased with approximately 500 ml of 10 mM Tris-HCl containing 0.1 M NaCl before it was packed into column followed by equilibrating with the same buffer for 10 column volume at a flow rate of 0.2 ml/min. The 1 ml protein solution (3.9546 mg protein) from 2.25.3.1 was applied into column

followed by equilibrating with the same buffer for 5-10 column volume at a flow rate of 0.2 ml/min. The fraction of 2 ml were collected, and the protein elution profile was monitored by measuring the absorbance at 280 nm whereas the enzyme activity was determined as described in 2.9.2. The active fraction at head profile were pooled. The pooled protein solution was dialyzed before determination of the enzyme activity and protein concentration.

2.26 Determination of enzyme purity by SDS-PAGE

.

The proteins from each step of purification were analyzed for purity by SDS-PAGE to determine the denature protein as described in 2.11.

2.27 Characterization of Agd97

2.27.1 Effect of pH on Agd97 activity

The effect of pH on the Agd97 activity was determined under the standard assay condition as described in 2.9.2 but at various pHs. The following buffer were used; 0.1 M of citrate buffer for 3.0 to 6.0, phosphate buffer for 6.0 to 7.0, and Tris-HCl buffer for pH 7.0 to 10.0 were used. The result was expressed as a percentage of the relative activity.

2.27.2 Effect of pH on Agd97 stability

The purified Agd97 was used to study pH stability. After the purified enzyme was incubated in various pHs buffer at 4^{o}C and collected to assay enzyme activity every day for 3 days. The following buffer were used; 0.02 M of citrate buffer for 3.0 to 6.0, phosphate buffer for 6.0 to 7.0, and Tris-HCl buffer for pH 7.0 to 10.0 were used. The result was expressed as a percentage of the relative activity.

2.27.3 Effect of temperature on Agd97 activity

The effect of temperature on the Agd97 activity was examined. The purified enzyme was determined for its activity as described in 2.9.2 but at various temperatures of 25-60^{o}C. The result was expressed as a percentage of the relative activity. The maximum activity was set as 100%. The presentage of relative activity was plotted against the temperature.

2.27.4 Effect of temperature on Agd97 stability

The effect of temperature on the stability of the enzyme was determined at 37^{o}C. The purified Agd97 was incubated at various temperatures of 30-60^{o}C. The result was expressed as a percentage of the relative activity. The highest activity was defined as 100%. The presentage of relative activity was plotted against the temperature.

2.28 Product hydrolytic produced by Agd97 from *E. coli* B21 (DE3)/pETAgd97-ChiA transformant

2.28.1 Production of hydrolytic by pET-Agd97 enzyme

The production of hydrolytic by co-hydrolysis of purified Agd97 (from *E. coli* BL21(DE3)/pETAgd97-ChiA transformant) with crude enzyme SK-1 (from *Bacillus licheniformis*) were determined by incubating the crude enzyme in 100 mM phosphate buffers, pH 6, and β-chitin at different temperatures hydrolytic 37, 50^{o}C overnight.

2.28.2 Detection of hydrolytic product

The product from enzymatic hydrolysis was analyzed by Thin Layer Chromatography (TLC). The method describe in 2.21.2 (Tanaka *et al*., 1999)

Table 2.1 The sequences of forward and reverse primer's for PCR Amplification

Primer (5'-3')	Nucleotide sequence	Length (bp)	Tm (^{o}C)
Forward primers			
Agd_pGF	5'-CCATATGAACTTGAAACATTCTCTGTTAG-3'	29	66
OChiA_proF	5'-GTTTTCCCTTGTTGTCTTC-3'	19	60
ChiA_RbsF	5'-CAAGGAGGAAAGGAGATGAAAATCGTGTTGATCAAC-3'	36	68
Reverse primers			
Agd_pGR	5'-CCGCTCGAGTCAGTTCAGCTCGGTGACGCG-3'	30	68
ChiA_R	5'-GCGCGAGGAGCAGCATACAAGATAA-3'	25	66
ChiA_R	5'-GCGCGAGGAGCAGCATACAAGATAA-3'	25	66

CHAPTER III

RESULTS

In this research, *chiA* and *agd* 97 were cloned and expressed together under the same promoter of plasmid pET 17b or under different promoter of pBSSK(-) vectors in heterologous host, *E. coli*. In this chapter, the identity confirmation of *A. caviae* using 16S rRNA was firstly presented followed by the cloning and expression of *agd97* and *chiA* genes from *A. caviae*, and *B. lichenniformis*, respectively. Finally, the expressed proteins of *agd97* and *chiA* genes in *E. coli* were then biochemically characterized.

3.1 Identification of *Aeromonas caviae* D6 bacteria

The bacterial strain D6 was isolated from soil in Nakhon Pathom province of Thailand. Its morphological and biochemical properties were characterized. D6 was a Gram negative, rod shape bacterium that could move by polar flagella. This bacterium can optimally grows at 30^{O}C. The biochemical characteristics of this bacterium were shown in Table 3.1**.** From such properties, D6 was identified as *Aeromonas caviae* (Department of Medical Sciences, 1997). In this study, the identity of this bacterium was confirmed by 16S rRNA gene sequence comparison. The 16S rRNA gene fragment was PCR amplified with specific primers using genomic DNA of the bacterium strain D6 as template DNA. Only a single band of PCR amplified product of 1.5 kb was obtained (Figure 3.1). DNA sequence analysis showed that the amplified fragment was 1**,**360 bp in length (Figure 3.2). By using BLAST program, the 16S rRNA gene of D6 showed 100 % identity to 16S rRNA gene of *Aeromonas caviae*. (accession no. X60408.1) (Figure3.3). Identification results by 16S rRNA and biochemical properties indicated that the bacterium stain D6 is *Aeromonas caviae*.

Table 3.1 Biochemical characteristics of the bacterium strain D6

Characteristics	Result
Shape	Rod
Gram staining	negative
Motility	+
VP + 1% NaCl	-
LIM	
- LDC	-
- Indole	+
- Motile	+
HIB + 1% NaCl	+
Mannitol	+
Inositol	-
Mannose	-
Arabinose	+
Urea	-
Citrate	-
Salicin	+
Cellobiose	-
Esculin + 1% NaCl	+
Dextrose acid	
- acid	+
- gas	-
Sucrose	+
Lactose	-
Lysine + 1% NaCl	-
Ornithine + 1% NaCl	-
Arginine + 1% NaCl	+
Ampicillin (10 µg)	R
Carbenicillin (100 µg)	R

Table 3.1 Biochemical characteristics of the bacterium strain D6(continued)

Characteristics	Result
Cephalothin (30 µg)	R
Colistin (10 µg)	S
NB + 0% NaCl	+
NB + 1% NaCl	+
NB + 3% NaCl	+
NB + 8% NaCl	-

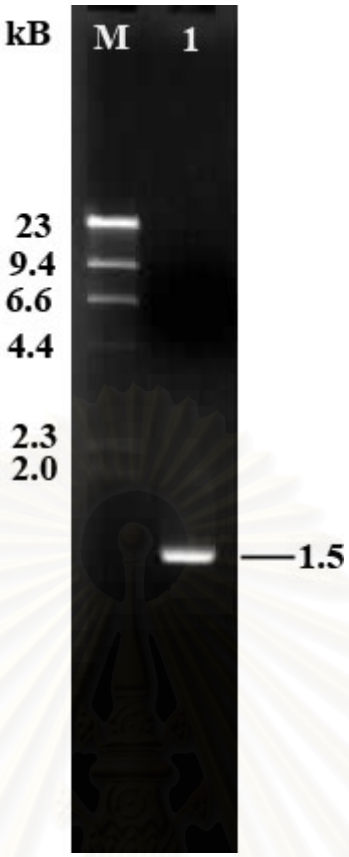


Figure 3.1 : 16S rRNA gene amplified product from the bacterium strain D6

Lane M = λ/*Hin*d III

Lane 1 = PCR products of 16S rRNA gene using genomic DNA of bacteria strain D6 as a template

pB

```
TAACACATGCAAGTCGAACGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGC 60
GGACGGGTGAGTAATGCCTGGGAAATTGCCCAGTCGAGGGGGATAACAGTTGGAAACGAC 120
TGCTAATACCGCATACGCCTACGGGGGAAAGCAGGGGACCCTCGGGCCTTGCGCGATTGG 180
ATATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCT 240
AGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG 300
GAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGT 360
GTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGTAGCTAAT 420
ATCTGCTGGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG 480
CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCG 540
GTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTC 600
CAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGAT 660
CTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAA 720
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATT 780
TGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGG 840
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG 900
CATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATC 960
CTGCAGAGATGCGGGAGTGCCTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTCGTCA 1020
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTG 1080
CCAGCACGTAATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGG 1140
GATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCG 1200
TACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGA 1260
TTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGT 1320
TGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT                       1360
```

pG

Figure 3.2 : Nucleotide sequence of 16S rRNA gene of the bacterium strain D6

The amplified 16s rRNA gene was sequenced. The result of 1,360 bp was shown above. The primers used for amplification were underlined.

```
D6          ----------------------------------------TAACACATGCAAGTCGAGCG 20
X60408.1    GAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCG 60
                                                    ********************

D6          GCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTG 80
X60408.1    GCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTG 120
            ************************************************************

D6          GGAAATTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCT 140
X60408.1    GGAAATTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCT 180
            ************************************************************

D6          ACGGGGGAAAGCAGGGGACCCTCGGGCCTTGCGCGATTGGATATGCCCAGGTGGGATTAG 200
X60408.1    ACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATATGCCCAGGTGGGATTAG 240
            ******************** ***************************************

D6          CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAT 260
X60408.1    CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAT 300
            ************************************************************

D6          CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT 320
X60408.1    CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT 360
            ************************************************************

D6          TGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTT 380
X60408.1    TGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTT 420
            ************************************************************

D6          GTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGTAGCTAATATCTGCTGGCTGTGACGTTA 440
X60408.1    GTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGTAGCTAATATCTGCTGGCTGTGACGTTA 480
            ************************************************************

D6          CTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA 500
X60408.1    CTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA 540
            ************************************************************

D6          GCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGA 560
X60408.1    GCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGA 600
            ************************************************************


D6          AAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGG 620
X60408.1    AAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGG 660
            ************************************************************

D6          GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 680
X60408.1    GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 720
            ************************************************************

D6          AAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 740
X60408.1    AAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 780
            ************************************************************
```

Figure 3.3 : Nucleotide sequence alignment of 16S rRNA gene of the bacterial strain D6 compared with *A. caviae* (X60408.1)

The highlighted letters indicated the different nucleotides

```
D6              TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGA 800
X60408.1        TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGA 840
                ************************************************************

D6              CGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAA 860
X60408.1        CGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAA 900
                ************************************************************

D6              AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGC 920
X60408.1        AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGC 960
                ************************************************************

D6              AACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGCAGAGATGCGGGAGTGC 980
X60408.1        AACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGCAGAGATGCGGGAGTGC 1020
                ************************************************************

D6              CTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG 1040
X60408.1        CTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG 1080
                ************************************************************

D6              GGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAA 1100
X60408.1        GGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAA 1140
                ************************************************************

D6              CTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATG 1160
X60408.1        CTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATG 1200
                ************************************************************

D6              GCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAG 1220
X60408.1        GCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAG 1260
                ************************************************************

D6              CGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT 1280
X60408.1        CGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT 1320
                ************************************************************

D6              CCATGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTTGCGGTGAATACGTTCCCGG 1340
X60408.1        CCATGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTTGCGGTGAATACGTTCCCGG 1380
                ************************************************************

D6              GCCTTGTACACACCGCCCGT---------------------------------------- 1360
X60408.1        GCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGATAGCTTA 1440
                ********************
```

Figure 3.3 : Nucleotide sequence alignment of 16S rRNA gene of the bacterial strain D6 compared with *A. caviae* (X60408.1) (continued)

The highlighted letters indicated the different nucleotides

3.2 Cloning and sequence analysis of the *agd97* gene from *A. caviae*D6

The chromosomal DNA of D6 was used as template. Primer pairs were designed with introduced *Nde*I, *Xho*I restriction sites, from the available nucleotide sequences of β-*N-acetylglucosaminidase* from *A. punctata* (accession no. AJ833914). PCR amplification was done at annealing temperature at 55^{o}C. Figure 3.4 shows the PCR product of *agd97* from agarose gel electrophoresis. The size of PCR product was about 2.6 kp of *agd97* without nonspecific bands.

The PCR product of *agd97* was ligated into pGEM®-T easy vector with *Nde*I and *Xho*I restriction sites and transformed into *E. coli* TOP10 by electroporation. The recombinant *E. coli* TOP 10/pGM-Agd97 was selected on LB medium containing 100 μg/ml ampicillin. Finally, the inserted DNA fragments in the recombinant plasmids were confirmed to be *agd97* gene by DNA sequencing.

The sequencing result *agd97* gene and primer positions are shown in Figure 3.5. The structural gene contained an open reading frame (ORF) of 2,664 bp, which encoded a polypeptide of 888 amino acids including a signal peptide of 22 amino acid residues with predicted molecular weight of 97.68 kDa. The ORF starts at the ATG codon and ends with the stop codon TGA, The G+C contents of was about 62.4%.

The nucleotide sequence and deduced amino acid sequence of the *adg97* were compared to those deposited in the EMBL-GenBank-DDBL database. The percentage of sequence similarity between the nucleotide sequences of *adg97* gene from *A.caviae* D6 and *β* -N-*acetylglucosaminidase* gene from *A. punctata* (accession no. AJ833914) was 96% (Figure 3.6) Percentage similarity of Agd97 to others in the database was 96, 95, 54, 52 and 15% when compared to *A. puncta*(AJ833914), *A. hydrophila*(YP_856066.1), *Vibrio parahaemolyticus* (ZP_01993789.1), *Yersinia pseudotuberculosis* (YP_001401865.1) and *Arthrobacter sp*. (CAB72127.1), respectively (Figure 3.7).

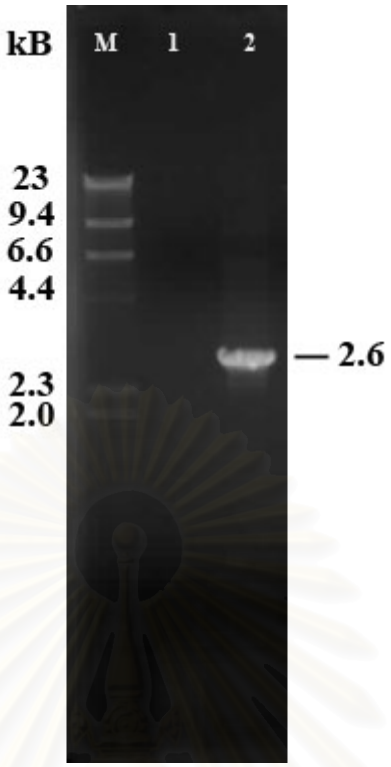


Figure 3.4 : PCR product of *agd97* gene from *A. caviae* D6 genome on 0.8% agarose gel.

PCR products of *agd97* on agarose gel electrophoresis and size of about 2,664 bp without nonspecific bands.

Lane M : λ/*Hin*dIII standard DNA marker
Lane 2 : negative control (no DNA template)
Lane 3 : PCR product as indicated

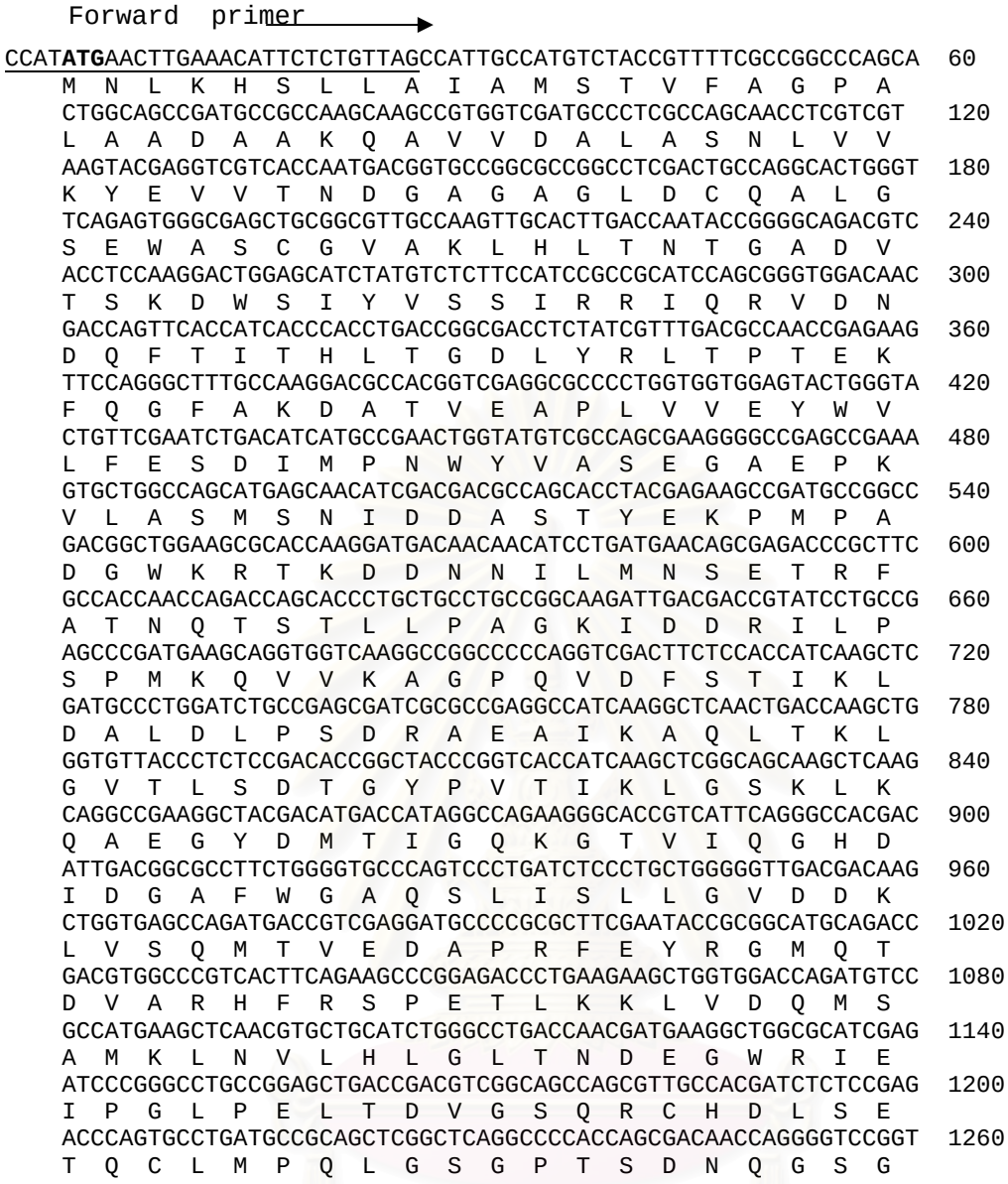


Figure 3.5 : Nucleotide and deduced amino acid sequence of *agd97* gene into pGEM®-T easy

The region and direction of primer were indicated by highlights and arrows.

```
TTCTACAGCAAGGCGGACTACATCGATCTCGTGCGCTATGCCAAGGCCCGCGGCGTGACC  1320
F  Y  S  K  A  D  Y  I  D  L  V  R  Y  A  K  A  R  G  V  T
GTGATCCCCGAGATCAACATGCCGGCCCACGCCCGTGCCGCCGTGGTCTCCATGGAGGCG  1380
V  I  P  E  I  N  M  P  A  H  A  R  A  A  V  V  S  M  E  A
CGCTACAAGCGTCTGATGGCGGAAGGCAAAGAGGCGGAAGCCAACCAGTTCCGACTGACC  1440
R  Y  K  R  L  M  A  E  G  K  E  A  E  A  N  Q  F  R  L  T
GACCCGGCCGATACCTCCAACGTCACCTCGGTGCAGTTCTACGACAAGATGTCCTTCATC  1500
D  P  A  D  T  S  N  V  T  S  V  Q  F  Y  D  K  M  S  F  I
AACCCCTGCCAGCCGGGTGCCGCCACCTTCGTGGCCAAGGTGATGGATGAAGTGGCCCAG  1560
N  P  C  Q  P  G  A  A  T  F  V  A  K  V  M  D  E  V  A  Q
ATGCACCAGGCCGCCGGTCAGCCCCTGACCGCATGGCACTACGGTGGTGACGAGGCGAAG  1620
M  H  Q  A  A  G  Q  P  L  T  A  W  H  Y  G  G  D  E  A  K
AACATCATGCAGGGCGGTGGTTACCAGGATCCGGCCGTCACCAAGAAAGAAGAGCTGGTC  1680
N  I  M  Q  G  G  G  Y  Q  D  P  A  V  T  K  K  E  E  L  V
GCCTGGAAGGGCAACGTCGACTCCAGCAAGCAGGACAAGCCGTTCGGCAAGTCACCGATG  1740
A  W  K  G  N  V  D  S  S  K  Q  D  K  P  F  G  K  S  P  M
TGCCAGAAGATGATCGACGATGGCAAGATCAAGGACGTGGCCGAGCTGCCGGTCCACTTT  1800
C  Q  K  M  I  D  D  G  K  I  K  D  V  A  E  L  P  V  H  F
GCCAAGGAAGTGAGCGAGATGGTCAAGGGCCACGGCTTCTCCACCCTGCAGGCGTGGGAA  1860
A  K  E  V  S  E  M  V  K  G  H  G  F  S  T  L  Q  A  W  E
GATGGTCTGAAGTACGCCACGGATGCCAGCGTGTTCGCCACCGACAAGACCCGGGTCAAC  1920
D  G  L  K  Y  A  T  D  A  S  V  F  A  T  D  K  T  R  V  N
TTCTGGGAAACCCTCTACTGGGGCGGCTTCAACGAGGCGATGAAGTGGGCGCACAAGGGC  1980
F  W  E  T  L  Y  W  G  G  F  N  E  A  M  K  W  A  H  K  G
TATGAGGTGGTGCTCTCGAACCCCGACTACCTCTACTTTGACTTCCCGAACGAGGTACAC  2040
Y  E  V  V  L  S  N  P  D  Y  L  Y  F  D  F  P  N  E  V  H
CCGGCCGAGCGCGGCTACTACTGGGCCACCCGCTTCAACGACACCCGCAAGGTGTTCGCC  2100
P  A  E  R  G  Y  Y  W  A  T  R  F  N  D  T  R  K  V  F  A
TTCGCCCCGGAAAACCTGCCGCAGAACGCCGAGACCTCGGTTGACCGCGACGGCAACGCC  2160
F  A  P  E  N  L  P  Q  N  A  E  T  S  V  D  R  D  G  N  A
TTCGTGGCCAAGGGTGACCATGATCCGGTCAAGTTCAAGGGGATCTCCGGTCAGCAGTGG  2220
F  V  A  K  G  D  H  D  P  V  K  F  K  G  I  S  G  Q  Q  W
AGCGAAACTGTGCGCACCGATGCCCAGTACGAGTACATGGTCTATCCGCGCATCTTCTCC  2280
S  E  T  V  R  T  D  A  Q  Y  E  Y  M  V  Y  P  R  I  F  S
GTGGCCGAGCGTGCCTGGCACAAGGGCGGCTTCGAGCTCGACTACGTGAAGAACCGCGAG  2340
V  A  E  R  A  W  H  K  G  G  F  E  L  D  Y  V  K  N  R  E
TTCTCCGGCACCACCAAGTTCGTCAACAAGGCCACCCTGAACAAGGAGTGGAACCAGTTC  2400
F  S  G  T  T  K  F  V  N  K  A  T  L  N  K  E  W  N  Q  F
GCCAACGTGCTGGGTCAGCGCGTGCTGCCGAAACTGGACCAGGCCGGGGTGGAATACCGC  2460
A  N  V  L  G  Q  R  V  L  P  K  L  D  Q  A  G  V  E  Y  R
CTCTCCGTACCGGGTGCCAAGGTGGTGAACGGCGTGCTTGAAGCGAACGTGGATCTGCCG  2520
L  S  V  P  G  A  K  V  V  N  G  V  L  E  A  N  V  D  L  P
```

Figure 3.5 : Nucleotide and deduced amino acid sequence of *agd97* gene into pGEM®-T easy

The region and direction of primer were indicated by highlights and arrows. (continued)

```
GGTCTGCCCATCCAGTACAGCCTGGACGGCACCAACTGGACTGCCTACGACGCCGCGGCC  2580
G  L  P  I  Q  Y  S  L  D  G  T  N  W  T  A  Y  D  A  A  A
AAGCCGAGCGTGAATGGCAAGGTCTGGCTGCGTACCACCAGCTTCGATGGCAAGCGCACC  2640
K  P  S  V  N  G  K  V  W  L  R  T  T  S  F  D  G  K  R  T
AGCCGCGTCACCGAGCTGAACTGACTGACTCGAGCGG                         2670
                  <-------------- Reverse primer
S  R  V  T  E  L  N  *
```

Figure 3.5 : Nucleotide and deduced amino acid sequence of *agd97* gene into pGEM®-T easy

The region and direction of primer were indicated by highlights and arrows. (continued)

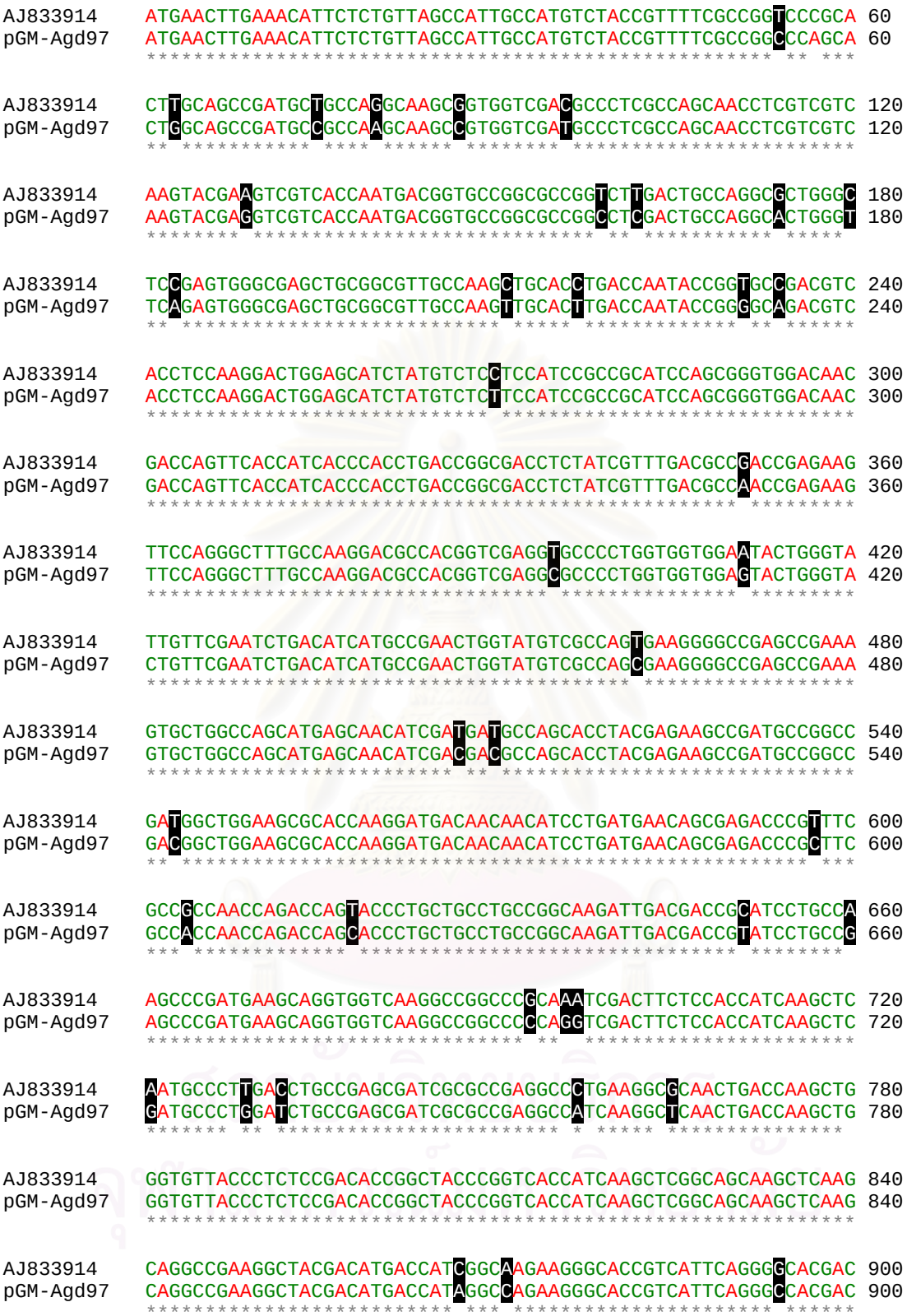


Figure 3.6 : Nucleotide sequence alignment of *agd97* gene from *A. caviae* D6 Compared with *A. punctata* (Accession no. AJ833914)

The highlighted letters indicated the different or missing nucleotides

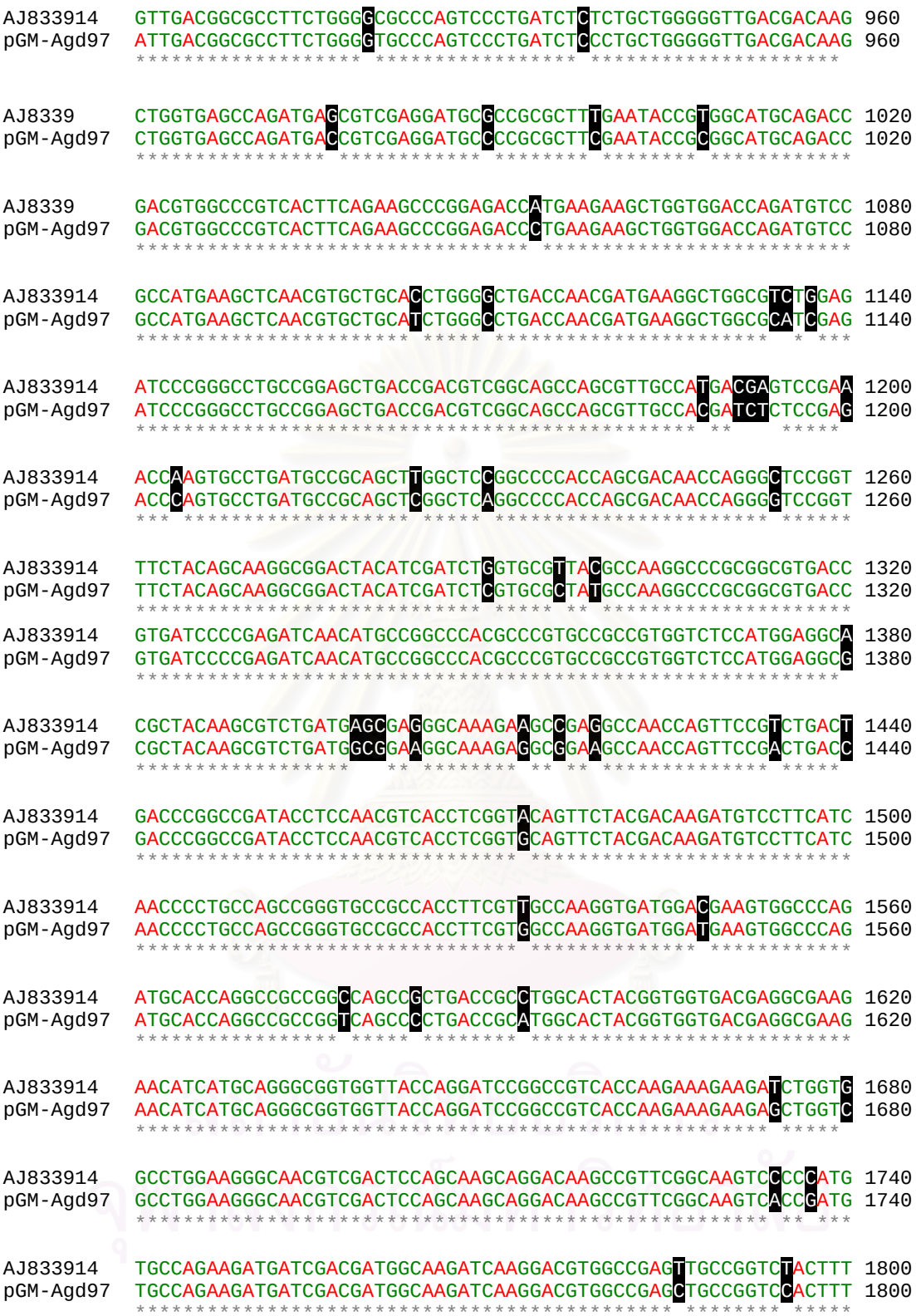


Figure 3.6 : Nucleotide sequence alignment of *agd97* gene from *A. caviae* D6 Compared with *A. punctata* (Accession no. AJ833914) (continued)

The highlighted letters indicated the different or missing nucleotides

```
AJ833914    GCCAAGGAAGTGAGCGAGATGGTCAAGGGCCACGGCTTCTCCACCCTGCAGGCCTGGGAA 1860
pGM-Agd97   GCCAAGGAAGTGAGCGAGATGGTCAAGGGCCACGGCTTCTCCACCCTGCAGGCGTGGGAA 1860
            ************************************************** ******

AJ833914    GATGGCCTGAAGTACGCCACGGATGCCAGCGTATTCGCCACCGACAAGACCCGGGTCAAC 1920
pGM-Agd97   GATGGTCTGAAGTACGCCACGGATGCCAGCGTGTTCGCCACCGACAAGACCCGGGTCAAC 1920
            ***** ************************ ************************** 

AJ833914    TTCTGGGAAACCCTCTACTGGGGTGGTTTCAACGAGGCGATGAAGTGGGCGCACAAGGGC 1980
pGM-Agd97   TTCTGGGAAACCCTCTACTGGGGCGGCTTCAACGAGGCGATGAAGTGGGCGCACAAGGGC 1980
            ********************** ** ********************************

AJ833914    TATGACGTGGTGCTCTCCAACCCGGATTACCTCTACTTCGACTTCCCGAACGAGGTACAC 2040
pGM-Agd97   TATGAGGTGGTGCTCTCGAACCCCGACTACCTCTACTTTGACTTCCCGAACGAGGTACAC 2040
            ***** *********** ***** ** *********** ********************

AJ833914    CCGGCCGAGCGCGGCTACTACTGGGCAACCCGCTTCAACGACACCCGCAAGGTGTTCGCC 2100
pGM-Agd97   CGGCCGAGCGCGGCTACTACTGGGCCACCCGCTTCAACGACACCCGCAAGGTGTTCGCC 2100
            ************************* ********************************

AJ833914    TTCGCCCCGGAAAACCTGCCGCAGAACGCCGAGACCTCGGTTGACCGCGATGGCAACGCC 2160
pGM-Agd97   TTCGCCCCGGAAAACCTGCCGCAGAACGCCGAGACCTCGGTTGACCGCGACGGCAACGCC 2160
            ************************************************* *********

AJ833914    TTCGTGGCCAAGGGTGACCAGGATCCGGTCAAGTTCAAGGGGATCTCCGGTCAGCAATGG 2220
pGM-Agd97   TTCGTGGCCAAGGGTGACCATGATCCGGTCAAGTTCAAGGGGATCTCCGGTCAGCAGTGG 2220
            ******************* ********************************** ***

AJ833914    AGTGAAACCGTGCGCACCGATGCCCAGTACGAATACATGGTCTATCCGCGCATCTTCTCC 2280
pGM-Agd97   AGCGAAACTGTGCGCACCGATGCCCAGTACGAGTACATGGTCTATCCGCGCATCTTCTCC 2280
            ** ***** ********************** ************************** 

AJ833914    GTGGCCGAGCGCGCCTGGCACAAGGGCGGCTTCGAGCTCGATTACGTGAAGGGTCGCGAG 2340
pGM-Agd97   GTGGCCGAGCGTGCCTGGCACAAGGGCGGCTTCGAGCTCGACTACGTGAAGAACCGCGAG 2340
            ********** ******************************** *********   ******

AJ833914    TTCTCCGGCACCACCAAGCACGTGAACAAGGCCACCCTGAACAAGGAGTGGAACCAGTTT 2400
pGM-Agd97   TTCTCCGGCACCACCAAGTTCGTCAACAAGGCCACCCTGAACAAGGAGTGGAACCAGTTC 2400
            ****************  *** ************************************

AJ833914    GCCAACGTGCTGGGTCAGCGCGTGCTGCCCAAACTGGACCAGGCAGGGGTGGAATACCGC 2460
pGM-Agd97   GCCAACGTGCTGGGTCAGCGCGTGCTGCCGAAACTGGACCAGGCCGGGGTGGAATACCGC 2460
            ***************************** ************ ***************

AJ833914    CTCTCCGTACCGGGTGCCAAGGTGGTGAACGGTGTGCTGGAAGCGAACGTGGATCTGCCG 2520
pGM-Agd97   CTCTCCGTACCGGGTGCCAAGGTGGTGAACGGCGTGCTTGAAGCGAACGTGGATCTGCCG 2520
            ********************************* ***** ********************

AJ833914    GGTCTGCCCATCCAGTACAGCCTGGATGGCAAGAGCTGGAGCGCCTACGACGCTGCCGCC 2580
pGM-Agd97   GGTCTGCCCATCCAGTACAGCCTGGACGGCACCAACTGGACTGCCTACGACGCCGCGGCC 2580
            *************************** ****  * *****  ********** ** ***

AJ833914    AAGCCGACCGTGGATGGCAAGGTCTACCTGCGTACCACCAGCTTCGATGGCAAGCGTACC 2640
pGM-Agd97   AAGCCGAGCGTGAATGGCAAGGTCTGGCTGCGTACCACCAGCTTCGATGGCAAGCGCACC 2640
            ******* **** ************ ******************************* ***

AJ833914    AGCCGCGTCACCGAGCTGAACTGA 2664
pGM-Agd97   AGCCGCGTCACCGAGCTGAACTGA 2664
            ************************
```

Figure 3.6 : Nucleotide sequence alignment of *agd97* gene from *A. caviae* D6 Compared with *A. punctata* (Accession no. AJ833914) (continued)

The highlighted letters indicated the different or missing nucleotides

```
pGM_Agd97       -MNLKHSLLAIAMSTVFAGPALAADAAKQAVVDALASNLVVKYEVVTNDGAGAGLDCQAL 59
A.punctata      -MNLKHSLLAIAMSTVFAGPALAADAARQAVVDALASNLVVKYEVVTNDGAGAGLDCQAL 59
A.hydrophila    -MNLKHSLLAIAMSTVFVSQVQAADAAKQAVVDALASNLVVKYEVVTNDGAGAGLDCQAL 59
Yersinia        -MNKFKLNALAAITATFGLIGYANGSATNQQVVDQLSTLKVNYKLLDNRAADNGVDCAKL 59
Vibrio          ------------------------------------------------------------
Arthrobacter    MWKKTLAMAVVAPAMLLSMAAPPALAAPGDPVSTNLALASAGATVTSSGDESVGSNGPDL 60

pGM_Agd97       GS--------------------------------EWASCGVAKLHLTNTGADVTSKDWS 86
A.punctata      GS--------------------------------EWASCGVAKLHLTNTGADVTSKDWS 86
A.hydrophila    GS--------------------------------EWASCGVAKLHLTNTGADVTSKDWS 86
Yersinia        GA--------------------------------DWASCNKVMITLTNTGDEIKGQDWA 86
Vibrio          ------------------------------------------------------------
Arthrobacter    AIDGGDTTRWSSEHSDTAQLTVKLAKPAAIDKIVIKWEKACAAQYKLQVSTDGVSFVDAT120

pGM_Agd97       IYVSSIRRIQRVDNDQFTITHLTGDLYRLT------------------------------ 116
A.punctata      IYVSSIRRIQRVDNDQFTITHLTGDLYRLT------------------------------ 116
A.hydrophila    IYVSSIRRIQRVDNDQFTITHLTGDLYRLT------------------------------ 116
Yersinia        IYFHSIRMILAVDNDQFTVTHLTGDLHKIE------------------------------ 116
Vibrio          ------------------------------------------------------------
Arthrobacter    DVISRANCAPETPDTQTIKSSLAGTKYQFVRMQGIAVTPIAGTKWGISLFEMEVWGVPAA 180

pGM_Agd97       ------------------------------------PTEKFQGFAKDATVEAPLVVE  137
A.punctata      ------------------------------------PTEKFQGFAKDATVEVPLVVE  137
A.hydrophila    ------------------------------------PTEKFQGFAKDATVEVPLVVE  137
Yersinia        ------------------------------------PTAKFAGFPANQTIEIPITGE  137
Vibrio          ------------------------------------------------------------
Arthrobacter    PAQNIALASAGATVSPSGQEVAGQWGPALVIDGDTDSTK NAQQSRWSSNTADSANITVK 240

pGM_Agd9        YWVLFESDIMPNWYVASEGAEPKVLASMSNIDDASTYEKPMPADGWKRTKDDNNILMNSE 197
A.punctata      YWVLFESDIMPNWYVASEGAEPKVLASMSNIDDASTYEKPMPADGWKRTKDDNNILMNSE 197
A.hydrophila    YWVLFESDIMPNWYVASEGAEPKVLASMSNIDDATTYEKPMPADGWKRTKDDNNILMNSE 197
Yersini         YWQLFATDFMPRWYATSGDAKPKVLASTD-TEDINAYLTPFTGDQWKRTKDDNNVLMTPE 196
Vibrio          ------------------------------------------------------------
Arthrobacter    AAPTLIDHVAIVWEKACAAKYKLQVSTDGITFVDATDVIAPTCNTRDVQKLKAGVAANA 300

pGM_Agd97      TRFATNQTSTLLPAGKIDDRILPSPMKQ-VVKAGPQVDFSTIKLDALDLPSDRAEAIKAQ 256
A.punctata     TRFAANQTSTLLPAGKIDDRILPSPMKQ-VVKAGPQIDFSTIKLNALDLPSDRAEALKAQ 256
A.hydrophila   TRFAANQTSSLLPAGKVDNQILPTPMKQ-VVKAGPQVDFSTIKLNALDLPSDRAEALKAQ 256
Yersinia       SRFVKNEAVKTLSAANLRGQIIPTPLDV-KVYPQDADLSLGVALELSALPKPASDAIQQR 255
Vibrio          ------------------------------------------------------------
Arthrobacter   YQYVRMQGIERTPIGARNTASRSGSFRSGMAKKSPLQHLCQSPVNLIPLPVNMETPDEAP 360
```

Figure 3.7 : Linear alignment of the deduced amino acid sequence of *agd97* from *A. caviae* D6 and those from various sources.

The linear alignment was made by ClustalW program.

Conserved residues were indicated by asterisks (*) (:) are amino acids which have the same group of side chains and similar size while (.) meaned amino acids which had the same group of side chains but different size

```
pGM_Agd97       LTKLG-------------------VTLSDTGYPVTIKLG-------------------- 276
A.puncenta      LTKLG-------------------VTLSDTGYPVTIKLG-------------------- 276
A.hydrophila    LTKLG-------------------VTLSDTGYPVTIKLG-------------------- 276
Yersinia        FELLG-------------------LALSATGYPIKTVIQPT--------------AFN 280
Vibrio          ------------------------------------------------------------
Arthrobacter    FKLGAGSRIVANNAVTAKSASFLAELFRTSTGLALPVVNGSTGDADDIVLLQTPGDIPNL 420


pGM_Agd97       SKLKQAEGYDMTIGQK-GTVIQGHDIDGAFWGAQSLISLLG--------VD-DKLVSQMT 326
A.puncenta      SKLKQAEGYDMTIGKK-GTVIQGHDVDGAFWGAQSLISLLG--------VD-DKLVSQMS 326
A.hydrophila    SKLKQAEGYDMTIGQK-GTVVQGHDIEGAFWGAQSLISLLG--------VN-DKLVSQMH 326
Yersinia        GDHAVSGAYELKIGEK-GAEVIGFDQVGVFYGLQSILSLVP--------IEGSKTIATLD 331
Vibrio          ------------------------------------------------------------
Arthrobacter    GAQLQAEAYTLSVDALTGAKITAATDDGIFNGVQTLRQLFPGIHCVQNQGQRHLDGSCVE 480


pGM_Agd97       VEDAPRFEYRGMQTDVARHFRSPETLKKLVDQMSAMKLNVLHLGLTNDEGWRIEIPGLPE 386
A.puncenta      VEDAPRFEYRGMQTDVARHFRSPETMKKLVDQMSAMKLNVLHLGLTNDEGWRLEIPGLPE 386
A.hydrophila    VEDAPRFEYRGMQTDVARHFRSPETLKKLVDQMSAMKLNVLHLGLTNDEGWRLEIPGLPE 386
Yersinia        AKDAPRFDYRGVSLDVGRNFKTKAAVLRLLDQMSAYKLNKFHFHLSDDEGWRIEIPGLPE 391
Vibrio          ------------------------------------------------------------
Arthrobacter    ISDAPRFDKRGMMLDVAREFKNPDEVKAIIDSLASYKISTLHMHLADDQGWRIEITNEGK 540


pGM_Agd97       LT-DVGSQRCHDLSETQCLMPQLGSGPTSDNQGSGFYSKADYIDLVRYAKARGVTVIPEI 445
A.puncenta      LT-DVGSQRCHDESETKCLMPQLGSGPTSDNQGSGFYSKADYIDLVRYAKARGVTVIPEI 445
A.hydrophila    LT-DVGSQRCHDESETKCLMPQLGSGPTSDNQGSGFYSKADYIDLVRYAKARGVTVIPEI 445
Yersinia        LT-DVGSQRCHDLTETTCLLPQLGSGPESNNLGSGYFTRADYIDILKYAKARQIDVIPEI 450
Vibrio            ----------------------------------------------------------
Arthrobacter    VAGDDIDYNQLTEISGKGGMTQFNRTYMDLLGNTGFYTQAEYKDLVAYAADRHIEIIPEI 600


pGM_Agd97       NMPAHARAAVVSMEARYKR----------LMAEGKEAEANQFRLTDPADTSNVTSVQFYD 495
A.puncenta      NMPAHARAAVVSMEARYKR----------LMSEGKEAEANQFRLTDPADTSNVTSVQFYD 495
A.hydrophila    NMPAHARAAVVSMEARYKR----------LMAEGKETEANQFRLTDPADTSNVTSVQFYD 495
Yersinia        DIPAHARAAVVSMEARYNN----------LMKQGKEKEANEFRLVDPTDDSNTTSVQFYE 500
Vibrio          ------------------------------------------------------------
Arthrobacter    DVPGHTSAILHAIPQLNTAGTKPNVDEWGVVPEDGTGNVGTSTLDVAAPQTWTFLEHVFG 660


pGM_Agd97       KMSFINP-----------CQPGAATFVAKVMDEVAQMHQAAGQPL--TAWHYGGDEAKN- 541
A.puncenta      KMSFINP-----------CQPGAATFVAKVMDEVAQMHQAAGQPL--TAWHYGGDEAKN- 541
A.hydrophila    KMSFINP-----------CQPGAATFVAKVMDEVAQMHQAAGQPL--TAWHYGGDEAKN- 541
Yersinia        RKSYLNP-----------CLDSSKHFVDKVIGEMAQMHKEAGMPL--TTWHFGGDEAKN- 546
Vibrio          ------------------------------------------------------------
Arthrobacter    QIAEMTTSEYIHIGGDESHVTGHDNYVEFITKAVKLIHDLDKKPIGWNEVAIGGLEAGGR 720


pGM_Agd97       ---------------------------------------------IMQGGGYQDPAVTKKE 557
A.puncenta      ---------------------------------------------IMQGGGYQDPAVTKKE 557
A.hydrophila    ---------------------------------------------IMQGGGYQDPAVTKKE 557
Yersinia        ---------------------------------------------IRLGAGYQD----KNG 558
Vibrio          ------------------------------------------------------------
Arthrobacter    HSVLDRRHRRHAEGHQDKGAKLMVSNGSTAYLDMKYNAKTPIGLTWAGMGDFPKYYDWNP 780
```

Figure 3.7 : Linear alignment of the deduced amino acid sequence of *agd97* from *A. caviae* D6 and those from various sources.

The linear alignment was made by ClustalW program.

Conserved residues were indicated by asterisks (*) (:) are amino acids which have the same group of side chains and similar size while (.) meaned amino acids which had the same group of side chains but different size

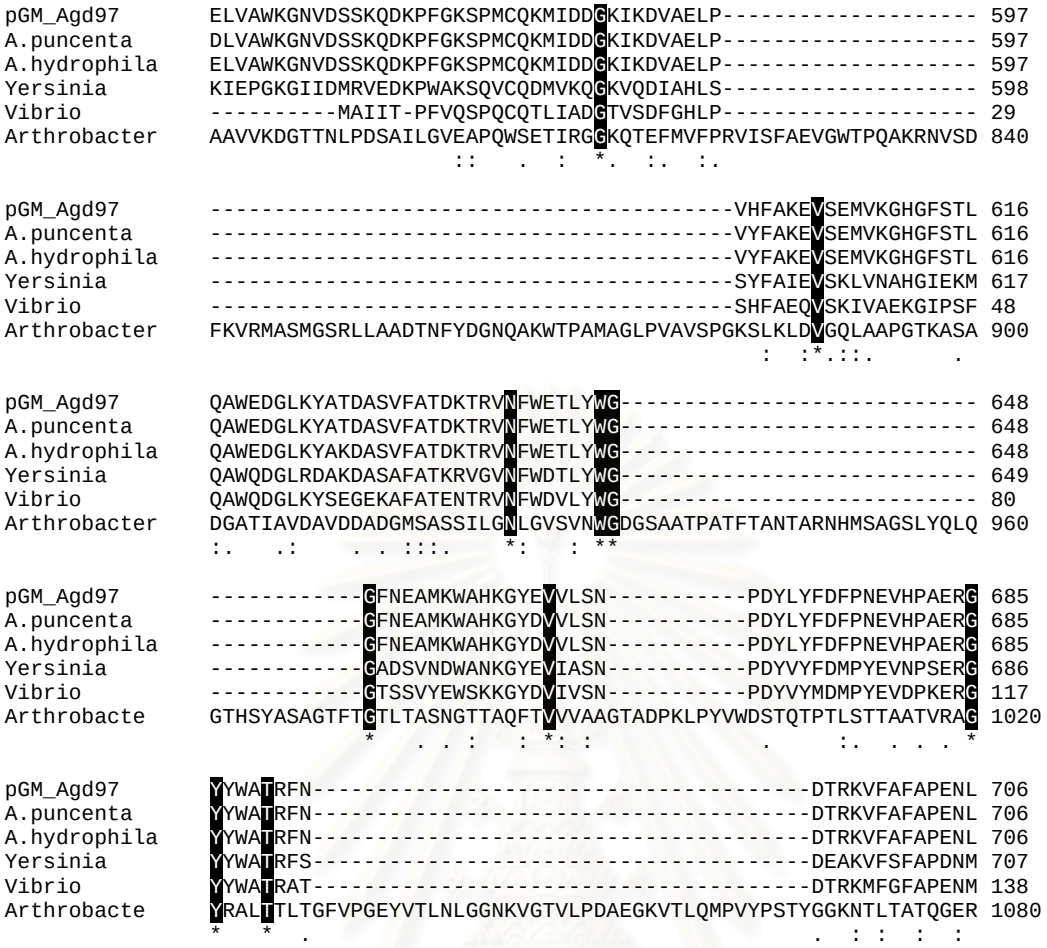


Figure 3.7 : Linear alignment of the deduced amino acid sequence of *agd97* from *A. caviae* D6 and those from various sources.

The linear alignment was made by ClustalW program.

Conserved residues were indicated by asterisks (*) (:) are amino acids which have the same group of side chains and similar size while (.) meaned amino acids which had the same group of side chains but different size

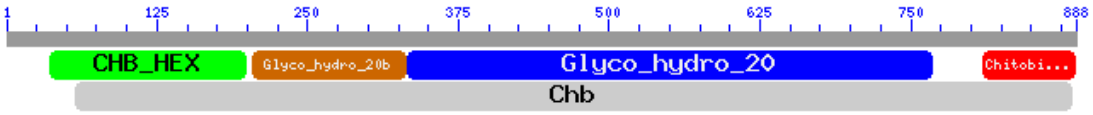


Figure 3.8 : **Domain mapping of Agd97**

The domain mapping was predicted by BLASTP.program.

3.3 Cloning Heterologus *chiA* and *agd97* gene into pBSSK

3.3.1 Subcloning *agd97* into pBSK60

Subcloning of *agd97* gene fragment from pGM-Agd 97 with *Nde*I and *Xho*I restriction sites was done by ligated into pBSSK$^-$ Chi60 promoter vector (pBSK60) with *Nco*I and *Xho*I restriction sites by *Nde*I and *Nco*I sites blunt end ligation. The ligation mixture was transformed into *E. coli* DH5α by electroporation and selected on LB agar with ampicillin. Recombinant pBSK60-Agd97 was characterized by digestion with restriction enzymes (*Xho*I, *Pst*I, *Bam*HI, and *Pst*I–*Xba*I), and detailed physical maps showed that plasmid insert was identical except that the *agd97* insert shown in Figure 3.9**.**

Sequencing result of recombinant pBSK60-Agd 97 was presented in two parts as shown in Figure 3.10. The first part shows nucleotide sequence of chi60 promoter and the second part shows nucleotide sequence of *agd97* gene. The nucleotide sequence of chi60 promoter consisting of 337 bases, ribosome binding site (RBS) at AAAGGAA sequence, the promoter sequence, TTGTTT for the -35 region and TATAGT for -10 region with 16-bp spacing between them. Part of *agd97* gene consisting of 2,664-bp start at ATG codon and ends with stop codon TGA, and predicted molecular weight of 97.68 kDa. The initiation codon (ATG) was preceded at a distance of 10-bp by the ribosome binding site (AAAGGAA) of Chi60 promoter and the orientation of the *agd97* gene is the same as that of the Chi60 promoter.

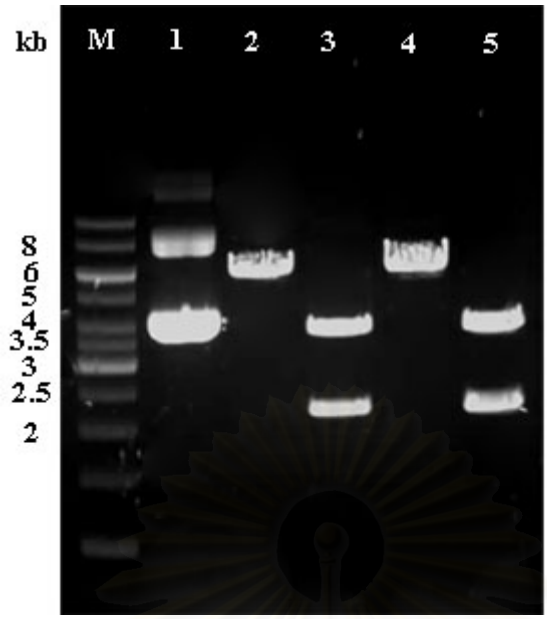


Figure 3.9 : Restriction pattern of pBSK60-Agd97 from selected recombinant clones

Lane M : 10 kb DNA ladder
Lane 1 : undigested plasmid.
Lane 2 : *Xho*I digested plasmid
Lane 3 : *Pst*I digested plasmid
Lane 4 : *Bam*HI digested plasmid
Lane 5 : *Pst*I-*Xba*I digested plasmid

```
TCCCCCGGGCTGCAGAGTGTGGTGCAATCCTGATAAATATTTATCTTTCCTTAATAAAAAAATTCACTA
TCCATATTTGTCGTGCTTTCTTTTATTTATATTAAAATAAATTCACGCTTGCTGAATAAAACCCAGTTG
ATAGCG
     -35                        -10
CTCTTGTTTTCACGCCTTTTTTATTTATATAGTCTGAATGTACGCGGTGGGAATGATTATTTCGCCACGTG
GAAAGACACTGTTGCTATTTATTGATTTTAATCTTCGAGGATTATTGCGGAATTTTTTCGCTTCGGCAA
                                                    SD
TGCATCGCGACGATTAACTCTTTTATGTTTATCCTCTCGGAATAAAGGAATCAGCCATGT
```

```
ATGAACTTGAAACATTCTCTGTTAGCCATTGCCATGTCTACCGTTTTCGCCGGCCCAGCA  60
M  N  L  K  H  S  L  L  A  I  A  M  S  T  V  F  A  G  P  A
CTGGCAGCCGATGCCGCCAAGCAAGCCGTGGTCGATGCCCTCGCCAGCAACCTCGTCGT  120
L  A  A  D  A  A  K  Q  A  V  V  D  A  L  A  S  N  L  V  V
AAGTACGAGGTCGTCACCAATGACGGTGCCGGCGCCGGCCTCGACTGCCAGGCACTGGGT  180
K  Y  E  V  V  T  N  D  G  A  G  A  G  L  D  C  Q  A  L  G
TCAGAGTGGGCGAGCTGCGGCGTTGCCAAGTTGCACTTGACCAATACCGGGGCAGACGTC  240
S  E  W  A  S  C  G  V  A  K  L  H  L  T  N  T  G  A  D  V
ACCTCCAAGGACTGGAGCATCTATGTCTCTTCCATCCGCCGCATCCAGCGGGTGGACAAC  300
T  S  K  D  W  S  I  Y  V  S  S  I  R  R  I  Q  R  V  D  N
GACCAGTTCACCATCACCCACCTGACCGGCGACCTCTATCGTTTGACGCCAACCGAGAAG  360
D  Q  F  T  I  T  H  L  T  G  D  L  Y  R  L  T  P  T  E  K
TTCCAGGGCTTTGCCAAGGACGCCACGGTCGAGGCGCCCCTGGTGGTGGAGTACTGGGTA  420
F  Q  G  F  A  K  D  A  T  V  E  A  P  L  V  V  E  Y  W  V
CTGTTCGAATCTGACATCATGCCGAACTGGTATGTCGCCAGCGAAGGGGCCGAGCCGAAA  480
L  F  E  S  D  I  M  P  N  W  Y  V  A  S  E  G  A  E  P  K
GTGCTGGCCAGCATGAGCAACATCGACGACGCCAGCACCTACGAGAAGCCGATGCCGGCC  540
V  L  A  S  M  S  N  I  D  D  A  S  T  Y  E  K  P  M  P  A
GACGGCTGGAAGCGCACCAAGGATGACAACAACATCCTGATGAACAGCGAGACCCGCTTC  600
D  G  W  K  R  T  K  D  D  N  N  I  L  M  N  S  E  T  R  F
GCCACCAACCAGACCAGCACCCTGCTGCCTGCCGGCAAGATTGACGACCGTATCCTGCCG  660
A  T  N  Q  T  S  T  L  L  P  A  G  K  I  D  D  R  I  L  P
AGCCCGATGAAGCAGGTGGTCAAGGCCGGCCCCCAGGTCGACTTCTCCACCATCAAGCTC  720
S  P  M  K  Q  V  V  K  A  G  P  Q  V  D  F  S  T  I  K  L
GATGCCCTGGATCTGCCGAGCGATCGCGCCGAGGCCATCAAGGCTCAACTGACCAAGCTG  780
D  A  L  D  L  P  S  D  R  A  E  A  I  K  A  Q  L  T  K  L
GGTGTTACCCTCTCCGACACCGGCTACCCGGTCACCATCAAGCTCGGCAGCAAGCTCAAG  840
G  V  T  L  S  D  T  G  Y  P  V  T  I  K  L  G  S  K  L  K
CAGGCCGAAGGCTACGACATGACCATAGGCCAGAAGGGCACCGTCATTCAGGGCCACGAC  900
Q  A  E  G  Y  D  M  T  I  G  Q  K  G  T  V  I  Q  G  H  D
ATTGACGGCGCCTTCTGGGGTGCCCAGTCCCTGATCTCCCTGCTGGGGGTTGACGACAAG  960
I  D  G  A  F  W  G  A  Q  S  L  I  S  L  L  G  V  D  D  K
CTGGTGAGCCAGATGACCGTCGAGGATGCCCCGCGCTTCGAATACCGCGGCATGCAGACC 1020
L  V  S  Q  M  T  V  E  D  A  P  R  F  E  Y  R  G  M  Q  T
GACGTGGCCCGTCACTTCAGAAGCCCGGAGACCCTGAAGAAGCTGGTGGACCAGATGTCC 1080
D  V  A  R  H  F  R  S  P  E  T  L  K  K  L  V  D  Q  M  S
```

Figure 3.10 : Nucleotide and deduced amino acid sequences of the *agd97* gene into pBSK60 vector

Red color and underlined = -35 regions of Chi60 promoter

Green color and underlined = -10 region of Chi60 promoter

Pink color and underlined = SD region of Chi60 promoter and

Black highlight = nucleotide sequence of start and stop *agd97* gene

```
GCCATGAAGCTCAACGTGCTGCATCTGGGCCTGACCAACGATGAAGGCTGGCGCATCGAG  1140
A  M  K  L  N  V  L  H  L  G  L  T  N  D  E  G  W  R  I  E
ATCCCGGGCCTGCCGGAGCTGACCGACGTCGGCAGCCAGCGTTGCCACGATCTCTCCGAG  1200
I  P  G  L  P  E  L  T  D  V  G  S  Q  R  C  H  D  L  S  E
ACCCAGTGCCTGATGCCGCAGCTCGGCTCAGGCCCCACCAGCGACAACCAGGGGTCCGGT  1260
T  Q  C  L  M  P  Q  L  G  S  G  P  T  S  D  N  Q  G  S  G
TTCTACAGCAAGGCGGACTACATCGATCTCGTGCGCTATGCCAAGGCCCGCGGCGTGACC  1320
F  Y  S  K  A  D  Y  I  D  L  V  R  Y  A  K  A  R  G  V  T
GTGATCCCCGAGATCAACATGCCGGCCCACGCCCGTGCCGCCGTGGTCTCCATGGAGGCG  1380
V  I  P  E  I  N  M  P  A  H  A  R  A  A  V  V  S  M  E  A
CGCTACAAGCGTCTGATGGCGGAAGGCAAAGAGGCGGAAGCCAACCAGTTCCGACTGACC  1440
R  Y  K  R  L  M  A  E  G  K  E  A  E  A  N  Q  F  R  L  T
GACCCGGCCGATACCTCCAACGTCACCTCGGTGCAGTTCTACGACAAGATGTCCTTCATC  1500
D  P  A  D  T  S  N  V  T  S  V  Q  F  Y  D  K  M  S  F  I
AACCCCTGCCAGCCGGGTGCCGCCACCTTCGTGGCCAAGGTGATGGATGAAGTGGCCCAG  1560
N  P  C  Q  P  G  A  A  T  F  V  A  K  V  M  D  E  V  A  Q
ATGCACCAGGCCGCCGGTCAGCCCCTGACCGCATGGCACTACGGTGGTGACGAGGCGAAG  1620
M  H  Q  A  A  G  Q  P  L  T  A  W  H  Y  G  G  D  E  A  K
AACATCATGCAGGGCGGTGGTTACCAGGATCCGGCCGTCACCAAGAAAGAAGAGCTGGTC  1680
N  I  M  Q  G  G  G  Y  Q  D  P  A  V  T  K  K  E  E  L  V
GCCTGGAAGGGCAACGTCGACTCCAGCAAGCAGGACAAGCCGTTCGGCAAGTCACCGATG  1740
A  W  K  G  N  V  D  S  S  K  Q  D  K  P  F  G  K  S  P  M
TGCCAGAAGATGATCGACGATGGCAAGATCAAGGACGTGGCCGAGCTGCCGGTCCACTTT  1800
C  Q  K  M  I  D  D  G  K  I  K  D  V  A  E  L  P  V  H  F
GCCAAGGAAGTGAGCGAGATGGTCAAGGGCCACGGCTTCTCCACCCTGCAGGCGTGGGAA  1860
A  K  E  V  S  E  M  V  K  G  H  G  F  S  T  L  Q  A  W  E
GATGGTCTGAAGTACGCCACGGATGCCAGCGTGTTCGCCACCGACAAGACCCGGGTCAAC  1920
D  G  L  K  Y  A  T  D  A  S  V  F  A  T  D  K  T  R  V  N
TTCTGGGAAACCCTCTACTGGGGCGGCTTCAACGAGGCGATGAAGTGGGCGCACAAGGGC  1980
F  W  E  T  L  Y  W  G  G  F  N  E  A  M  K  W  A  H  K  G
TATGAGGTGGTGCTCTCGAACCCCGACTACCTCTACTTTGACTTCCCGAACGAGGTACAC  2040
Y  E  V  V  L  S  N  P  D  Y  L  Y  F  D  F  P  N  E  V  H
CCGGCCGAGCGCGGCTACTACTGGGCCACCCGCTTCAACGACACCCGCAAGGTGTTCGCC  2100
P  A  E  R  G  Y  Y  W  A  T  R  F  N  D  T  R  K  V  F  A
TTCGCCCCGGAAAACCTGCCGCAGAACGCCGAGACCTCGGTTGACCGCGACGGCAACGCC  2160
F  A  P  E  N  L  P  Q  N  A  E  T  S  V  D  R  D  G  N  A
TTCGTGGCCAAGGGTGACCATGATCCGGTCAAGTTCAAGGGGATCTCCGGTCAGCAGTGG  2220
F  V  A  K  G  D  H  D  P  V  K  F  K  G  I  S  G  Q  Q  W
AGCGAAACTGTGCGCACCGATGCCCAGTACGAGTACATGGTCTATCCGCGCATCTTCTCC  2280
S  E  T  V  R  T  D  A  Q  Y  E  Y  M  V  Y  P  R  I  F  S
GTGGCCGAGCGTGCCTGGCACAAGGGCGGCTTCGAGCTCGACTACGTGAAGAACCGCGAG  2340
V  A  E  R  A  W  H  K  G  G  F  E  L  D  Y  V  K  N  R  E
TTCTCCGGCACCACCAAGTTCGTCAACAAGGCCACCCTGAACAAGGAGTGGAACCAGTTC  2400
F  S  G  T  T  K  F  V  N  K  A  T  L  N  K  E  W  N  Q  F
GCCAACGTGCTGGGTCAGCGCGTGCTGCCGAAACTGGACCAGGCCGGGGTGGAATACCGC  2460
A  N  V  L  G  Q  R  V  L  P  K  L  D  Q  A  G  V  E  Y  R
CTCTCCGTACCGGGTGCCAAGGTGGTGAACGGCGTGCTTGAAGCGAACGTGGATCTGCCG  2520
L  S  V  P  G  A  K  V  V  N  G  V  L  E  A  N  V  D  L  P
GGTCTGCCCATCCAGTACAGCCTGGACGGCACCAACTGGACTGCCTACGACGCCGCGGCC  2580
G  L  P  I  Q  Y  S  L  D  G  T  N  W  T  A  Y  D  A  A  A
AAGCCGAGCGTGAATGGCAAGGTCTGGCTGCGTACCACCAGCTTCGATGGCAAGCGCACC  2640
K  P  S  V  N  G  K  V  W  L  R  T  T  S  F  D  G  K  R  T
AGCCGCGTCACCGAGCTGAACTGAACTGACTCGAGCGG                        2670
S  R  V  T  E  L  N  *
```

Figure 3.10 : Nucleotide and deduced amino acid sequences of the *agd97* gene into pBSK60 vector (continue)

Black highlight = nucleotide sequence of stop *agd97* gene

3.3.2 Expression of the *agd97* gene in *E. coli*

The expression plasmid pBSK60-Agd97 for the *agd97* gene under the control of chi60 promoter in *E. coli* was constructed from pBSK60. Transformants were grown aerobically for 7 day at 37^{o}C in LB medium containing ampicillin.(100 μg/ml) and cells were harvested after centrifugation. The supernatant (extracellular Agd97) was assayed for Agd97 activity. Enzyme activity from high to low was observed in *E. coli* XL-1 blue, DH5α, TOP10 and JM109 respectively. The optimum condition for Agd97 production was at day 3 of production and when the ratio of culture media to the total container volume was at 1:2.5. The profile of the time course of enzyme production was shown in Figure 3.11**.** *E. coli* XL-1 blue transformants produce Agd97 at 207.269 mU/ml of culture broth. This value corresponds to an approximately 3.55 fold lower than that of Agd 97 activity in the culture of the native host, D6. SDS-PAGE analysis of Agd97 expression in *E. coli* XL-1 blue with pBSK60-Agd97 was shown in Figure 3.12. The estimated molecular weight of the expressed Agd97 on SDS-PAGE was 97 kDa.

3.3.3 Chitin hydrolysis to GlcNAc by Agd97

To produce GlcNAc from digestion β-chitin, the enzyme Agd97 was used in combination with CHI60 from *Serratia sp*. (see Chapter II) that was able to release chitobiose. The reaction was done at 37^{o}C overnight with various units of Agd97 (62.1, 41.4 and 20.7 mU/ml) and was analyzed by thin-layer chromatography after the specific time course (Figure 3.13). The substrates from hydrolysis of chi60 (*N*-acetylchitobiose) were all hydrolyzed and GlcNAc was produced as a final product by used 62.1 mU/ml of Agd97.

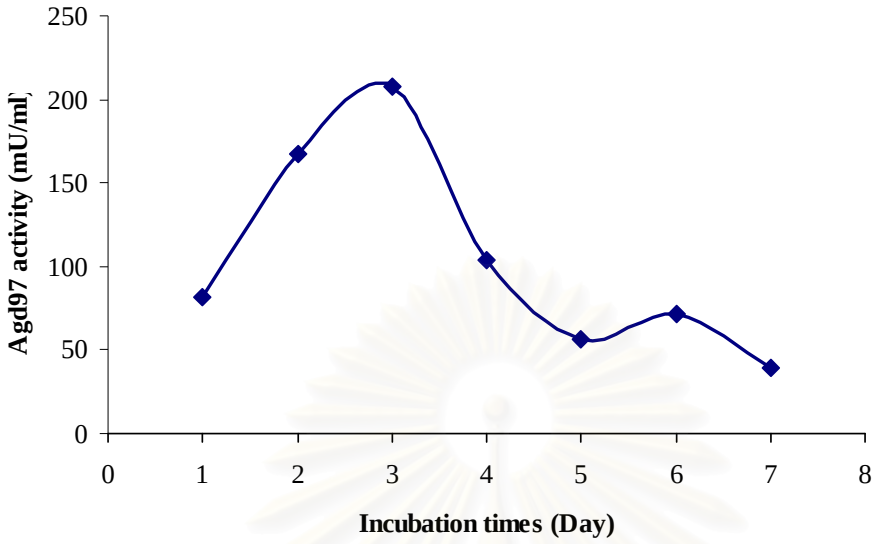


Figure 3.11 : The profile of the time course of Agd 97 production from *E. coli* XL-1 blue / pBSK60-Agd97 at 37^{o}C

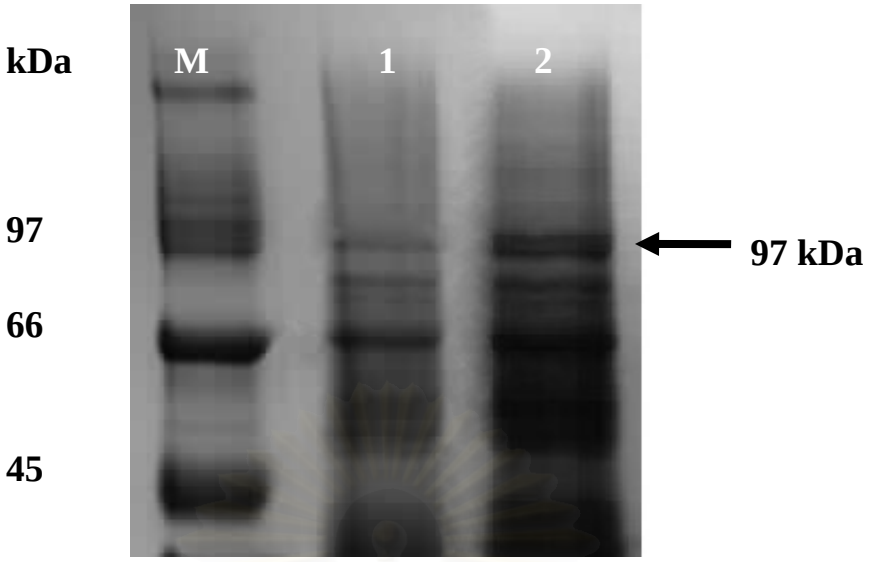


Figure 3.12 : SDS-PAGE analysis of Agd97 expression in *E. coli* XL-1 blue / pBSK60-Agd97.

Lane M : protein MW marker
Lane 1 : *E. coli* XL-1 blue / pBSK60
Lane 2 : *E. coli* XL-1 blue / pBSK60-Agd97

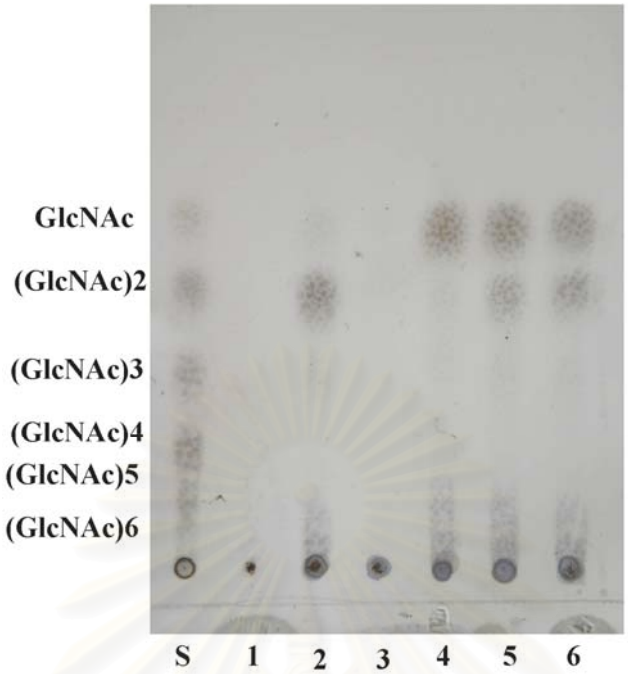


Figure 3.13 : Analysis of enzymatic hydrolysates by TLC.

The reactions were done at 37^{o}C overnight using enzyme mixture of Chi60 and Agd 97 by various units of Agd97 and using β-chitin as substrate.

Lane S contained a mixture of *N*-acetylchitooligomers ranging from GlcNAc to *N*-acetylchitohexose, $(GlcNAc)_6$

Lane 1 The hydrolysis of β -chitin without enzyme

Lane 2 The hydrolysis of β -chitin with enzyme Chi60

Lane 3 The hydrolysis of β -chitin with enzyme Agd97

Lane 4-6 The hydrolysis of β -chitin with enzyme mixture (62.1, 41.4 and 20.7 mU of Agd97 and 9 mU of Chi60).

3.3.4 Cloning *chiA* gene into pBSK60-Agd97

B. licheniformis SK-1 to produce chitinase enzyme. The chitinase enzyme good properties hydrolysis beta-chitin and was produced main products diacetylchitobiose, $(GlcNAc)_2$ and monosaccharide, GlcNAc. Hence, we isolated chitinase (*chiA*) gene from SK-1, cloning and expression in pBSK60-Agd97 vector. *chiA* gene fragment was obtained through PCR using *B. licheniformis* genomic DNA as the template and primer pairs were designed base on nucleotide sequences of *chiA* from SK-1 with introduced promoter of chiA (OChiA_proF+ChiA_R). The PCR reaction of *chiA* gene was done at annealing temperature at 61^{o}C for 30 sec. PCR product fragment was 2,068 bp in length (Figure 3.14).

pBSK60-Agd97 vector was digested by *Xba*I and then made to blunt end liner vector. The *chiA* gene fragment which contain its promoter was ligated with liner pBSK60-Agd97 vector and the ligation mixture were transformed into *E. coli* XL-1 blue by electroporation. The physical maps by recombinant plasmids digested with *Eco*RI, *Xho*I and *Pst*I. showed that the *agd97* and *chiA* genes insert into pBSSK^{-} was about 7,690 bp. The orientation of the *chiA* gene fragment was opposite to the chi60 promoter (Figure 3.15)

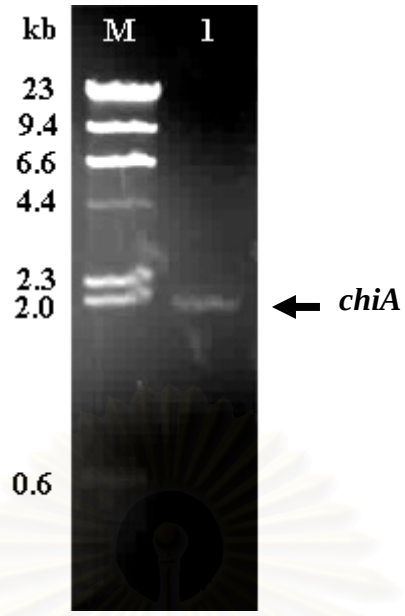


Figure 3.14 : PCR product of *chiA* (chiA promoter) gene from *B. licheniformis* SK-1 genome on 0.8% agarose gel.

Lane M : λ/*Hin*dIII standard DNA marker

Lane 1 : PCR product as indicated

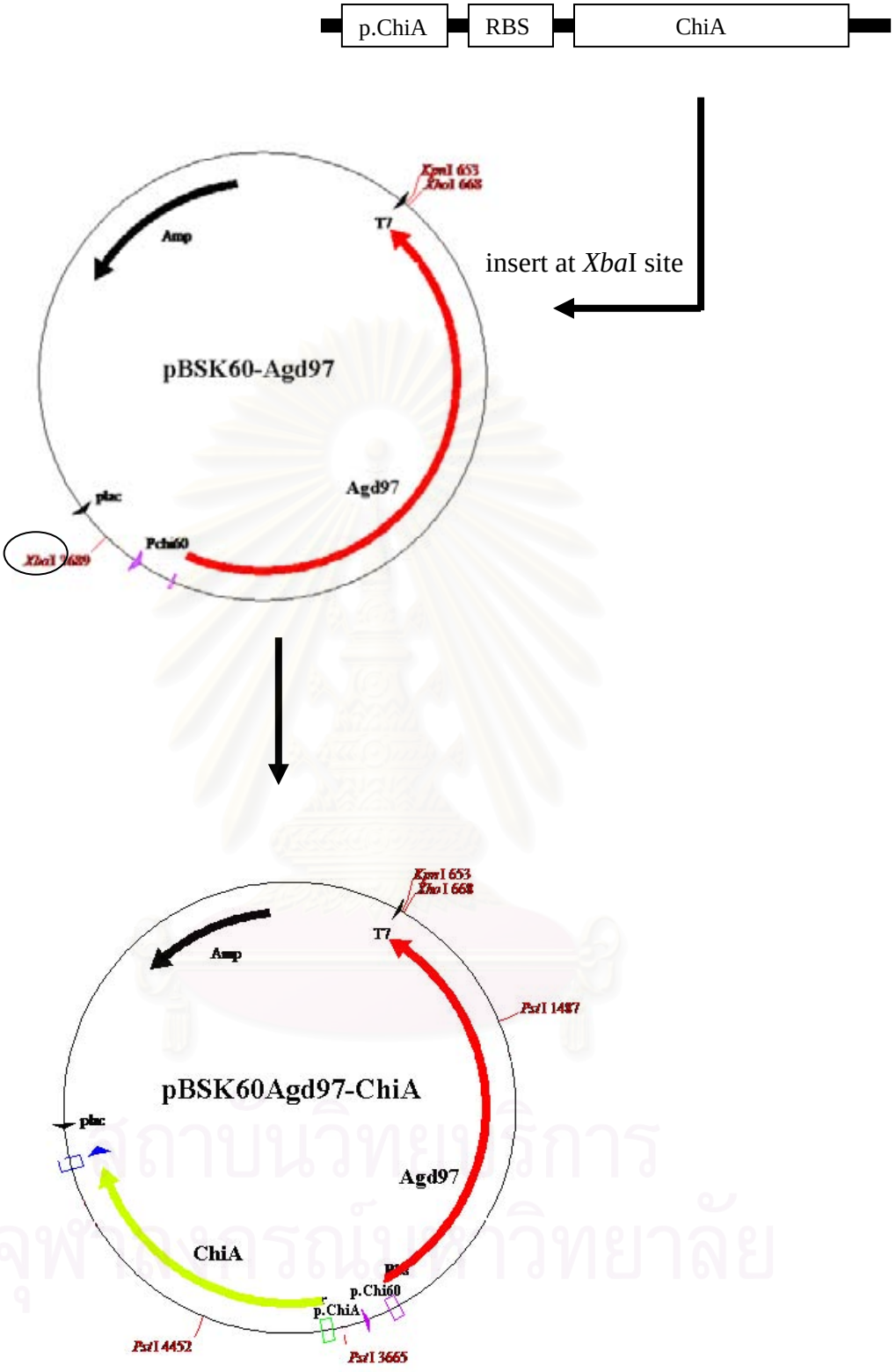


Figure 3.15 : Construction of recombinant pBSK60Agd97-ChiA

3.3.5 Expression of recombinant pBSK60Agd97-ChiA

The pBSK60Agd97-ChiA contains the *agd97* gene with chi60 promoter, ribosome binding site sequence of chi60 promoter, the *chiA* gene with chiA promoter and ribosome binding site sequence of chiA promoter. The orientation of two promoters is opposite**.** To express the *chiA* and *agd97* genes in *E. coli* XL-1 blue under separated promoters of each gene. The recombinant clones were detected *chiA* gene phenotype by grown on colloidal chitin minimum medium-ampilcilin agar plate at 37^{o}C for 5 days. From the result, the recombinant clones could not growth on colloidal chitin minimum medium-ampicillin agar plate. They could growth on LB-Ampicillin plate only. The recombinant clones on LB-ampicilin plate were picked for culture LB medium at 37^{o}C shaking condition. Preparation supernatant culture were assayed using ChiA and Agd97 activity. Supernatant culture from *E. coli* XL-1 blue with and without plasmid pBSK60-Agd97 were used as control. The result was shown could not be detected ChiA and Agd97 activity.

3.4 Cloning heterologus *chiA* and *agd97* gene into pET-17b

3.4.1 Subcloning *agd97* fragment from pGM-Agd97

The *agd97* gene fragment from recombinant plasmid pGM-Agd97, was subsequently cloned into pET17b vector. The recombinant plasmid was transformed into *E. coli* DH5α by electroporation. *E. coli* transformants were randomly picked for plasmid extraction and digestion with *Nde*I-*Xho*I. The confirmation by restriction patterns indicated that *agd97* gene fragment was successfully cloned into recombinant pET-Agd97. The result is shown in Figure 3.16

The recombinant plasmids were confirmed to consist of *agd97* gene by DNA sequencing. The sequencing result *agd97* gene indicated the same in nucleotide sequence of recombinant pGM-Agd97.

The recombinant pET-Agd97 was transformed into different expression hosts (*E. coli* BL21 (DE3), BL21 (pLysS), and Rosetta) by electroporation. The transformant were selected on LB agar with ampicillin or chloramphenicol (for *E. coli* Rosetta) containing 0, 0.5 and 1% glucose. Extracted plasmid from all transformants was digested with *Nde*I–*Xho*I. Figure 3.17 shows result of recombinant plasmid without *agd97* gene fragment.

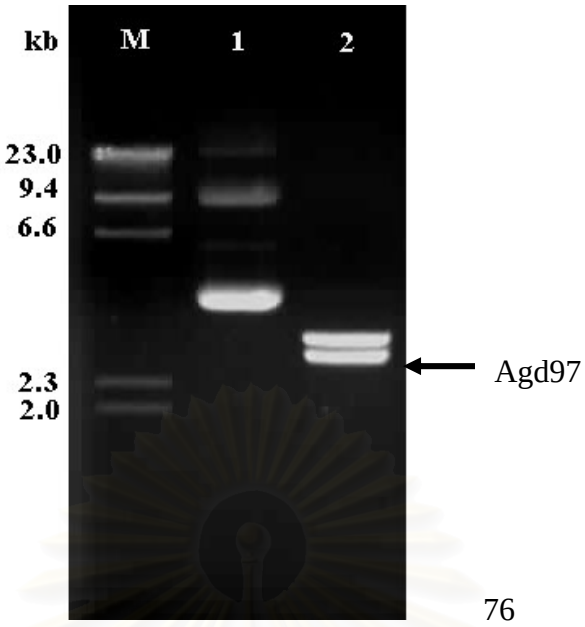


Figure 3.16 : The recombinant plasmid pET-Agd 97 into *E. coli* DH5α

The recombinat pET-Agd97 in *E. coli* DH5α was digested with *Nde*I-*Xho*I.

Lane M : λ/*Hin*dIII standard DNA marker

Lane 1 : undigested plasmid.

Lane 2 : *Nde*I-*Xho*I digested plasmid

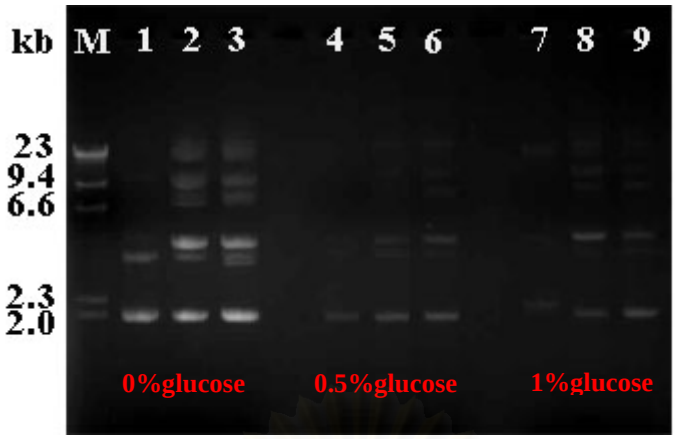


Figure 3.17 : The recombinant plasmid pET-Agd 97 into *E. coli* BL21(DE3), BL21 (pLysS), and Rosetta

The recombinat pET-Agd97 in *E. coli* DH5α were retransformed into *E. coli* expression host by selected on / in LB-ampicillin containing 0, 0.5 and 1% glucose and were digested with *Nde*I-*Xho*I.

Lane M : λ/*Hin*dIII standard DNA marker

Lane 1,4,7 : recombinant plasmid into *E. coli* BL21(DE3)

Lane 2,5,8 : recombinant plasmid into *E. coli* BL21(pLysS).

Lane 3,6,9 : recombinant plasmid into *E. coli* Rosetta.

3.4.2 Cloning of *chiA* gene

chiA gene from SK-1 with introduced ribosome binding site of pET-17b fragment for cloning and expression under pET system. It were obtained through PCR using SK-1 genomic DNA as the template and primer pairs were designed base on nucleotide sequences of *chiA* from SK-1 with introduced RBS of pET17b (ChiA_RbsF+ChiA_R). The PCR reaction was done at annealing temperature at 65^{o}C for 30 sec. DNA fragment was 1951 bp in length was showed in Figure 3.18.

PCR product of *chiA* gene (RBS of pET17b) fragment was blunt end ligated into pETAgd97 vector at *Xba*I restriction site upstream of the *agd97* gene and transformed into *E. coli* TOP10 and BL21 (DE3). Restriction patterns of recombinant pETAgd97-ChiA revealed that *chiA* was actually insert into pETAgd97 but was in the opposite orientation to *chiA* gene (Figure 3.19). As a result, *chiA* gene could not be expressed in this recombinant vector as it was in the opposite direction when put under T7 promoter.

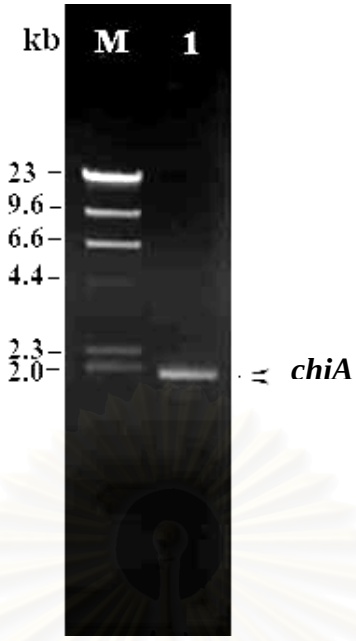


Figure 3.18: PCR product of *chiA* (RBS of pET17b) gene from *B. licheniformis* SK-1 genome on 0.8% agarose gel.

Lane M : λ/*Hin*dIII standard DNA marker

Lane 1 : PCR product as indicated

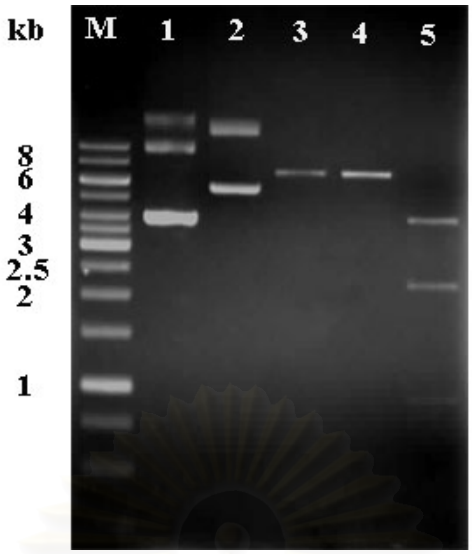


Figure 3.19: Restriction pattern of pETAgd97-ChiA from selected recombinant clones

Lane M : 10 kb DNA ladder
Lane 1 : undigested plasmid without *chiA* gene
Lane 2 : undigested plasmid pETAgd97-ChiA
Lane 3 : *Eco*RI (cut *chiA*) digested plasmid pETAgd97-ChiA
Lane 4 : *Xho*I (cut *agd97*) digested plasmid pETAgd97-ChiA
Lane 5 : *Eco*RI-*Sal*I (cut *agd97* and *chiA*) digested plasmid pETAgd97-ChiA

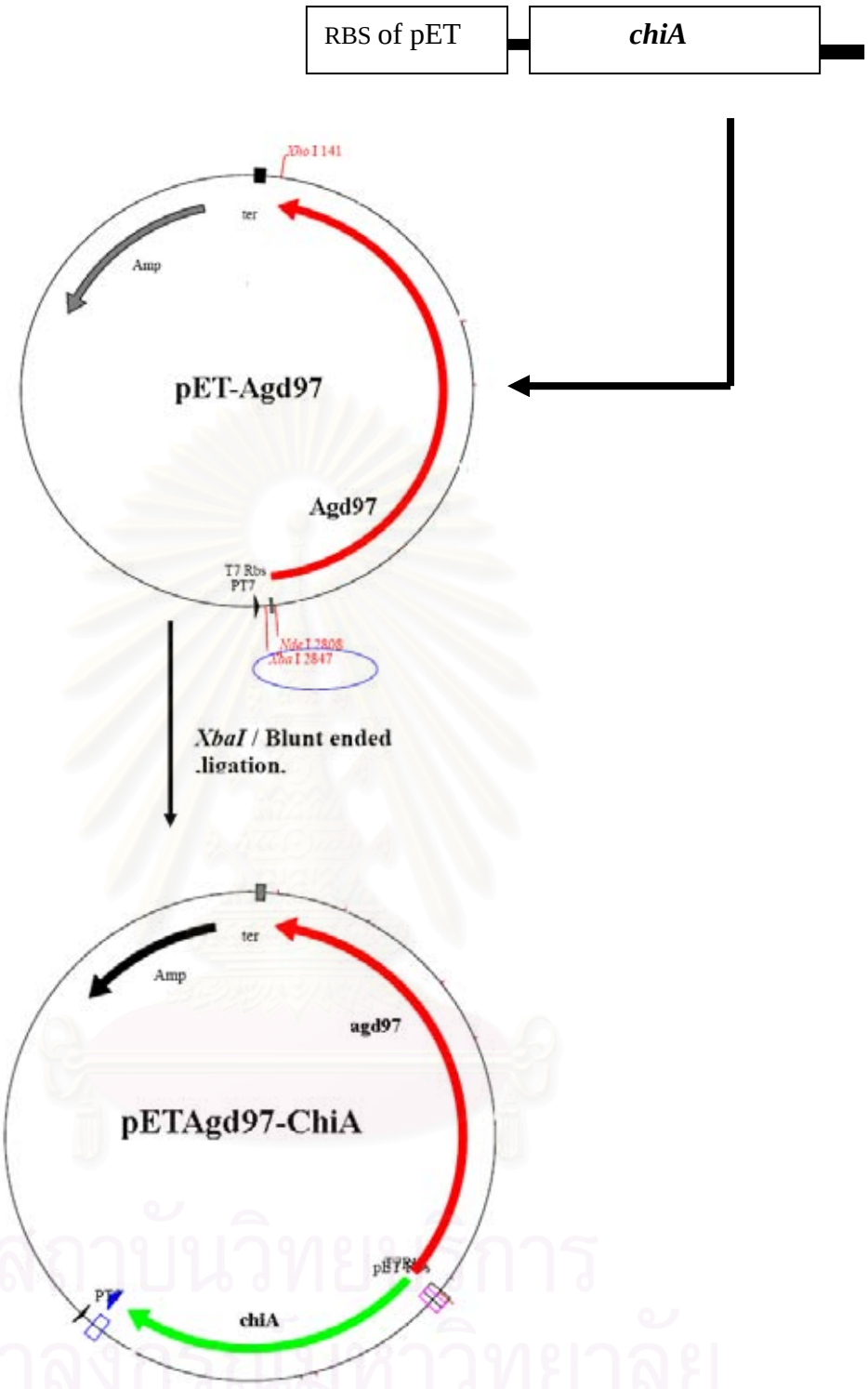


Figure 3.20: Construction of recombinant pETAgd97-ChiA

3.4.3 Expression of the recombinant *agd97* gene from *E. coli* BL21 (DE3)

To express the recombinant agd97 in *E. coli*, the expression plasmid pETAgd97-ChiA was previously constructed so that *agd97* gene was under the control of T7 promoter. The *E. coli* BL21 (DE3) / pETAdg97-ChiA was grown in LB medium was induced by IPTG at each of following concentration of 0, 0.1, 0.2, 0.6 and 1 mM. The culture from each IPTG concentration added was sampled at 0, 3, 6, 9, 12, 15, 18, 21 and 24 hours after the induction at 37^{o}C aerobic condition. Culture broth were harvested and assayed for Agd97 activity following the standard method. The highest activity was observed at 6 hours after induction using 0.2 mM IPTG. The Agd97 activity of *A. caviae*D6, pBSK60-Agd97 and pET-Agd97 was shown in Table 3.2

When the recombinant clone was cultured without IPTG induction, the activity of Agd97 not detected all through cultivation.

Ten micrograms protein from culture broth was separated on 10% SDS-polyacrylamide gel. The results in Figure 3.21 showed that the intensity of a major protein band of about 97 kDa in culture broth from each harvested time corresponded to the level of Agd97 activity.

Table 3.2 : The Agd97 activity from clone and native D6

	Agd97 activity (Unit/ml)
A. caviae(D6)	0.7
pBSK60-Agd97	0.207
pETAgd97-ChiA	15.656

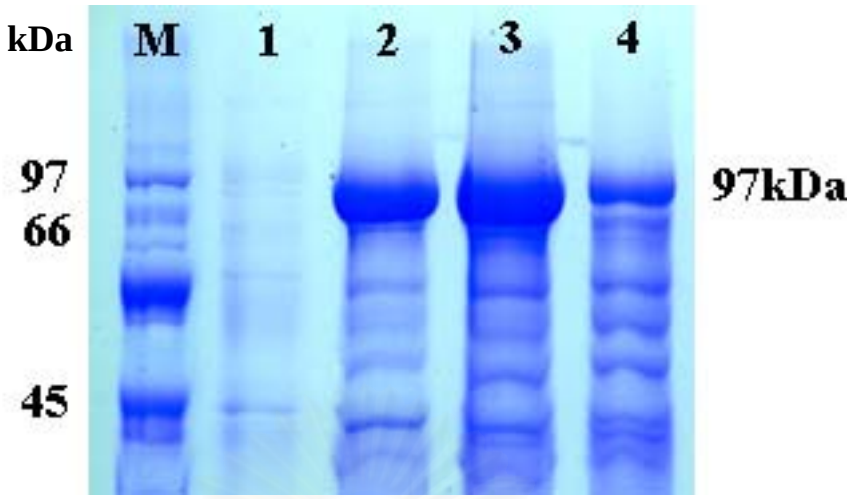


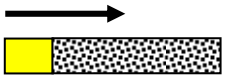
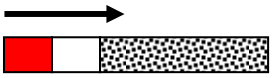
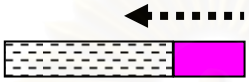
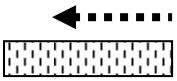
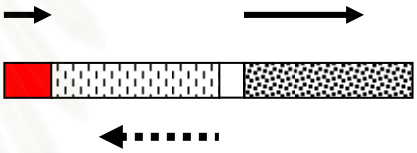
Figure 3.21 : SDS-PAGE of culture broth crude enzyme of the *E. coli* BL21 (DE3) transformant containing pETAgd97-ChiA induced by 0.2 mM IPTG at various times

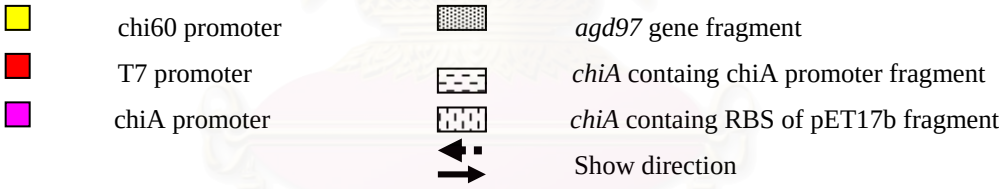
Lane M : protein MW marker

Lane 1 : crude proteins of *E. coli* BL21 (DE3) transformant harboring pETAgd97-ChiA non-induced

Lane 2-4 : crude proteins of *E. coli* BL21 (DE3) transformant harboring pETAgd97-ChiA at 3, 6 and 9 hours after induction, respectively

Table 3.3 :Construction of heterologus *chiA* and *agd97* genes into pBSK60 and pET-17b

Gene fragment	pBSK60	pET-17b
agd97		
chiA (Endogenous promoter)		
chiA(SD of pET17b)		
chiA with *agd97*		



3.5 Purification and characterization of Agd97 from *E. coli* BL21 (DE3)

The Agd97 crude enzyme was purified by a two-step procedure, using anion exchange (DEAE-cellulose) followed by gel filtration (sephadex-G100).

Crude enzyme was prepared from 2 liters of culture broth by reducing the total volume of culture broth to 10 ml, The concentrated culture broth exhibited 10.6989 mg protein, 21,487 unit of Agd97 activity. This made the specific activity of Agd97 at with 2,008 unit/mg protein.

The anion-exchange chromatography yielded four protein peaks. The unbound proteins were eluted from the column by 0.01 M Tris-HCl, pH 7.0. The bound proteins were then eluted with a linear gradient of 0-0.5 M NaCl in the same buffer. The specific peak with Agd97 activity was the second one, which was eluted at 0.1 M NaCl as indicated in the profile **(**Fig 3.22**).**

When gel filtration chromatography was performed, five different peaks of protein were present Agd 97 activity was observed in the first one. **(**Fig3.23**).**

The summary of purification of Agd97 was shown in Table 4.4 The purification of the Agd97 was 2.48-fold, with overall yield of 1.4%. The final amounts of Agd97 obtained were 0.06 mg. The specific activity of this Agd97 was 4,989.87 U/mg of protein.

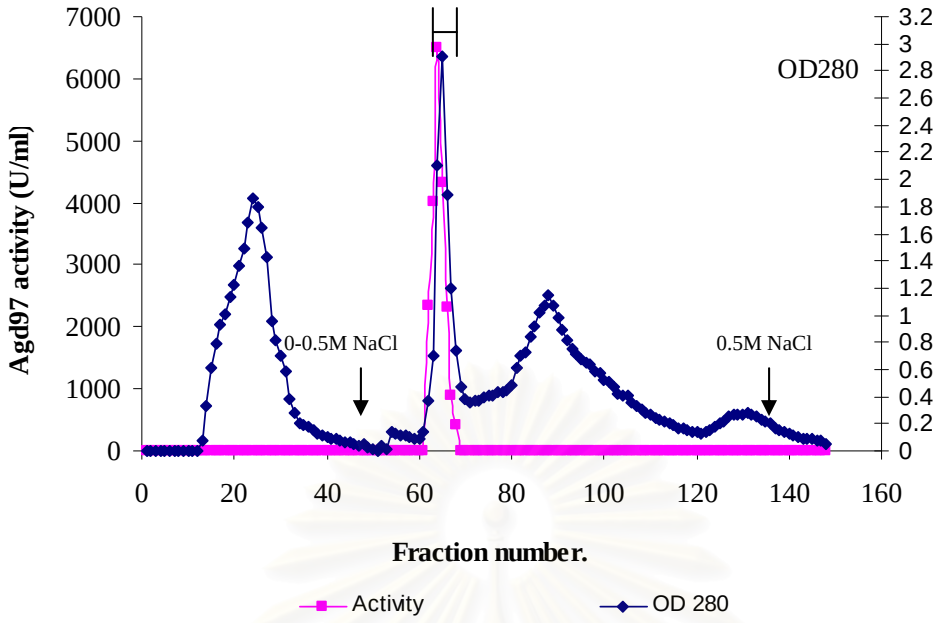


Figure 3.22 : Purification profile of Agd97 from *E. coli* BL21(DE3) transformant by DEAE-cellulose column chromatography.

The enzyme was applied to DEAE-cellulose column and washed with 10 mM Tris-HCl buffer, pH 7 containing 10% glycerol and 1% 2-mercaptoethanol until the absorbance at 280 mm of eluent decreased to base line. The bound protein elution was made by a linear gradient of 0-0.5 M NaCl in the same buffer at a flow rate of 1 ml/min. Fractions of 3 ml each were collected. The arrow indicated where each gradient was started. Active proteins with activity peaks from the fraction number 62-68 were pooled as indicated by (⊢⊣)

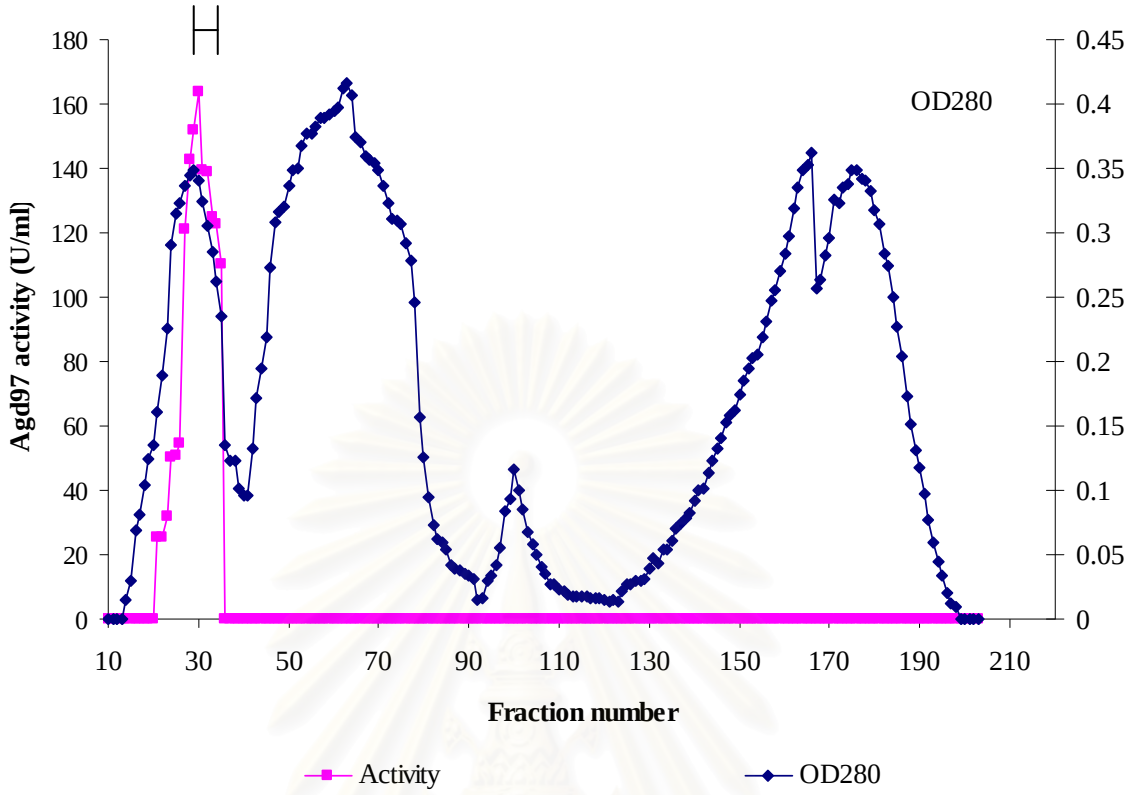


Figure 3.23 : Purification profile of Agd97 from the *E. coli* BL21 (DE3) transformant by Sephadex-G100 column chromatography.

The enzyme was applied to Sephadex-G100 column and washed with 10 mM Tris-HCl buffer, pH 7 containing 0.1M NaCl until the absorbance at 280 mm of eluent decreased to base line at a flow rate of 0.2 ml/min. Fractions of 2 ml each were collected. The unbound protein was active protein. Active proteins with activity peaks from the fraction number 29-30 were pooled as indicated by (⊢)

Table. 3.4 :Purification of Agd97 from *E. coli* BL21(DE3) recombinant clone

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	% Recovery
crude enzyme	10.6989	21487	2008	1	100
DEAE-cellulose	3.9546	6550.6296	1656.4582	0.8249	30.4864
Sephadex-G100	0.0632	315.36	4989.8734	2.4849	1.4676

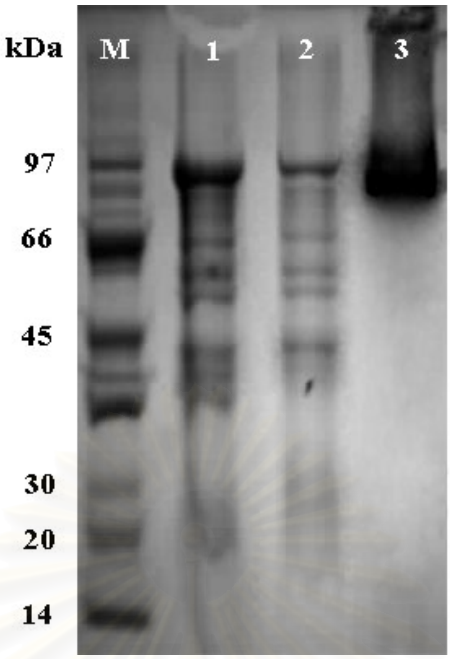


Figure 3.24 : SDS-PAGE analysis of purification fraction of the recombinant Agd97.

Proteins were stained with coomassie brilliant blue.

Lane M : Protein MW marker (molecular weights indicated in kDa)

Lane 1 : Crude protein solubilized Agd97

Lane 2 : Protein in the active peak eluted from DEAE-cellulose column

Lane 3 : The purified Agd97 eluted from Sephadex G-100 column

3.5.1 Electrophoresis and molecular weight determination of the purified enzyme

The molecular weight of the enzyme was calculated on the basis of semi-logarithmic plots of the mobility of the bands on SDS-PAGE, using a standard curve established with protein of known molecular weight. The purified Agd97 migrated as a single band on a SDS-PAGE with estimated molecular weight of 97 kDa.**(**Fig 3.25**).**

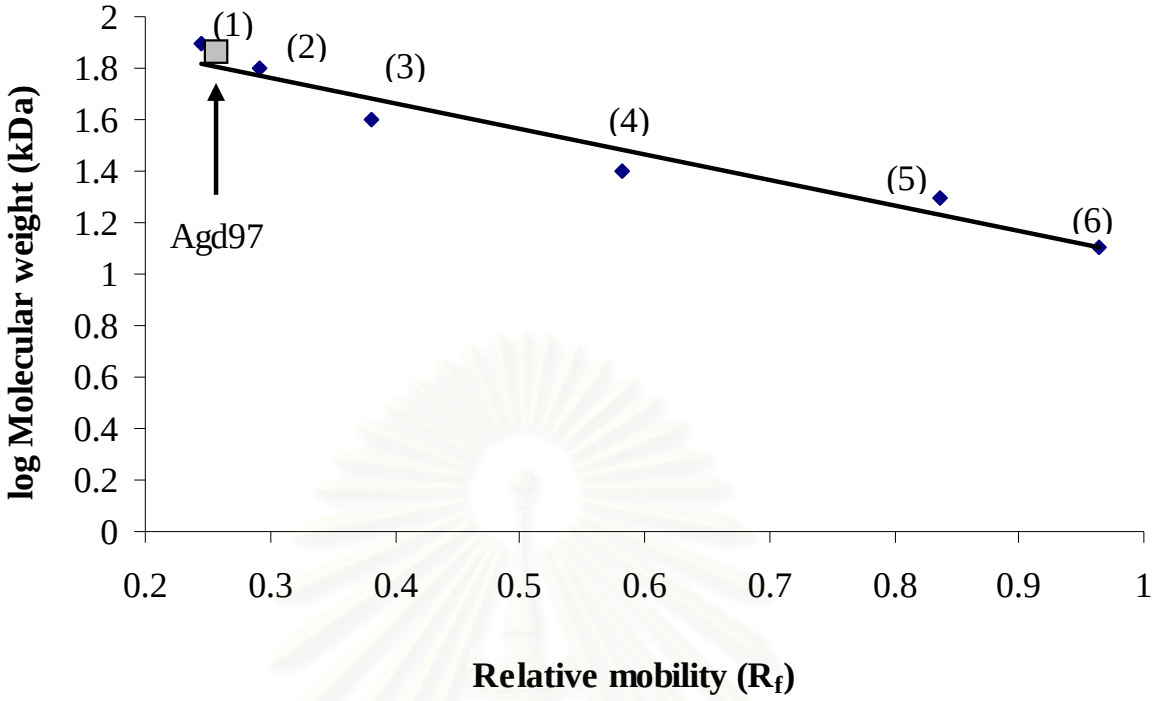


Figure 3.25 : Molecular weight calibration curve of standard proteins by 10% SDS-PAGE for molecular weight determination of Agd97 from *E. coli* BL21 (DE3) / pETAgd97-ChiA

(1)	Phosphorylase b	97 kDa
(2)	Albumin	66 kDa
(3)	Ovalbumin	45 kDa
(4)	Carbonic anhydrase	30 kDa
(5)	Trypsin inhibitor	20 kDa
(6)	α-Lactalbumin	14 kDa

3.5.2 Enzyme characterization

The recombinant Agd97 purified from *E. coli* Bl21 (DE3) harboring pETAgd97-ChiA was used for several biochemical analyses as follows. Firstly, the effect of pH on activity of purified Agd97 were examined at 37^{o}C using pNP(GlcNAc) as the substrate; The optimum pH of the enzyme activity was at pH 6 (phosphate buffer) (Fig 3.26).

Secondly, the effect of pH on the stability of the purified enzyme was examined after incubation for 3 day at 4^{o}C in various buffer of pH 3 to10.The enzyme was active from pH 6 to 10 by maintaining at least ~98% activity. (Fig3.27).

Thirdly, enzyme activities at various temperature (25-60^{o}C) were analyzed with the same substrate, and the optimum temperature was found to be 45^{o}C for hydrolysis of *p*NP(GlcNAc) (Fig 3.28)**.** It was completely inactivated at 60^{o}C.

Fourthly, the thermal stability of the enzyme was shown in (Fig 3.29). After preincubation for 10 min at pH 7, the enzyme was stable below 40^{o}C but was completely inactivated at 50^{o}C.

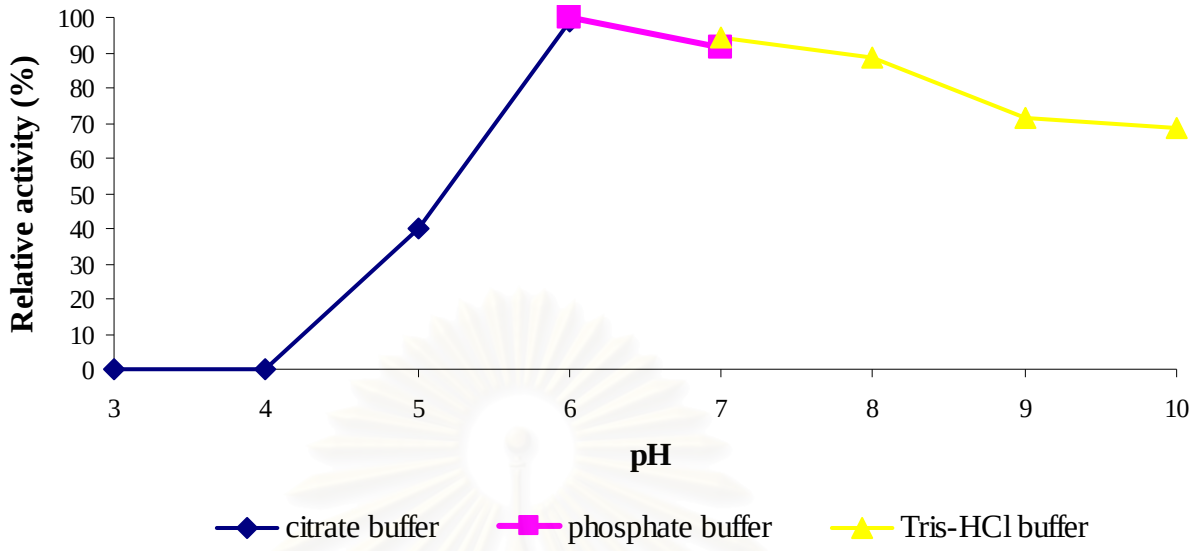


Figure 3.26 : Effect of pH on Agd97 activity

The reactions were assayed followed by the standard enzyme assayed condition in each various pHs at 37^{o}C for 30 minutes. Relative activity of various pHs were compared with the maximum activity (100%)

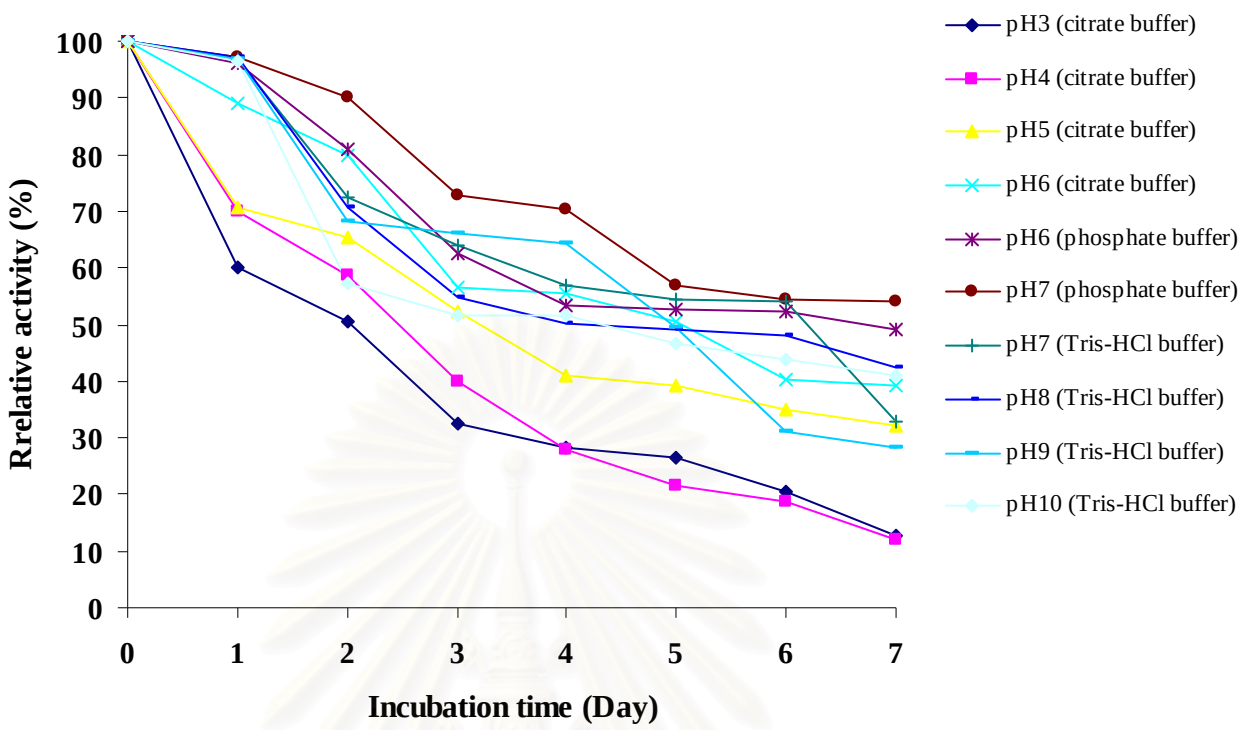


Figure 3.27 : Effect of pH on Agd97 stability

The purified enzyme solution was kept for 3 day at 4^{o}C in 0.02M buffer of various pH values (3-10) and the remaining activies were assayed under the standard condition, pH 3-6, citrate buffer; pH 6-7 phosphate buffer; pH 7-10, Tris-HCl buffer.

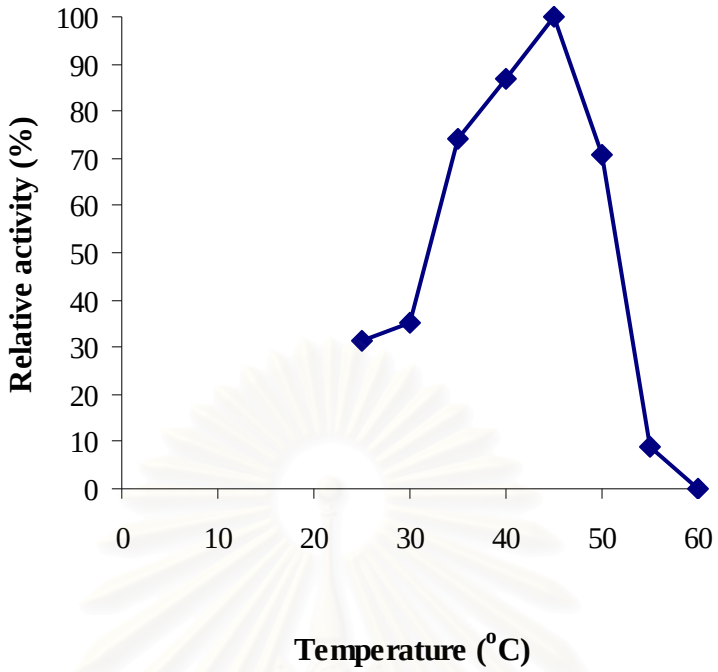


Figure 3.28 : Effect of temperature on the Agd97 activity.

The activity of the purified Agd97 was measured by spectrophotometer with 2.5 mM *p*-nitrophenyl GlcNAc as a substrate at pH 6.0 and various temperatures 25-60°C

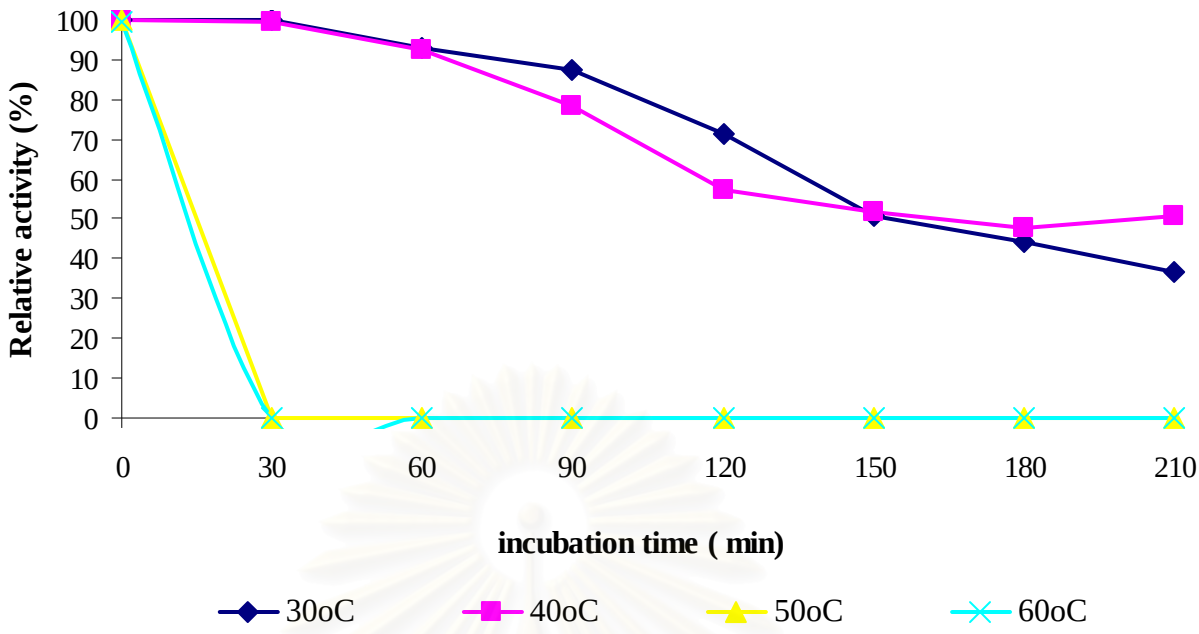


Figure 3.29: Effect of temperature on the Agd97 stability

The purified enzyme solution was kept at various temperature (30-60^{o}C) in 0.02 M Tris-HCl buffer (pH7) and the remaining activity for various time were assayed under standard condition.

3.5.3 Product hydrolytic produced by purified Agd97 from *E. coli* BL21 (DE3)

The hydrolysates of *N*-acetylchitooligomers $(GlcNAc)_n$, β-chitin) by combination with another chitinase (ChiA from *B. licheniformis*) that able to release *N*-acetylchitobiose. The reaction was done at 37^{o}C overnight with various units of purified Agd97 (100, 200, 300 and 400 mU/ml) and was analyzed by thin-layer chromatography after the specific time course (Figure 3.30). The substrates from hydrolysis of ChiA (*N*-acetylchitobiose and GlcNAc) were all hydrolyzed and GlcNAc was produced as a final product.

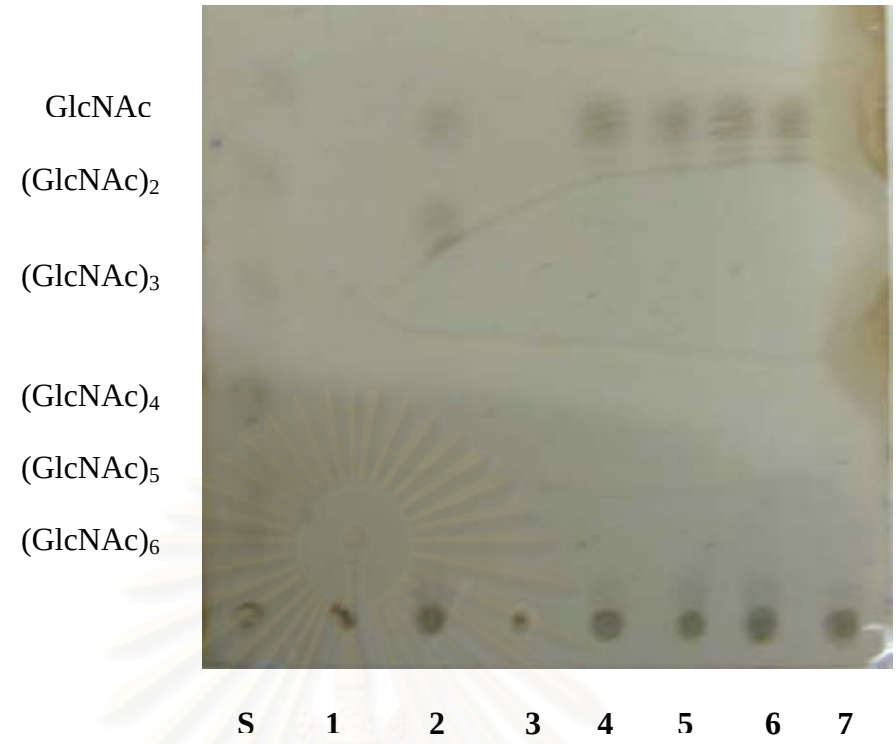


Figure 3.30 : Analysis of enzymatic hydrolysates by TLC

The reaction mixture contained two enzyme of purified Agd97 and ChiA by various unit of purified Agd97.

The reaction were done at 37^{o}C overnight using β -chitin as Substrate.

Lane S contained a mixture of *N*-acetylchitooligomers ranging from GlcNAc to *N*-acetylchitohexose, $(GlcNAc)_6$.

Lane 1 The production of β -chitin without enzyme

Lane 2 The production of β -chitin with enzyme ChiA(100 mU)

Lane 3 The production of β -chitin with enzyme Agd97

Lane 4-7 The production of combination of Agd97 with ChiA (units Agd97 were 100, 200, 300 and 400 mU, respectively) using β-chitin as substrate.

CHAPTER IV

DISCUSSION

The enzymatic degradation from chitin polymer to *N*-acetylglucosamine (GlcNAc) is a chitinolytic system, which has been found in microorganisms, plants and animals. The chitinolytic enzymes are generally induced as a multi-enzyme complex and are traditionally divided into two main classes: endo-chitinase and β-*N*-acetylglucosaminidase Inbar and Chet, 1991 demonstrated that *A. caviae* has high level of chitinolytic activity with impressive β-*N*-acetylglucosaminidase activity. The chitinase enzyme from *B. lichenniformis* (SK-1) showed the high ability to produce chitobiose from chitin digestion (Kudan, 2006). In this study, we cloned β-*N*-acetylglucosaminidase (*agd97*) from *A. caviae* (D6) and Chitinase (*chiA*) from *B. lichenniformis* (SK-1) in order to obtain the combination of these two chitinolytic enzyme, which are essential for the complete hydrolysis of chitin to GlcNAc in one step.

4.1 Identification of *A. caviae*

A. caviae and *B. lichenniformis* was previously isolated and identified as a chitinolytic enzyme producing organism in our laboratory. *A. caviae* isolated from soil in Nakhon Pathom province and was initially identified by biochemical test. The other identification method was 16S rRNA. The principle of using rRNA sequences to characterize micro-organisms has gained wide acceptance (Johnson *et al* and Murray *et al*., 1984, Clarridge, 2004). The approach described here rapidly provides complete, unambiguous and contiguous sequence determination of 16S rRNA. The analysis of nucleic acid sequences coding for 16S rRNA is particular useful for phylogenetic analysis (Lane *et al*., 1985, Paster *et al*., 1988 and Jonhson *et al*., 1989, Tortoli, 2003, Clarridge, 2004) and for characterization of an organism of uncertain affiliation. Identification based on the 16S rDNA sequence is of interest because ribosomal small subunit exists universally among bacteria and includes regions with species-specific variability, which makes it possible to identify bacteria to the genus or species level by comparison with databases in the public domain (Vandamme *et al.*, 1996).

In this research, identification of this bacterium was confirmed by 16S rRNA gene sequence. DNA sequence analysis by using BLAST program, the 16S rRNA gene of D6 showed 100 % identity to 16S rRNA gene of *A. caviae*. (Genblank accession no. X60408.1). Identification results by 16S rRNA and biochemical properties were indicated bacterium stain D6 is *A. caviae*.

4.2 *agd97* gene

β-*N*-acetylglucosaminidase (EC3.2.1.30) is required by chitinolytic bacteria together with chitinase to digest chitin to β-*N*-acetylglucosaminidase to be used as carbon and nitrogen source (Cohen, Chet ., 1998 and Keyhami, Roseman, 1999). In this thesis, we report the isolation of gene coding for β-*N*-acetylglucosaminidase (*agd97*) and Chitinase (*chiA*), expression. Purification and characterization of *agd97* gene product (pETAgd97-ChiA) was performed in *E. coli*.

PCR method is used for *in vitro* synthesis the interesting gene or gene fragment. The method requires a pair of primers which is specific to the target gene. In this experiment, a pair of primer was designed according to the nucleotide sequence from *A. punctata* CB101 *agd97* gene database. The PCR result (Fig 3.4) shows that expected size of PCR product was produced, suggesting that primers were specific to band *agd97* gene from *A. punctata*. Sequencing result of the nucleotide sequences of the PCR product (2,664 bp) from *agd97* was showed 96% similarity to *agd97* of *A. puntata*.

The *agd97* ORF encodes a polypeptide of 888 amino acid residues with a calculated molecular mass of 97 kDa. The 22 amino acid residues N terminal were predicted to be a signal peptide with high fidelity when analyzed using SignalP software (http://www.cbs.dtu.dk/services/SignalP). The existence of a signal peptide in a precursor peptide of Agd97 suggests that Agd97 can be exported across the cellular membrane. This finding may indicate the localization of Agd97 in the outer membrane (secreted in culture broth). In contrast to the membrane–associated β-*N*-acetylglucosaminidase, which has been reported from *Altermonas sp*. 0-7 (Tsujibo *et al*., 2000) and *Porohyromonas gingivalis* (Lovatt and Roberts, 1994). Whose enzymes

possessed a lipoprotein signal sequence that restricted the protein to the cell membrane.

The deduced amino acid sequence of the *agd97* from *A. caviae* (D6) was compared with those of the β-GlcNAcase. The Agd97 sequence showed similarity to those of *A. puncta* (96% identity), *A. hydrophila* (95% identity), *Vibrio parahaemolyticus* (54% identity), *Yersinia pseudotuberculosis,* (52% identity) and *Arthrobacter sp*. (15% identity). The amino acid sequence of Agd97 from *A. caviae*. showed high similarity to those of β-GlcNAcase from gram-negative bacteria. These bacterial Agd97 belong to family 20 of glycosyl hydrolases (BLASTP). Among the primary sequence of β-GlcNAcase, three-dimensional structure was reported only for β-*N*-acetylglucosaminidase (formerly chitobiase) from *S. marcescens*. Tews *et al*.(1996) reported that β-*N*-acetylglucosaminidase of *S. marcescens* uses an acid-base reaction mechanism with glutamic acid 540 as the catalytic amino acid as the catalytic amino acid (Tews *et al*., 1996). This residue is well conserved in all members of family 20 of glycosyl hydrolases.

4.3 Expression of pETAgd97-ChiA under pET system

Expression systems used for *agd 97* in this study were pET and pBSSK$^-$ Chi60 promoter vector. Expression systems of pET were designed to produce many copies of a desired protein within a host cell. In order to accomplish this, the expression vector containing all of the genetic coding necessary to produce the protein, including an appropriate promoter to the host cell, a sequence which terminates transcription, and a sequence which codes for ribosome binding was inserted into a host cell. The pET expression system was developed in 1986 (Studier and Moffatt, 1986). It is widely used because of its ability to mass-produce proteins, the specificity involved in the T7 promoter which only binds T7 RNA polymerase, and also the design of the system which allows for the easy manipulation of how much of the desired protein is expressed and when that expression occurs. Therefore, pET-17b vector (Appendix D) was selected to express *agd97* gene. To insert *agd97* gene fragments into pET-17b at the right position, the gene fragment was subcloned from pGM-Agd97 with the 5’ end containing *NdeI* restriction site and 3’ end containing *Xho*I restriction site.

The pET expression system, pET vector contain the highly efficient ribosome binding site from the phage T7 major capsid protein and are used for the expression of target genes without their own ribosome binding site (Novagen 2003). *chiA* gene was expressed under pET-17b system containing ribosome biding site of pET-17b.

chiA was cloned in pETAgd97 vector at upstream of *agd97*. The transcription of *agd97* and *chiA* used T7 promoter. The *agd97* was success to clone in pET-17b vector in cloning host *E. coli* DH5α. However, when the pETAgd97 vector was transformed to expression host (*E. coli* BL21 (DE3), BL21 (pLysS), and Rosetta), the vector can not maintain the inserted DNA fragment. The restriction fragment length of pETAgd97 extracted from the expression hosts are different from pETAgd97 extracted from the cloning host. The Agd97 may be toxic to the *E. coli* cell so the host cells may rearrange the inserted Agd97 such that the expression could not occur. Surprisingly, the pETAgd97-ChiA vector containing *agd97* and *chiA* gene could be maintained in the cloning host and the three expression hosts. It would be possible to suggest that the expression level of *agd97* in pETAgd97-ChiA vector was less than that in pETAgd97 vector, which contained *agd97* gene alone. The distance between the T7 promoter and *agd97* may have caused the significant reduced level of expressed *agd97*, which made the expressed protein less toxic to the host cells.

E. coli strain containing a chromosomal copy of the gene for T7 RNA polymerase, *E. coli* BL21 (DE3) was used for host expression because this strain has gene encoding bacteriophage T7 RNA polymerase, which is integrated into the chromosome of BL21. Since the gene fragment did not have their own promoter, they were expressed under T7/*lac* promoter on the pET-17b vector. In the pET system, T7 polymerase was under the control of *lac*UV5 promoter, and the plasmid vector equipped with a bacteriophage T7/*lac* promoter upstream of the gene. Both promoters contain the *lac* operator (*lac*O) in such position that binds to the repressor which results in the transcription of T7 RNA polymerase. Therefore, the target gene in the vector was transcribed by adding IPTG. A final concentration of 1 mM IPTG is recommended for full induction with vector having T7/*lac* promoter (Novagen 2003). Final IPTG concentration should be optimized because of its great contribution to recombinant protein expression and potential harm to cell growth (Lu and Mei, 2007).

In this study, IPTG was added to final concentration of 0.1, 0.2, 0.6 and 1 mM, respectively. The highest Agd97 activity of pETAgd97-ChiA was obtained when induced with 0.2 mM at 6 hours after induction. At high IPTG concentrations Agd97 activity were decreased because high IPTG concentration may be harm to cell growth resulting in gene expression inhibition (Zhong *et al*., 2006).

4.4 Expression of pBSK60-Agd97

As the pET expression system has relatively stronger promoter (T7/*lac* promoter), than that in pBSSK$^-$ chi60 promoter expression systems therefore the expression of *agd97* gene under pBSSK$^-$ chi60 promoter (Fig 3.12) was much less than the T7/lac. As a result expressed *agd97* gene could be maintained in pBSK60-Agd97 vector.

The medium volumn is an important parameter reflection the dissolved oxygen level in flask, and can influence the metabolic process of the cell and hence influence the target proteins expression (Lu and Mei, 2007). To study the effect of medium volumn on the enzyme expression, cultivation was carried out in a 250-ml flasks with various LB medium volume (25, 50, 75, 100 and 125 ml). From the activity result, the highest Agd97 activity was obtained when the medium volume was 100 ml. This result indicated that the optimal culture volumn was 100 ml in which dissolved oxygene was sufficient for broth cell growth and expression protein. It was also seen that the 25 ml medium volume could not improve but even reduced the expression level. One possible reason was that the fermentation broth would become very thick due to water evaporation when very little media were in the flask, which might bring about some inhibition effects on cell growth and protein expression (Zhong *et al*., 2006). It is indicated that gene expression in pBSK60-Agd97 system required low amount of dissolved oxygen.

Chitinolytic enzyme mainly show two types: endo-type chitinase (EC 3.2.1.14) and exo-type β-*N*- acetylglucosaminidase (NAG) (EC 3.2.1.52). First, chitinase hydrolyzes a chitin polymer into small oligosaccharides mainly composed of a diacetylchitobiose, $(GlcNAc)_2$. Successively, NAG hydrolyzes the resulting oligosaccharides into a monosaccharide, GlcNAc (Xiqan *et al*., 2008). In this study,

GlcNAc were produced as final product from hydrolysis of chitin by combination of Agd97 with chitinase Moreover, the resulting *N*-acetylchitobiose produced from another chitinase (Chi60 from *Serratia marcescens*, ChiA from *Bacillus licheniformis*) changed successively to GlcNAc. These results clearly indicate an exo-type cleavage of the Agd97 enzyme, releaseing GlcNAc residue.

4.5 Purification and characterization of cloned Agd97

The Agd97 culture broth from *E. coli* BL21 (DE3)/pETAgd97-ChiA was purified by two-step procedure, using anion exchange follow by gel filtration.

The development of techniques and methods for the separation and purification of proteins has been an essential pre-requisite for many of the recent advancements in bioscience and biotechnology research. The global aim of a protein purification process is not only the removal of unwanted contaminants, but also the concentration of the desired protein and its transfer to an environment where it is stable and in a form ready for the intened application (Queiroz *et al.,* 2001).

The principal properties of enzymes that can be exploited in separation methods are size, charge, solubility, density and the possession of specific binding sites. Most purification protocols required more than one step to achieve the desired level of product purity. This includes any conditioning steps necessary to transfer the product from one technique into conditions suitable to perform the next technique. Each step in the process will cause some loss of product. Consequently, the key to successful and efficient protein purification is to select the most appropriately techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required (Amersham pharmacia biotech, 1999).

Since the disruption of cells results in the release of proteases from subcellular compartment (Cooper, 1977), the effect of the free proteases must be eliminated. In this work, a reagent containing a thiol group such as β-mercaptoethanol in the buffer will minimize the oxidation damage (Bollag *et al.,* 1996).

The purification of the Agd97 was 3 fold, with over all yields of 4.81 %. The specific activity of this Agd97 was 4989.87 U/ml.

The molecular weight (97 kDa) estimated for the Agd97 purified from BL21(DE3) is similar to that (92 kDa) estimate for enzyme from *Enterobacter sp*. G-1

(Matsuo *et al*., 2000), *Aeromonas hydrophila* (Xiqian *et al*., 2004). But smaller than the molecular weights of the enzyme from *Pycnoporus cinnabrinus* (Akira , 1981) and greater than that of the enzyme from *Stachybotrys elegans* (Greg *et al*., 2002). *Streptomyces thermoviolaceus* (Takahiro *et al*., 2004) and *Trichoderma harzianum* (Cirano *et al*., 2005).

This Agd97 was purified from culture broth by a comparatively simple procedure with high recovery of total activity. The enzyme showed high specific activites as compared with the purified preparations from *Aeromonas sp*. (Mitsuhiro *et al*., 2000), *Aeromonas hydrophila* (Xiqian *et al*., 2004) and *Streptomyces thermoviolaceus* (Takahiro *et al*., 2004).

The properties of purified Agd97 are optimum temperature and pH at 45^{o}C, pH 6 (phosphate buffer) and pH and temperature stability ranging 6-10, below 40^{o}C respectively. When compared the enzymatic properteies of the native and cloned Agd97, the optimum pH, pH stability and thermal stability shared the same characters, while optimum temperature showed slightly different. The native is 37^{o}C. Some properties of the purified Agd97 are similar to those of other enzyme: the optimum pH, temperature in the neutrual rang of the enzyme from Agd97 was similar to that of enzymes from *Enterobacter sp*.(Matsuo *et al*., 1999), *Streptomyces thermoviolaceus* (Takahiro *et al*., 2004). While many other enzymes show pH optimum in acid range (Kenji *et al*., 1985) and the enzyme was stable up to about 45^{o}C (Lisboa *et al*., 2004).

CHAPTER V

CONCLUSIONS

The bacterium strain D6, isolated from soil samples, with the ability to produce a chitinolytic enzyme, β-*N*-acetylglucosaminidase, was identified from its 16S rRNA sequence as *A. caviae*.

In this study, the *agd97* gene, which encoded β-*N*-acetylglucosaminidase was isolated from *A. caviae* D6 genome. The sequencing result showed that the *agd97* gene was 2,664 bp in size, which encoded a polypeptide of 888 amino acids with predicted molecular weight of 97.68 kDa. The putative signal peptide of 22 amino acid residues was found at the N-terminal of Agd97.

The gene was cloned into pET-17b and pBSK60 vector for heterologous expression in *E. coli*. As a result, *agd97* from two recombinant clones, *E. coli* BL21 (DE3)/pETAgd97-ChiA and *E. coli* XL-1 blue/pBSK60-Agd97 was successful produced. The Agd97 from BL21 (DE3)/pETAgd97-ChiA was found to shorten the time needed for enzyme production from 5 days to only 6 hours. Moreover, the obtained Agd97 activity was 21 fold higher when compared with that from native host. However, Agd97 activity from XL-1 blue/pBSK60-Agd97 revealed weaker activity than that from native host. The advantage that was found was that the crude enzyme from this host was suitable for subsequent applications as much less polysaccharide was produced in the culture broth.

In addition, the properties of purified Agd97 were also studied. Agd97 was isolated and purified from *E. coli* BL21 (DE3)/pETAgd97-ChiA. The procedures involved column chromatography on DEAE-cellulose followed by sephadex G-100 gel filtration. Disk electrophoresis indicated that the purified enzyme was homogeneous. The molecular weight of Agd97 purified from molecular weight calibration curve by 10% SDS-PAGE was 97 kDa. The purification of the Agd97 was 3-fold, with overall yield of 4.81%. The specific activity of Agd97 was 4,989.87 U/mg of protein. The optimum pH and temperature of the Agd97 was 6 (in phosphate buffer) and 45^{o}C, respectively. The buffer pH within the range of 6 to 10 could maintain Agd97 activity and the enzyme was also stable at temperature below 40^{o}C.

The combined expression of *chiA* gene from SK-1 and gene from D6 was not successful in the same recombinant vector. Further study is needed in order to achieve the recombinant clone with co-expression of both genes, which will lead to the streamlined production of *N*-acetylglucosamine.

REFERENCES

Aiba, S. (1994). "Preparation of *N*-acetylchitooligosaccharides from lysozymic hydrolysates of partially *N*-acetylated chitosans." Carbohydr. Res. **267**: 297-306.

Akira Ohtakara, M. Y., Motoharu, M. and Takako, I. (1981). "Purification and characterization of *N*-aetylhexosaminidase from *Pycnoporus cinnabarinus*." Agric. Biol. Chem. **45**: 239-247.

Alam, M. M., Nikaidou, N., Tanaka, H., and Watanabe, T. (1995). "Cloning and sequencing of chic gene of *Bacillus circulans* WL-12 and relationship of its product to some other chitinases and chitinase like proteins. " J. Ferment. Bioeng. **80**: 454-461.

Aloise, P. A., Lumme, M., and Haynes, C. A. (1996). "β-*N*-acetyl D-glucosamine production from chitin waste using chitinase from *Serratia marcescens*. Ferment Bioeng J **80**: 454-461

Andersen, M. D., Jensen, A., Robertus, J. D., Leah, R., and Skriver, K. (1997). "Heterologous expression and characterization of wild-type and mutant forms of a 26 kDa endochitinase from *Hordeum vulgare* L. " Biochem. J. **322**: 815-822.

Biotech., A. P. (1999). Protein purification handbook. Sweden.

Bollag, D. M., Rozycki, M. D., and Edelstein, S. J. (1996). Protein Methods. 2nd ed. New York: Wiley-Liss.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal. Biochem. **72**: 248-254.

Brameld, K. A., and Goddard III, W. A. (1998a). "Substrate distortion to a boat conformation at subsite-1 is critical in the mechanism of family 18 chitinases." J. Am. Chem. Soc. **120**: 3571–3580.

Brameld, K. A., and Goddard III, W. A. (1998b). "The role of enzyme distortion in the single displacement mechanism of family 19 chitinases. " Proc. Natl. Acad. Sci. USA. **120**: 4276-4281.

Cabib, E. (1987). "The synthesis and degradation of chitin." Adv. Enzymol. Relat. Areas. Mol. Biol. **59**: 59-101.

Chernin, L. S., De la, Fuente, L., and Sobolev, V. (1997). "Molecular cloning structural analysis and expression in Escherichia coli of a chitinase gene from *Enterobacter agglomerans*." Appl. Environ. Microbiol. **63**: 834-839.

Clarridge, J. E. C., III. (2004). "Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious disease." Clinical Microbiol. Rev. **17**: 840–862.

Claus, D., and Berkeley, R. C. W. (1986). "Genus *Bacillus Cohn* **1872**." Bergey's Manual of Systematic Bacteriology, 2: 1105-1139.

Cohen-Kupiec, R., Chet I. (2006). "The molecular biology of chitin digestion." Enzyme and Microbial. Technology. **38**: 765-771.

Cooper, T. G. (1977). "The tools of biochemistry." New York: John Wiley & Sons. pp. 47-50.

Dahlquist, F. W., Borders, Jr., C. L., Jacobson, G., and Raftery, M. A. (1969). "The stereospecificity of human, hen, and papaya lysozymes." Biochemistry. **8**: 694-700.

Davies, G., and Henrissat, B. (1995). "Structures and mechanisms of glycosyl hydrolases." Structure. **3** (9): 853-859.

Deshpande, M. (1986). "Enzymatic degradation of chitin & its biological applications" J. Sci Ind. Res. **45**: 273-281.

Flach, J., Pilet, P. E., and Jolles, P. (1992). "What's new in chitinase research." Experimentia. **48**: 701-706.

Fuchs, R. L., Mcpherson, S. A., and Drahos, D. J. (1986). "Cloning of *Serratia marcescens* gene encoding chitinase." Appl. Environ. Microbiol. **51**: 504-509.

Fukamizo, T., Koga, D., and Goto, S. (1995). "Comparative biochemistry of chitinases anomeric form of the reaction products." Biosci. Biotech. Biochem. **59**: 311-313.

Gooday, G. W. (1990). "The ecology of chitin degradation." Adv. Microb. Ecol. **11**: 387-430.

Gooday, G. W. (1999). "Aggressive and defensive roles of chitinases." EXS. **87**: 157-169.

Gordon, R. E, Haynes, W. C., and Pang, C. H. (1973). The Genus *Bacillus* for Microbiology, Washington DC., U. S. Department of Agriculture.

Greg Taylor, J. H., Charest, P. M., and Khan, W. (2002). "Purification and

characterization of extracellular exochitinase, *N*- acetylhexosaminidase from the fungal mycoparasite Stachybotrys elegans." Candida Journal of Microbiology. **48**: 311-319.

H. K. and Meyers, S. P. (1995). "Preparation and characterization of chitin and chitosan." J. Aquatic. Food Prod. Techmol. **4**: 27-52.

Hart, P. J., Pfluger, H. D., Monzingo, A. F., Hoihi, T., Robertus, J. D. (1995). "The refined crystal structure of an endochitinase from *Hordeum vulgare* L. seeds at 1.8 Å resolution." J. Mol. Biol. **248**: 402-413.

Henrissat, B. (1991). "A classification of glycosyl hydrolases based on amino acid sequence similarities." Biochem. J. **280**: 309-316.

Henrissat, B., and Bairoch, A. (1993). "New families in the classification of glycosyl-hydrolases based on amino acid sequence similarities." Biochem. J. **293**: 781-788.

Henrissat, B., and Bairoch, A. (1996). "Updating the sequence-based classification of glycosyl hydrolases." Biochem. J. **31**: 695-696.

Hollis, T., Honda, Y., Fukamizo, T., Marcotte, E., Day, P. J., and Robertus, J.D. (1997). "Kinetic analysis of barley chitinase." Arch. Biochem. Biophys. **344**: 335-342.

Huang, C. J., and Chen, C. Y. (2005). "High level expression and characterization of two chitinases ChiCH and ChiCW of *Bacillus cereus* 28-9 in *Escherichia coli*." Biochem. Biophys. Res. Commun. **372**: 8-17.

Inbar, J., and Chet, I. (1991). "Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soli-borne plant pathogens by this bacterium." Soil Biochem. **23**: 973-978.

Iseli, B., Armand, S., Boller, T., Neuhaus, J. M.,Henrissat, B. (1996). "Plant chitinases use two different hydrolytic mechanisms." FEBS Lett. **382**: 186-188.

Itoh, Y., Kawase, T., Nikajdou, N., Fukada, H., Mitsutomi, M., Watanabe, T., and and Itoh, Y. (2002). "Functional analysis of the chitin binding domain of a family 19 chitinase from *Streptomyces griseus* HUT6037: substrate-binding affinity and cis-dominant increase of antifungal function." Biosci. Biotechnol. Biochem. **66**: 1084-1092.

Jian Lin, X. X., Xiang Zeng, Fengping Wang. (2006). "Expression, characterization

and mutagenesis of the gene encoding *N*-acetylglucosaminidase from *Aeromonas caviae* CB101." Enzyme Microb. Technol. **38**: 765-771.

Johnson, A. M., and Baverstock, P. R. (1989). "Rapid ribosomal RNA sequencing and the phylogenetic analysis of protists." Parasit. Today. **5:** 102-105.

Kadowaki, S., Saskiawan, I., Watanabe, J., Yamamoto, K., Bunno, M., Ichihara, Y., and Kumagai, H. (1997). "Transglycosylation activity of β-*N*-acetyl-hexosaminidase from *Penicillium oxalicum* and its application to synthesis of a drug carrier." J. Ferment. Bioeng. **83**: 341-345.

Kenji Yamamoto, K. M. L., and Hidehiko, K. (1985). "Purification and characterization of β-*N*-acetylhexosaminidase from *Penicillium oxalicum*." Biol. Chem. **49** (3): 611-619.

Keyhami, N. O., and Roseman, S. (1999). "Physiological aspects of chitin catabolism in marine bacteria." Biochim. Biophys. Acta. 1473: 108-122.

Kobayashi, S., Kashiwa, K., Kawasaki, T., and Shoda, S. (1991). "Novel method for polysaccharide synthesis using an enzyme: the first in vitro synthesis of cellulose via non-biosynthetic path utilizing cellulase as catalyst." J. Am. Chem. Soc. **113**: 3079-3084.

Kobayashi, S., Kashiwa, K., Kawasaki, T., and Shoda, S. (2005). "Highlight article the challenge of synthetic cellulose." J. Polym. Sci. Chem. Ed. **43**: 693-710.

Kobayashi, S., and Ohmae, M. (2006). "Enzymatic ring-opening polymerization of lactones by lipase catalyst: mechanistic aspects." Adv. Polym. Sci. **194**: 159-210.

Kobayashi, S., Sakamoto, J., and Kimura, S. (2001a). "In vitro synthesis of cellulose and related polysaccharides." Prog. Polym. Sci. **26**: 1525-1560.

Kobayashi, S., Shoda, S., and Uyama, H. (1995). "Enzymatic polymerization and oligomerization." Adv. Polym. Sci. **121**: 1-30.

Kobayashi, T., Otsuka, S., and Yugari, Y. (1979). "Effect of chitosan on serum and liver cholesterol levels in cholesterol-fed rats." Nutr. Rep. Int. **19**: 327-334.

Koga, D., Sasaki, Y., Uchiumi, Y., Hirai, N., Arakane, Y., and Nagamastsu, Y. (1997). "Purification and characterization of Bombyx mori chitinase." Insect Biochem. Mol. Biol. **27**: 759-767.

Kramer, K. J., and Koga, D. (1986). "Insect chitin: physical state, synthesis, degradation and metabolic regulation." Insect Biochem. **16**: 851-877.

Kudan, S. (2006). "Protein engineering of bacterial chitinase for *N*-acetylchitooligosaccharide production." Doctoral degree of Biotechnology. Faculty of Science, Chulalongkorn university.

Kuranda, M. J., and Robbins, P. W. (1991). "Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*." J. Biol. Chem. **266**: 19707-19758.

Kuroki, R., Weaver, L. H., and Matthews, B. W. (1995). "Structure-based design of a lysozyme with altered catalytic activity." Nature Struct. Biol. **2**: 1007-1011.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. and Pace, N. R. (1985). "Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses." Proc. Natl. Acad. Sci. **82** (20): 6955–6959.

Lisboa De Marco, J., Valadarres, I. M. C. and Felix, C. R. (2004). "Purification and characterization of an *N*-acetylglucosaminidase produced by *Trichoderma harzianum* which controls *Crinipellis perniciosa*." Appl. Microbiol. and Biotechnol. 64: 70-75.

Lovatt, A., and Roberts, I. S. (1994). "Cloning and expression in *Escherichia coli* of the *nahA* gene from *Porphyromonas gingivalis* indicates that *N*-acetylhexosaminidase is an outer-membrane-associated lipoprotein." Microbiology. **140**: 3399-3406.

Lowe, G., Sheppard, G., Sinnot, M. L., and Williams, A. (1967). "Lysozyme-catalyzed hydrolysis of some b-aryl di-*N*-acetylchitobiosides." Biochem. J. **104**: 893-899.

Makino, A., Kurosaki, K., Ohmae, M., and Kobayashi, S. (2006). "Chitinase-catalyzed synthesis of alternating *N*-deacetylated chitin: a chitin-chitosan hybrid polysaccharide." Biomacromolecules. **7**: 950-957.

Matsuo, Y., Masako K., PARK, J. K., Katsunori T., Tsuyoshi N., Makoto K., and Hideyuki, M. (1999). "Purification, characterization and gene analysis of *N* -acetylglucosaminidase from *Enterobacter* sp. G-1." Biosci. Biotechnol. Biochem., **63**: 1261-1268.

Minke, R., and Blackwell, J. (1978). "Structure of a-chitin." J. Molec. Biol. **120**: 167-181.

Mitsutomi, O., Isono, M., Uchiyama, A., Nikaidov, N., Ikegami, T., and Watanabe, T. (1998). "Chitosanase activity of the enzyme previously reported as β-1,3, α-1,4-glucanase from *Bacillus circulans* WL 12." Biosci. Biotechnol. Biochem. **62**: 2107-2114.

Mitsuhiro Ueda, Y. F., Takashi, K., and Motoo, A. (2000). "Cloning, nucleotide sequence and expression of the *N*-Acetylglucosaminidase gene from *Aeromonas* sp. NO. 10S-24." Bioscience and Bioengineeing. **89**: 164-169.

Miyashita, K., Fujii, T., and Sawada, Y. (1991). "Molecular cloning and characterization of chitinase gene from *Streptomyces lividans."* J. Microbiol. **137**: 2065-2072.

Monzingo, A. F., Marcotte, E. M., Hart, P. J., and Robertus, J. D. (1996). "Chitinase, chitosanases, and lysozymes can be divided into prokaryotic and eukaryotic families sharing a conserved core." Nature Struct. Biol. **3**: 133-140.

Murao, S., Kuwada, T., Itoh, H., Oyama, H., and Shin, T. (1992). "Purification and characterization of a novel type of chitinase from *Vibrio alginolyticus* TK-22." Biosci. Biotechnol. Biochem. **56**: 368-369.

Muzzarelli, R. A. A. (1977). Chitin. Pergamon. Oxford.

Muzzarelli, R. A. A. (ed) "Chitin enzymology." Eur. Chitin Soc, Grottammare. **2**: 581-594.

Muzzarelli, R. A., Mattioli-Balmonte, M., Pugnaloni, A., and Biagini, G. (1999). "Biochemistry, histology and clinical uses of chitins and chitosans in wound healing." In Chitin and Chitosan. 251-264.

Nanjo, F., Sakai, K., Ishikawa, M., Isobe, K., and Usui, T. (1989). "Properties and transglycosylation reaction of a chitinase from *Nocardia orientalis*." Agric. Biol. Chem. **53**: 2189-2195.

Novagen (2003). pET System Manual. United States.

Okazaki, K., Kato, F., Watanabe, N., Yasuda, S., Masui, Y., and Hayakawa, S. (1995). "Purification and properties of two chitinases from *Streptomyces* sp. J-13-3." Biosci. Biotechnol. Biochem. **59**: 1586-1587.

Paster, B. J. a. Dewhirst, F. E. (1988). "Phylogeny of campylobacter, wolinellas, *Bacteroides garcilis* and *Bacteroides ureolyticus* by 16S ribosomal ribonucleic acid sequencing." Int. Syst. Bacteriol. **38**: 56-62.

Patil, S. R., Ghormade, V., and Deshpande, M. V. (2000). "Chitinolytic enzymes: an

exploration." Enzyme Microb. Technol. **26**: 473-483.

Cirano, J. U., and John F. P. (2005). "Purification and characterization of an extracellular chitobiase from *Trichoderma harzianum*." Current Microbiology. **3**: 285-289.

Pichyangkura, R., Kudan, S., Kuttiyawong, K., Sukwattanasinitt, M., and Aiba, S. (2002). "Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase." Carbohydr. Res. **337**: 557-559.

Queiroz, J. A., Tomaz, C. T., and Cabral, J. M. S. (2001). "Hydrophobic interaction chromatography of proteins." J. Biotechnol. **87**: 143-159.

Stephen, N. (1991). "Life after death for empty shells." New Sci. **129**: 46-48.

Saito, A., Fujii, T., Yoneyama, T., Redenbach, M., Ohno, T., Watanabe, T., and Miyashita, K. (1999). "High multiplicity of chitinase genes in *Streptomyces coelicolor* A3 (2)." Biosci. Biotechnol. Biochem. **63**: 710-718.

Sakai, K., Uchiyama, T., Matahira, Y., and Nanjo, F. (1991). "Immobilization of chitinolytic enzymes and continuous production of *N*-acetyl D-glucosamine with the immobilized enzymes." J. Ferment. Bioeng. **72**: 168-172.

Sambrook, J., and Ruddell, W. D. (2001). Molecular cloning: A laboratory manual. New York, Cold Spring Harbor Laboratory, Cold Spring Harbor.

Sashiwa, H., Fujishima, S., Yamano, N., Kawasaki, N., Nakayama, A., Muraki, E., and Hiraga, K., Oda, K., and Aiba, S. (2002). "Purification of *N*-acetyl D-glucosamine from alpha-chitin by crude enzymes from *Aeromonas hydrophila* H2330." Carbohydr. Res. **337**: 761-763.

Shimono, K., Matsuda, H., and Kawamukai, M. (2002). "Functional expression of chitinase and chitosanase, and their effects on morphologies in the yeast *Schizosaccharomyces pombe*." Biosci. Biotechnol. Biochem. **66**: 1143-1147.

Shiro, M., Ueda, M., Kawaguchi, T. and Arai, M. (1996). "Cloning of a cluster of chitinase genes from *Aeromonas* sp. NO 10S-24." Biochem. Biophys. Acta. **1305**: 44-48.

Sitrit, Y., Vorgias, C. E., Chet, I. and Oppenheim, A. B. (1995). "Cloning and primary structure of the *chiA* gene from *Aeromonas caviae*." Bacteriol. **177**: 4187-4189.

Stoyachenko, I. A., Varlamov, V. P., and Davankov, V. A (1994). "Chitinases of *Streptomyces kurssanovii*: purification and some properties." Carbohydr. Polym. **24**: 47-54.

Studier, F. W. (1991). “Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes.” J. Mol. Biol. **189**: 113-130.

Suzuki, K., Sugawara, N., Suzuki, M., Uchiyama, T., Katouno, F., Nikaidou, N. and and Watanabe, T. (2002). "Chitinases A, B, and C1 *of Serratia marcescens* 2170 produced by recombinant *Escherichia coli*: enzymatic properties and synergism on chitin degradation." Biosci. Biotechnol. Biochem. **66** (5): 1075-1083.

Suzuki, K., Suzuki, M., Taiyoji, M., Nikaidou, N., and Watanabe, T. (1998). "Chitin binding protein (CBP21) in the culture supernatant of *Serratia marcescens* 2170 " Biosci. Biotechnol. Biochem. **62**: 128-135.

Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Henrissat, B., and Watanabe, T. (1999). "The third chitinase gene (chiC) of *Serratia marcescens* 2170 and the relationship of its product to other bacterial chitinases." Biochem. J. **343**: 587-596.

Takahiro Kubota, K. M., Masahide, Y., Yoshihiko, I., and Hiroshi, T. (2004). "Molecular characterization of an intracellular *N*-Acetylglucosaminidase involed in the chitin degradation system of *Streptomyces thermoviolaceus*." Biosci. Biotechnol. Biochem. **68**: 1306-1314.

Tarabay, T. A. (1979). "*Bacillus licheniformis* corneal ulcer." Am. J. Ophthalmol. **87**: 717-719.

Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Lsogai, A., Henrissat, B., and Dijkstra, B. W. (1995). "Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and X-ray structure of a complex with allosamidin: evidence for substrate assisted catalysis." Biochemistry. **34**: 15619-15623.

Tews, I., Perrakis, A., Oppenheim, A., Dauter, Z., Wilson, K. S., and Vorgias, C. E. (1996). "Bacterial chitobiase structure provides insight into catalytic mechanism and the basis of Tay-Sachs disease." Nature Structural Biology. **3**: 638-648.

Tews, I., Scheltinga, A. C., Terwisscha, V., Perrakis, A., Wilson, K. S., and Dijkstra, B. W. (1997). "Substrate assisted catalysis unifies two families of chitinolytic enzymes." J. Am. Chem. Soc. **119**: 7954-7959.

Tharanathan, R. N. and Kittur F. S. (2003). "Chitin-the undisputed biomolecule of great potential." Crit. Rev. Food Sci. Nutr. **43** (1): 61-87.

Tortoli, E. (2003). "Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s." Clin. Microbiol. Rev. **16**: 319-354.

Tsujibo, H., Miyamoto, J., Kondo, N., Miyamoto, K., Baba, N., and Inamori, Y. (2000). "Molecular cloning of the gene encoding an outer-membrane-associated N-acetylglucosaminidase involved in chitin degradation system of *Alteromonas* sp. strain O-7." Biosci. Biotechnol. Biochem. **64**: 2512-2516.

Tsujibo, H., Okamoto, T., Hatano, N., Miyamoto, K., Watanabe, T., Mitsutomi, M., and Inamori, Y. (2001). "Family 19 chitinases from *Streptomyces thermoviolaceus* OPC-520: molecular cloning and characterization." Biosci. Biotechnol. Biochem. **64**: 2445-2453.

Usui, T., Matsui, H., and Isobe, K (1990). "Enzymatic synthesis of chitooligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate." Carbohydr. Res. **203**: 65-77.

Vandamme, P., Vandamme, B., Pot, M., Gillis, P., De Vos, K., Kersters and Swings, J. (1996). "Polyphasic taxonomy, a consensus approach to bacterial systematics." Microbiol. Rev. **60**: 407–438.

Wang, Q., Graham, R. W., Trimbur, D., Warren, R. A. J., and Withers, S. G. (1994). "Changing enzymatic reaction mechanisms by mutagenesis: conversion of a retaining glycosidase to an inverting enzyme." J. Am. Chem. Soc. **116**: 11594-11595.

Wang, S. L., and Chang, W. T. (1997). "Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in shrimp and crab shell powder medium." Appl. Environ. Microbiol. **63**: 380-386.

Watanabe, T, Agiga, Y., and Kobayashi, S. (2001). "Ring-opening glycosylation of a chitobiose oxazoline catalyzed by a non-chitinolytic mutant of chitinase." Chem. Lett. **30**: 1180-1181.

Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uschida, M., and Tanaka, H. (1993). "Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity." J. Biol. Chem. **268**: 18567-18572.

Watanabe, T., Uchida, M., Kobori, K., and Tanaka, H. (1994). "Site-directed mutagenesis of Asp-197 and Asp-202 residues in chitinase A1 of *Bacillus circulars* WL-12." Biosci. Biotech. Biochem. **58**: 2283-2285.

Xiqian Lan, N. O., Naohide, N., Ritsuko, K., Mitsuo, O., and Makoto, S. (2004). "Purification, cloning, and sequence analysis of *N*-Acetylglucosaminidase from the chitolytic bacterium *Aeromonas hydrophila* Strain SUWA-9." Biosci. Biotechnol. Biochem. **68**: 1082-1090.

Xiqian Lan, X. Z., Ritsuko, K., Zeyang, Z. and Makoto, S. (2008). "Gene cloning, expression, and characterization of a second *N*-Acetylglucosaminidase from the chitinolytic bacterium *Aeromonas hydrophila* Strain SUWA-9." Biosci. Biotechnol. Biochem. **72**: 492-498.

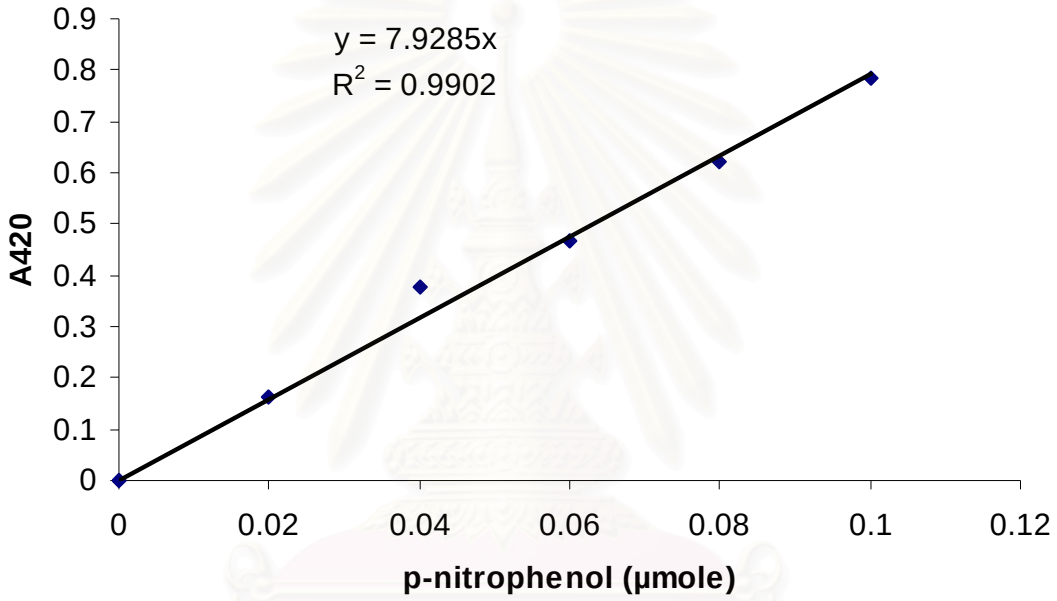
Yamanoi, T., Tsutsumida, M., Oda, Y., Akaike, E., Osumi, K., Yamamamoto, K., and Fujita, K. (2004). "Transglycosylation reaction of Mucor hiemalis endo-β-*N*-acetylglucosaminidase using sugar derivatives modified at C-1 or C-2 as oligosaccharide acceptors." Carbohydr. Res. **339**: 1403-1406.

Yannis, P., Gali, P., Giorgos, T., Constantionus, E. V., Amos B. O., and Kyriacos, P. (2001). "High solution structure analyses of mutant chitinase A complexes with substrates provide new insight into the mechanism of catalysis." Biochemistry. **40**: 11338-11343.

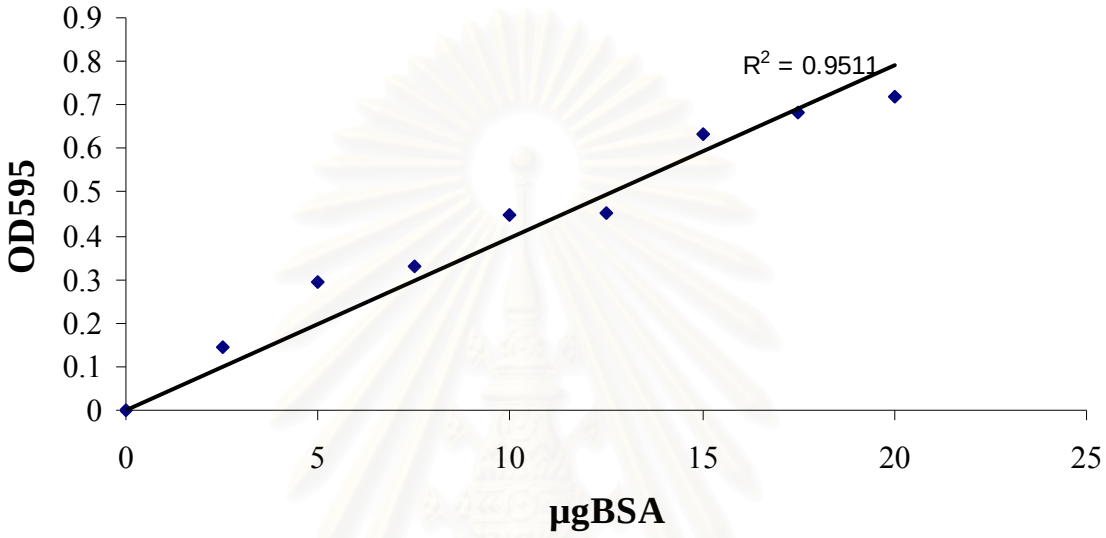
Zhong, Z., Xu, Z., Peng, L., Huang, L., Fang, X. and Cen, P. (2006). "Tandem repeat mhBD2 gene enhance the soluble fusion expression of hBD2 in *Escherichia coli*." Appl. Microbiol. and Biotechnol. **71**: 661-667.

APPENDICES

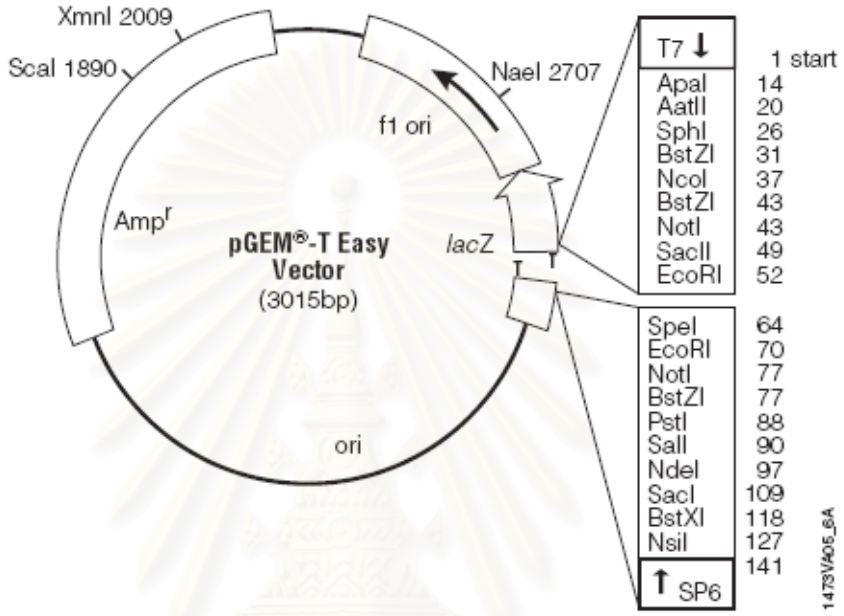
APPENDIX A : Standard curve of *p*-nitrophenol for chitinolytic enzyme assay by colorimetric method at 37 ^{O}C



APPENDIX B : Standard curve for protein determination by Bradford method



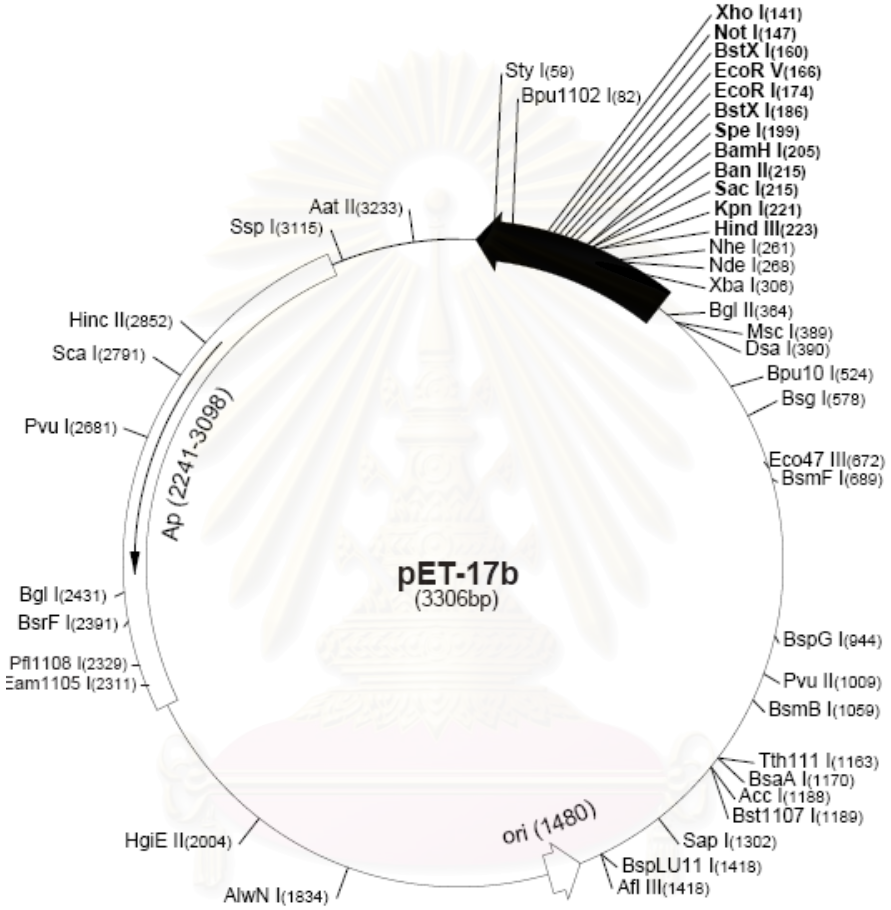
APPENDIX C : Restriction map of pGEM-T easy vector (Promega)



Specialized application of pGEM-T easy.

- Cloning PCR products.
- Construction of unidirectional nested deletion with the Erase-a Base system.
- Production of ssDNA.
- Blue/white screening for recombinants.
- *In vitro* transcription from dual opposed promoter.

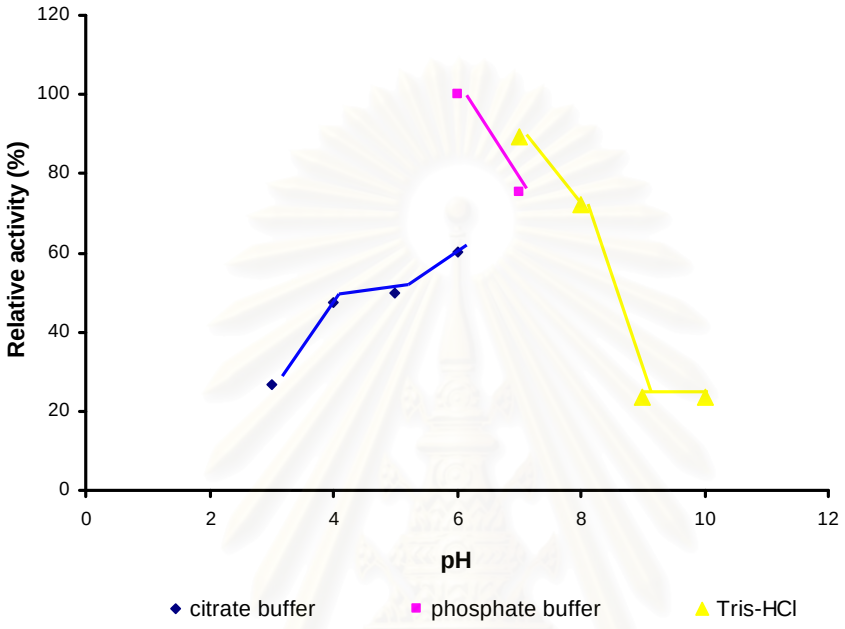
APPENDIX D : Restriction map of pET-17b (Novagen)



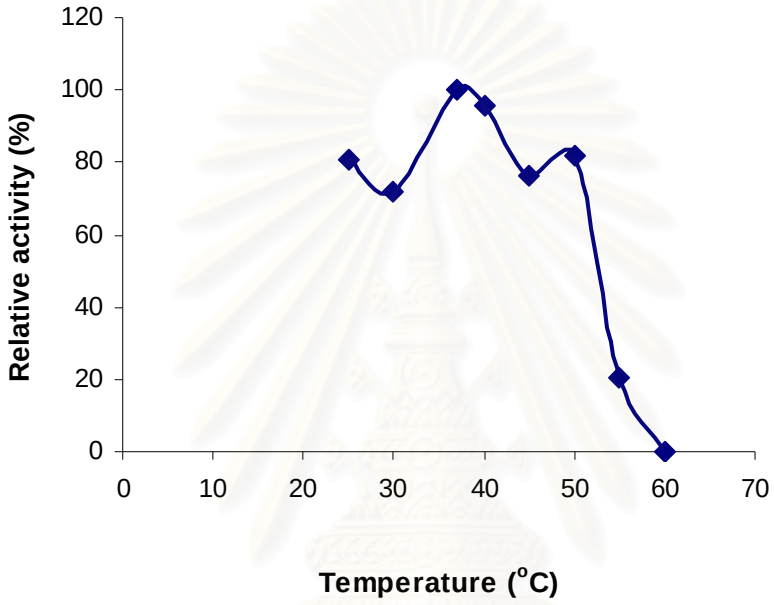
pET-17b vector characteristics

- T7 lac promoter
- Expression in *E. coli* BL21 (DE3)

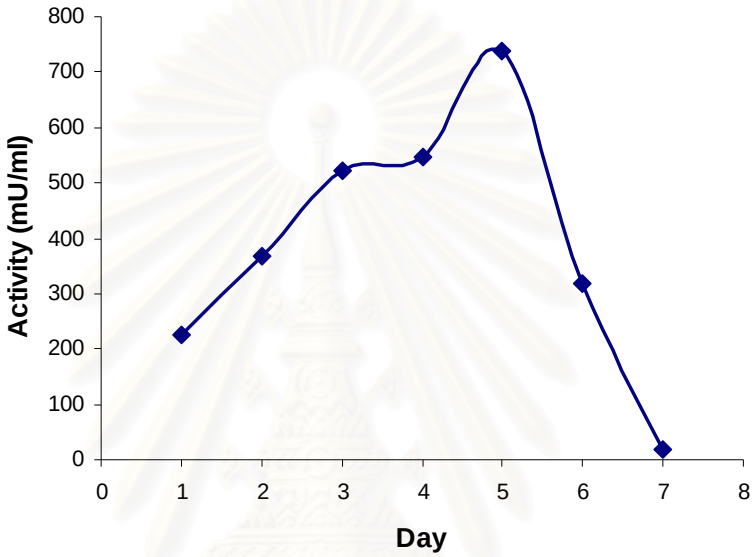
APPENDIX E : Optimum pH on β-*N*-acetylglucosaminidase activity from *Aeromonas caviae* D6



APPENDIX F : Optimum temperature on β-*N*-acetylglucosaminidase activity from D6



APPENDIX G : Time course for β-*N*-acetylglucosaminidase production at 37°C from *D6*



BIOGRAPHY

Miss Srisuda Trakulnaleamsai was born in Bangkok, Thailand. She obtained a B.Sc. degree in Food Technology Department, Faculty of Science. In 2004 she was studied for Master Degree of Science in Biochemistry at Chulalongkorn University.