การหาลำดับนิวคลีโอไทด์และการแสดงออกของยีนไคทิเนสจาก *Bacillus* sp. PP8

นางสาวสันทนา นาคะพงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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NUCLEOTIDE SEQUENCING AND EXPRESSION OF CHITINASE GENE FROM *Bacillus* sp. PP8

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สันทนา นาคะพงศ์ : การหาลำดับนิวคลีโอไทด์และการแสดงออกของยีนไคทิเนสจาก *Bacillus* sp. PP8. (NUCLEOTIDE SEQUENCING AND EXPRESSION OF CHITINASE FROM *Bacillus* sp. PP8.) อ.ที่ปรึกษา: อ.ดร.รัฐ พิชญางกูร, หน้า 143. ISBN 974-17-6350-6.

จากการศึกษาไคทิโนไลติกเอนไซม์ของ *Bacillus* sp. PP8 ซึ่งแยกได้จากดินใน ประเทศไทย พบว่า ไคติน และ GlcNAc สามารถเหนี่ยวนำให้ผลิตไคทิเนสได้ ในขณะที่ไคโตซานและไคตินเหนี่ยวนำให้ผลิตไคโทซานเนส สภาวะที่เหมาะสมในการเร่งปฏิกิริยาของไคติเนสและไคโตซานเนสคือ pH 8.0 / 50°ซ และ pH 7.0 / 50° ซ ตามลำดับ ผลิตภัณฑ์ที่ได้จากการย่อยคอลลอยด์ดอลไคตินด้วยเอนไซม์หยาบคือ GlcNAc และ $(GlcNAc)_2$ เมื่อนำเอนไซม์หยาบมาทำ SDS-PAGE และย้อมแอกติวิตี พบโปรตีนขนาด 145, 66, 55, 45 และน้อยกว่า 15 kDa แสดงไคทิเนสแอกติวิตี นอกจากนี้ยังพบโปรตีนขนาด 47.5 kDa แสดงไคโทซานเนสแอกติวิตี เมื่อโคลนยีนไคทิเนสโดยวิธี shot gun แล้วทำการคัดเลือกบนอาหารแข็งที่มีคอลลอยด์ไคทิน พบโคลนที่ทำให้เกิดวงใสรอบโคโลนี 3 โคลน โดยเลือก pST847 ซึ่งเป็นโคลนที่มีแอกติวิตี้ของไคทิเนสสูงที่สุดมาศึกษาต่อ พลาสมิด pST847 มีชิ้น insert ขนาด 6.3 กิโลเบส แทรกอยู่ใน pBS/SK$^-$ เมื่อทำ deletion ของ pST847 เป็นพลาสมิด 4 ชนิด คือ pSN*XX*-3.0, pSN*PX*-6.3, pSN*BB*-1.8 และ pSN*BP*-4.5 พบว่า pSN*XX*-3.0 เท่านั้นที่ทำให้เกิดวงใสรอบโคโลนี จากการศึกษายีนไคทิเนสใน pSN*XX*-3.0 พบ open reading frame ขนาด 1797 bp ถอดรหัสเป็นโปรตีน ขนาด 66.2 kDa มี signal peptide อยู่ทางปลายด้านอะมิโน และมีค่า pI เท่ากับ 4.5 ลำดับของกรดอะมิโนที่ได้มีความคล้ายคลึงกับลำดับกรดอะมิโนของไคทิเนสจาก *Bacillus licheniformis* และ *Bacillus subtillis* โดยเอนไซม์ที่ได้จาก pSN*XX*-3.0 หรือ CHI66 นี้มีสมบัติเหมือนกับเอนไซม์จาก *Bacillus* sp. PP8 เมื่อทำการแสดงออก CHI66 ใน *E. coli* โดยอาศัย overexpression vector 2 ชนิด คือ pET19b และ pTrcHis2-C พบว่า CHI66 แสดงออกได้ดีใน pET19b (+) โดยสภาวะที่เหมาะแก่การผลิตไคทิเนสคือ เขย่า 300 รอบ/นาที ที่ 30°ซ เป็นเวลา 60 ชั่วโมง หลังจากเหนี่ยวนำให้ผลิตไคทิเนสด้วย 1 mM IPTG ไคทิเนสที่ได้มีแอกติวิตี้สูงกว่าไคทิเนสจาก *Bacillus* sp. PP8 ประมาณ 6 เท่า และมีสมบัติเหมือนไคทิเนสจาก *Bacillus* sp. PP8 เมื่อนำไคทิเนสที่ได้จากการแสดงออกใน *E. coli* มาทำให้บริสุทธิ์บางส่วนด้วยวิธีการดูดซับแบบจำเพาะกับคอลลอยด์ดอลไคทิน พบว่า ได้ไคทิเนสประมาณ 90% จากการดูดซับแบบจำเพาะนี้

ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2004.....

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SANTHANA NAKAPONG: NUCLEOTIDE SEQUENCING AND EXPRESSION OF CHITINASE FROM *Bacillus sp.* PP8.
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Bacillus sp. PP8 was isolated from PP Island, Krabi, Thailand. When *Bacillus* sp. PP8 was grown in liquid medium containing colloidal chitin, flake chitin, or powdered chitin, chitinase and chitosanase activities were detected However, when it was grown in mediun containing colloidal chitosan or 100%deacetylated flake chitosan, only chitosanase activity was detected. The best inducer for chitinase and chitosanase activity was colloidal chitin and colloidal chitosan, respectively. Chitinase and chitosanase activity was determined by modified Schale's method. Chitinase and chitosanase activities reached their maximum level at 84 and 60 hours of cultivation, respectively. Both activities gradually decreased after further cultivation. GlcNAc can induce low level of chitinase production from PP8, however GlcN cannot induce either chitinase or chitosanase production. The optimum pH and temperature of the chitinase and chitosanase was pH 8.0 / 50^{o}C and pH 7.0 / 50^{o}C, respectively. GlcNAc and $(GlcNAc)_2$ are major products when colloidal chitin was used as substrate for hydrolysis by PP8 chitinase. When crude enzyme from PP8 was analyzed by SDS-PAGE followed by activity staining, we observed five bands of 145, 66, 55, 45 and less than 15 kDa in size containing chitinase activity. On the other hand, only a single band of 47.5 kDa with chitosanase activity was observed. A chitinase encoding gene was previously cloned using shot gun cloning. Three colonies producing different clear zones on the selective agar designated, pST24, pST847 and pST1691 were selected. The highest chitinase activity was found in clone pST847 containing the 6.3 kb inserted fragment. pST847 was chosen for further analysis. There are four derivatives; pSN*XX*-3.0, pSN*PX*-6.3, pSN*BB*-1.8 and pSN*BP*-4.5 of pST847 but only pSN*XX*-3.0 had chitinase activity. Nucleotide sequence of pSN*XX*-3.0 revealed an open reading frame of 1797 bp encoding for 599 amino acids with signal peptide corresponding to 66.20 kDa. The chitinase gene expression was performed using pET19b and pTrcHis-2C. The chitinase activity was found in the culture medium and confirmed by SDS-PAGE followed by activity staining. The optimum pH, temperature, and products of colloidal chitin hydrolysis of the recombinant chitinase corresponded to the 66 kDa native chitinase from *Bacillus* sp. PP8. The enzyme did not degrade when kept in pH 5-8, at 4^{o}C for 2 months. Partial purified of the enzyme was performed by chitin adsorption and about 90% yield were recovery.

Department…. Biochemistry…… Student's signature…….

Field of Study..Biochemistry…….Advisor's signature.....

Academic year.2004……..

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Finally, the greatest indebtedness is expressed to my father, my mother and my younger sister for their unlimited love, understanding and encouragement.

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ABBREVIATION

A	Absorbance
Å	Aungstrom
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CCMM	Colloidal Chitin Minimal Medium
CDM	Chitosanase Detection Medium
°C	Degree Celsius
DD	Deacetylation Degree
IPTG	Isopropyl-β-D-thiogalactopyranoside
L	Litre
mL	Millilitre
µL	Microlitre
M	Molar
mM	Millimolar
µM	Micromolar
µg	Microgram
rpm	Revolution per minute
nm	Nanometre
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

CHAPTER I
INTRODUCTION

Chitin

I Occurrence

Chitin, is one of the most abundant natural polysaccharide next to cellulose, with annual production 10^{10}-10^{11} tons per annum. It is synthesized by different organisms with different chitin contents, which are given in Table 1.1 (Knoor, 1984). Chitin is well known to consist of 2-acetamido-2-deoxy-β-D-glucose connected though a β-1,4 linkage. It was discovered in 1811 by the French scientist Henri Barconnot, as a substance occurring in mushrooms. In 1823 Odier found this compound in the cuticles of insects and named it chitin from the Greek word for tunic or envelope.

II Structure of chitin

Chitin is a cationic amino polysaccharide, composed of *N*-acetyl-D-glucosamine (GlcNAc) linked by β-1,4 linkage as shown in Figure 1.1 a. Chitin, derivative, chitosan is a heteropolymer of D-glucosamine (GlcN) (~80%) and GlcNAc (~20%) units, which is shown in Figure 1.1 b. It is a product derived from deacetylation of chitin in the hot alkaline milieu. The physical structure of chitin at the molecular level has been the subject of continuous study since 1920s, and the primary methods of analysis have been X-ray crystallography and infrared spectroscopy. Comparison of the X-ray data for chitins from different sources has revealed the existence in nature of more than one polymorphic form. Generally, the individual chain assumes an essentially linear structure, which undergoes one full twist, every 10.1-10.5 Å along the chain axis. Because each glycosidic unit in the chain is chiral, and all units are connected by an oxygen atom that C-1 of one glycosidic unit to C-4 of a next unit, a distinct left and right direction can be assigned to each polymer chain. Most chitin, including those from crustaceans, insects and fungi are in the so-called α-form, in which the unit cell is orthorhombic and the individual chains are arranged in antiparallel manner. The hydrophobic forces between the surfaces of the glucose rings further stabilize the stacking of the chains. Furthermore the arrangement of the CH_2OH side chains, dictating the type of

intermolecular bonding along the chitin chain and resulting in the conformation of chitin.

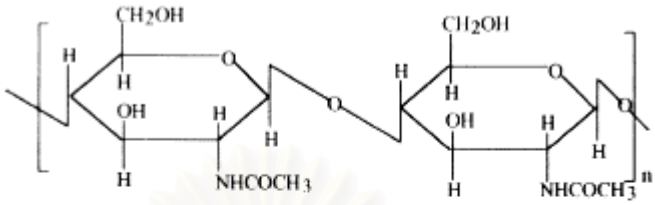
In α-chitin, half of the -CH_2OH groups are bonded to carbonyl with in the same stack of chains, and half are bonded to the –CH_2OH groups on an adjacent stack. The existence of this intersheet bonding is probably responsible for the stability of the α-chitin structure, specifically its inability to swell in water. However, a rare second form known as β-chitin has been identified in four sources, the spines of the polychaete *Aphrodite*, the pen of the squid *Loligo*, the tubes of *Pogonophora* and the spines of certain marine diatoms. This chitin conformation has a monoclinic unit cell with the polymer chain arranged in a same sense, different from the α-chitin; the –CH_2OH groups are all hydrogen bonded to carbonyl group in the same stack of chain. Hence there is no hydrogen bonding between the stacks of the chains. A third form, γ-chitin has been reported from the stomach lining of *Loligo* only. It has a three-chain unit cell in which two up chains are followed by the down chain. However, this form has not been subjected to detailed analysis as in α and β forms, and it has yet to be established that this is truly a third structure, and not a distorted version of one of the others. The diagrammatic structure of all conformations of chitins are shown in Figure 1.2 (Blackwell, 1989)

Table 1.1 Chitin content of selected crustacean, insect, molluscan organs and fungi.

Type	Chitin content (%)	Type	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 (truefly)	54.8[c]
Crangon (shrimp)	69.1[c]	Pieris (sulfer 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*	42.0[d]
Molluscan organs		*Penicillium notatum*	18.5[d]
Clamshell	6.1	*Penicillium chrysogenum*	20.1[d]
Oyster shell	3.6	*Saccharomyces cereviseae*	2.9[d]
Squid, skeleton pen	41.0	*Mucor rouxii*	44.5
Krill, deproteinized shell	40.2	*Lactarius vaiiereus* (mushroom)	19.0

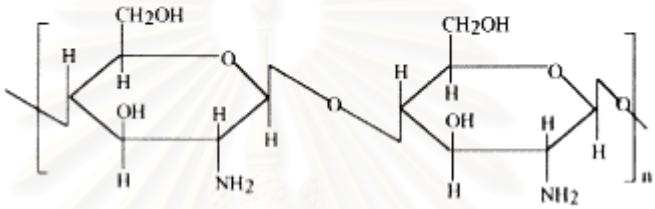
a. Wet body weight
b. Dry body weight or total dry weight of cuticle
c. Organic weight of cuticle
d. Dry weight of cell wall

a)



Chitin

b)

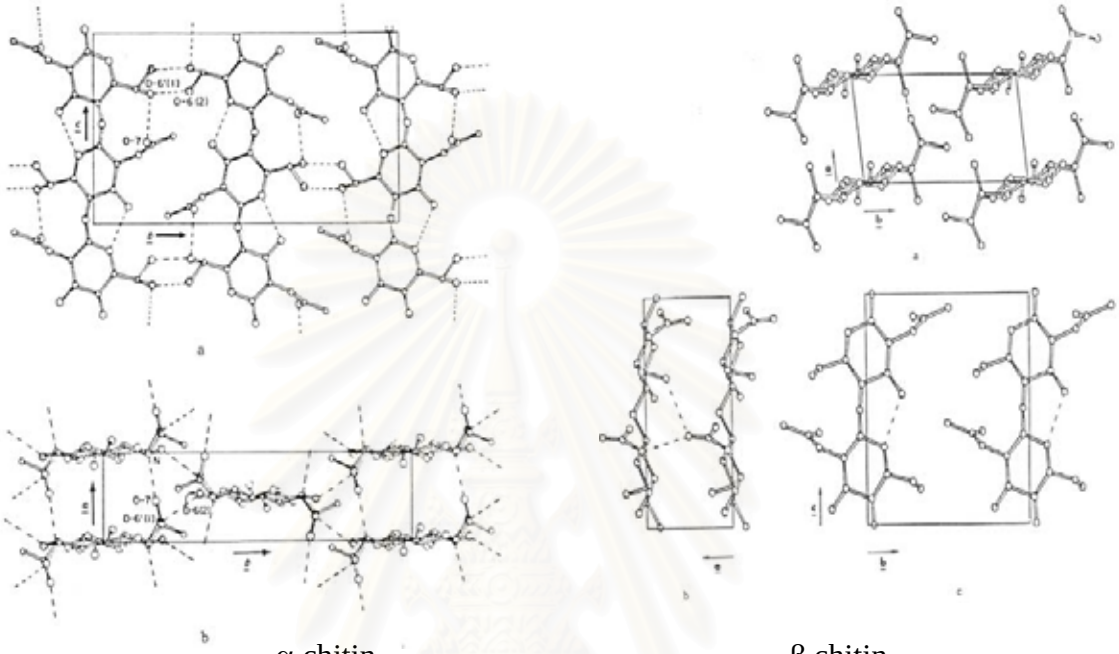


Chitosan

Figure 1.1 The chemical structure of chitin and chitosan

a) chitin

b) chitosan



α-chitin β-chitin

Figure 1.2 Structure of α-chitin and β-chitin

Structure of α-chitin and β-chitin and hydrogen bond between C=O-NH group.

III Chitin and chitosan biosynthesis

In crustacean and insect, Chitin biosynthesis is involved in ecdysis (molting) to allow growth and development of those organisms. The biosynthesis of chitin starts with a common sugar in insect, trehalose. The non-reducing disaccharide is converted to glucose by trehalase, and the glucose unit is converted to glucose-6-phosphat hexokinase. Glucose-6-phosphate is isomerized to frucose-6-phosphate which, is converted to glucosamine-6-phosphate and *N*-acetyl-glucosamine-6-phosphate by an aminotransferase and *N*-acetyltransferase, respectively. In this stage *N*-acetyl-glucosamine-6-phosphat is converted to UDP-*N*-acetylglucosamine by the action of several enzyme. In the terminal polymerization step is still fragmentary. Little is known about this step of chitin biosynthesis in any organism. One intermediate step might be transfer of GlcNAc on to a lipid to form dilichyldiphospho-N-acetylglucosamine, which is transported outside the cell and attached to specific residue, possible asparagine on the receptor protein. The resultant primer is extended by addition of GlcNAc units by chitin synthetase. However, the current evidence for the occurrence of lipid-GlcNAc intermediates or acceptors during chitin assemble is weak and therefore not included in the depicted pathway, which is shown in Figure 1.3 (Merzendorfer and Zimoch, 2003)

In fungi system, two main types of cytoplasmic vesicles appear to be associated with cell wall construction. The polymer, which comprised the amorphous phase of the wall, is secreted in macrovesicles whereas chitin synthetase is separately delivered in microvesicle called chitosome. By packaging chitin synthetase into chitosome, the fungi can distribute this enzyme to specific regions on the cell surface where the cell wall synthesis. UDP-*N*-acetylglucosamine is found in free in cytosol, and probably plays no role in the spatial regulation of chitin biosynthesis. Seemingly, the regulation is achieved through the deployment and activation of chitin synthetase. Since chitosomal chitin synthetase is a zymogen, chitosome can move through a cytoplasm laden with substrate without making chitin prematurely. Chitin is synthesized *in situ*, i.e. assembled outside the cytoplasm by enzyme delivered by chitosome. Presumable, when the chitosome reaches its destination, proteases on the

cell surface (periplasm) activate the zymogen, and assembly of GlcNAc units begins. (Garcia, 1989)

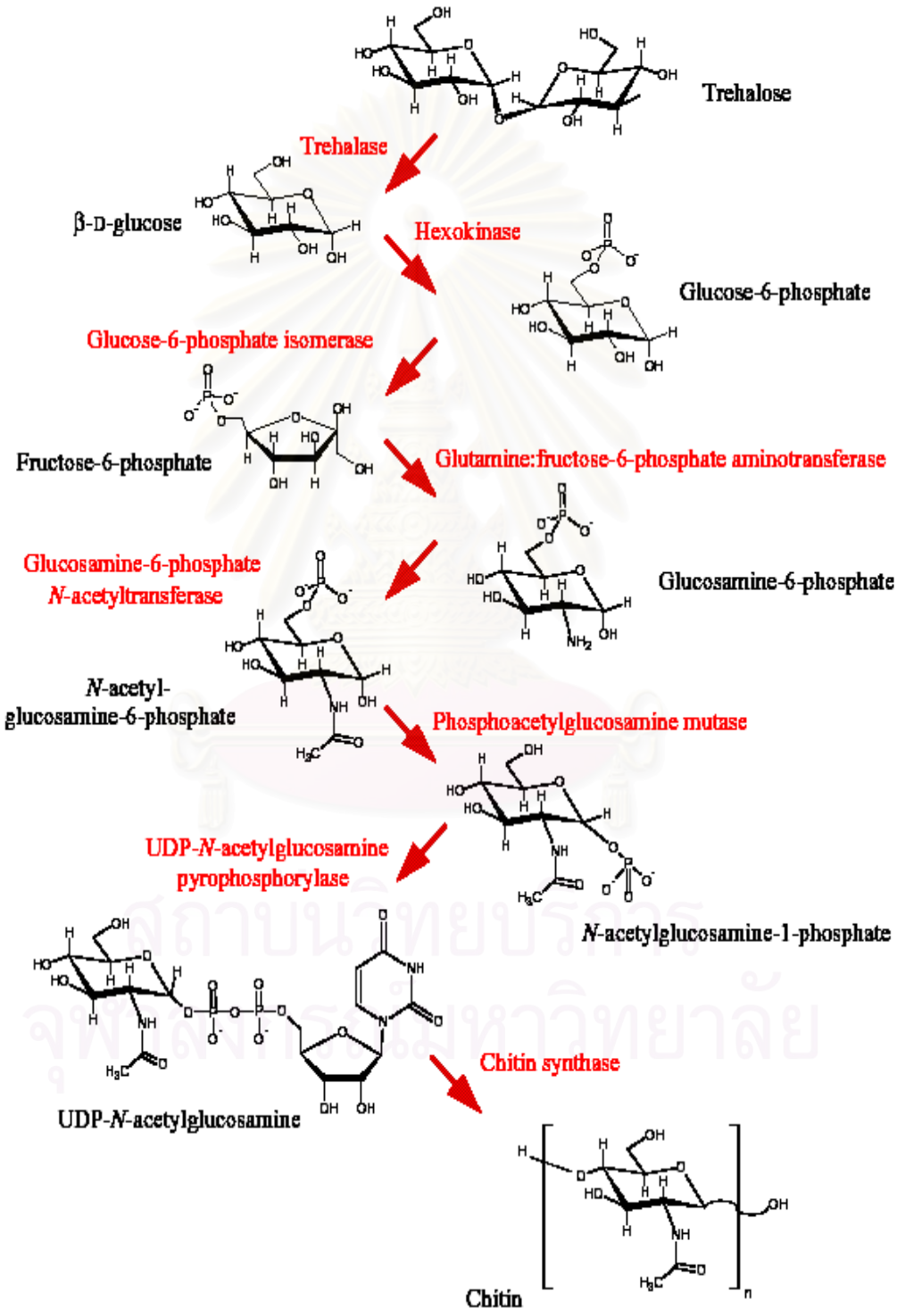


Figure 1.3. **Insects biosynthesis**

The diagrammatic representation insects chitin biosynthesis is based on previously published pathways (Kramer and Koga, 1989; Cohen, 2001)

An extensive research for an independent pathway for chitosan biosynthesis, specifically one via direct polymerization of unacetylated glucosamine residues from uridine diphosphate D-glucosamine into chitosan proved fruitless. Despite the fact that authentic chitin from various sources is not a good substrate for enzymatic deacetylation, indirect evidence strongly suggested that chitosan was derived from UDP-GlcNAc, the substrate for chitin biosynthesis. The enigma was resolved when Davis and Garcia discovered that the deacetylase be allowed to act on chitin chains as they were being formed; i.e. the simultaneous presence and operation of chitin synthetase and chitin deacetylase were required for chitosan synthesis. Seemingly the two enzymes operate in tandem, one polymerizing GlcNAc units from UDP-GlcNAc, the other removing acetate moieties from the nascent chains. (Davis and Garcia, 1984)

IV Chitin and chitosan production

Today several companies are producing chitin and chitosan on a commercial scale. The majority of the companies are located in the U.S. and Japan, where large amounts of chitin and chitosan are manufactured each year from the shells of crabs and shrimps. An effective methods used for chitin isolation and purification should assure the effective removal and if possible utilization of waste shell components. Thus, various procedures have been adapted for chitin recovery. Traditionally, shell wastes are deproteinized by treating with either sodium hydroxide or by digestion with proteolytic enzymes such as papain, pepsin, trypsin and acalase. The effectiveness of alkali deprotenization depends on the temperature of processing, base concentration and the ratio of the amount of base to the shells whereas the enzymatic deproteinization depends on the selectivity of the enzyme used and process conditions. Demineralization can usually be achieved by extraction with hydrochloric acid. Full demineralization of the shells is possible when the amount of acid is greater than the mineral content. Pigment residues are removed by with the extraction with acetone, chloroform, ethyl acetate or ethanol and ether mixture.

Chitosan production is usually prepared by N-deacetylation of chitin. The process is carried out either at room temperature or at elevated temperature using concentrated sodium or potassium hydroxide solution. Alkali concentration, time and temperature of the process should be controlled because of their influences on degree of deacetylation, molecular weight as well as the distribution of deacetylated units along the chain. These properties reflect the usefulness of chitosan for many applications. Chitosan can also be isolated from some fungi. The isolation of fungal chitosan can be accomplished by extraction from mycelia previously deproteinized with diluted alkali solution or by enzymatic digestion. The use of chitin deacetylase for chitosan production was intensively investigated. The study shows that insoluble crystalline chitin is resistant to enzymatic deacetylation. However, partially chemical deacetylated chitin can be further deacetylated by chitin deacetylase. The enzymatic process is more efficient in the case of deacetylation of chitin oligomers, which are soluble in water and therefore are more accessible for enzyme action. (Synowiecki and Khateeb, 2003)

V Chemical and physical characteristics

Chitin and chitosan have reactive hydroxyl and amino groups. They can be considered to be weak base. They have a high charge density, one cationic charge per glucosamine units. In many reactions, the nonbonding pair of electrons on the primary amino groups of GlcN units plays the role of acceptor protons. Thus, the primary amine group becomes cationically charged. These nonbonding pairs of electrons on the primary amino group also make chitosan a potent nucleophile, ready to react with most aldehydes to form imines. However, it is possible to selectively modify the hydroxyl group. This can be achieved by protect on of the amino groups through the formation of polysaccharide formate or acetate and subsequent reaction of the salt with the electrophile. The hydroxyl group at C-6 is more reactive than at C-3 and therefore is preferentially derivatized. Chitosan is modified to many variety derivatives that find widespread utilization in many applications, as shown in Figure 1.4.

An important parameter to determine closely between chitin and chitosan is the degree of *N*-acetylation, i.e. the ratio of 2-acetamido-2-deoxy-D-glucopyranose to 2-amino-2-deoxy-D-glucopyranose structural units. This ratio has a striking effect on the solubility of chitin and chitosan. A typical degree of *N*-deacetylation of chitin is

0.90, while chitosan is the fully or partially *N*-acetylated with a typical degree of *N*-acetylation of less than 0.35. The average molecular weight of chitin is $1.03x10^6$ to $2.5x10^6$, but the *N*-deacetylation reaction reduces this to $1x10^5$ to $5x10^5$(Wu, 1988).

Chitin is highly crystalline, only a limited number of solvents are known which are applicable as reaction solvents. Chitin and chitosan degrade before melting, which is typical for polysaccharides with extensive hydrogen bonding. This problem make is necessary to dissolve chitin and chitosan in an appropriate solvent system to impart functionality. In the case of chitosan, it is usually less crystalline than chitin, which presumable make chitosan more accessible to reagents. There are relatively a few solvents for chitin, while nearly all-aqueous acids dissolve chitosan (see table 1.2).

Table 1.2 Commonly used solvents for chitin and chitosan

Chitin	Chitosan
Dimethylformamide+lithium chloride	Aqueous formic acid
Hexafluoroisopropanol	Aqueous acetic acid
Hexafluoroacetone sesquihydrate	Aqueous lactic acid
1,2-Chloroethanol	

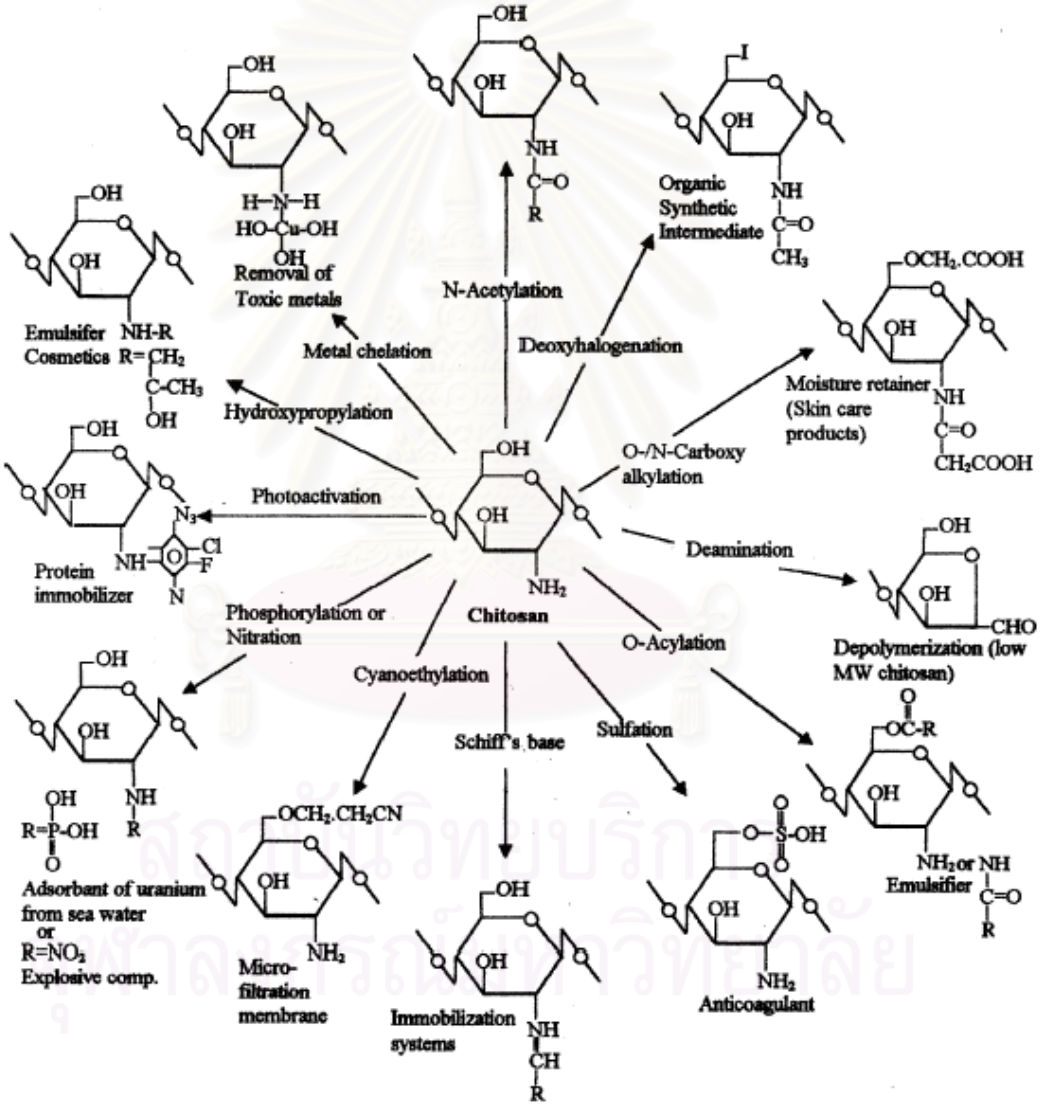


Figure 1.4 Chitin derivatives

Chitin derivatives and theirs applications

V Current applications of chitin and chitosan

The characteristics of chitin and chitosan for use as potential broad range industrial applications, 1) they are from natural resource and renewable materials, 2) they are biodegradable and do not pollute the environment, 3) they are biocompatible to humans and most of the animals, 4) they are almost nontoxic, 5)they have biological function, and 6) they have changeable structures. For these reasons, there are a lot of emphases on devising methods of adopting the biomolecule for versatile applications.

Biomedical applications

Chitin and chitosan are nontoxic and biodegradable polysaccharide that attracts much attention in medical and pharmaceutical fields. This is due to its polymeric cationic character, and its gel and film forming properties. Chitosan has been modified for utilization in controlled release drug delivery system. Such systems should allow the control of the rate of drug administration and prolong the duration. The drug is either physically blended or covalently linked to the amino group of chitosan, and generally is released from the chitosan metrix by erosion of the polymer after contact with body fluids (Illum, 1998). Studies have shown the antitumorigenic properties of chitin and chitosan oligomer in growth inhibition of tumor cell via an immuno-enhancing effect on Met-A solid tumor transplants into BALB/C mice. The antitumor was assumed to involve the increased production of lymphokines including interleukine 1 and 2, sequentially, leading to the manifestation of antitumor affect through proliferation of cytolytic-T lymphocytes. (Tokaro, 1988) Not only antitumor activity but chitosan also have an anticancer activity, chitosan at 6-50 naq mL-1 in concentration has been shown to aggregate L-1210 leukemia cell in vitro. Therefore, the growth and invasive procession of cancer cells could be selectively inhibited by chitosan (Sivica and Woodman, 1971). In other cases the introduction of chitin and chitosan into the human or animal body is helpful for healing of different diseases or for preventing sickness. Some other activities are shown in Table 1.3

Table 1.3 Physiological influence of chitin derivatives in the human body.

Significance	Kind of activity
1. Antimicrobial activity	Reaction with anionic component of the cell wall, chelation of ions in metalo-enzymes, changes in bacterial adhesion, inhibition of enzyme linking glucans to chitin
2. Immuno Stimulation	Activation of macrophage secretion and synthesis of interferons and interleukin
3. Chemotactic action	Stimulation of migration of fibroplasts and other stromal cells
4. Action as a source of GlcN and GlcNAc	Rebuilding of extracellular matrix
5. Enhanced reconstruction of connective tissue	Healing of ulcers, meniscal lessions, osteoinduction, and other wounds, influence on assemble and orientation of collagen fibers
6. Dietary significance	Anticholesterolemic and antiulcer activity lowering of body overweight
7. Growth stimulation	Molecular recognition and entrapment of growth factor, stimulation of lectin type activity

Biotechnology applications

The consumer demand for food without harmful chemical additive has focused efforts in the discovery of novel material antimicrobials. Because of the positive charge on C-2 of the GlcN monomers at pH below 6, chitosan is more soluble and has a better antimicrobial activity than chitin. The exact mechanism of their action is still unknown. It is possibly the formation of chitosan complexes with anionic components of the cell walls, such as N-acetylmuramic, sialic acid and neuraminic acid, as well as an inactivation of microbial metaloenzymes through chelation of metal ions. The minimal inhibitory concentration of chitosan and its derivatives vary significantly for different bacterial cultures (see Table 1.4). These variations were suggested to be due to the existing difference in the molecular weight and degree of acetylation of chitosan. (Chen, 1998). Chitosan and its derivatives were also investigated as a component of cosmetics, toothpaste and moisturizer. Chitosan has a moisturizing effect on the skin that is dependent on the molecular weight and degree of deacetylation, as well as offers protection from mechanical hair damage and exhibits an antielectrostatic effect on hair. High-molecular weight chitosan increases the water resistance of emulsions protecting against irradiation. A cosmetic cream supplemented with chitosan increased an ability of bioactive, lipophilic ingredients such as vitamins, which better penetrate the outer layer of skin. This benefit is also caused by the activation of fibroplasts and improved collagen deposition (Horner, 1997). Dental fluids and toothpaste containing chitosan can seal dentinal tubules and protect against microbial infection but maintain diffusion of ions and water (Lawska, 1997). These biopolymers can also be used in textile industry. The addition of chitin into the coating of waterproof textile causes a large increase in its water vapor permeability. Moreover, the finishing of wool fibers with chitin derivatives improves their dye ability and colorfastness (Hudson, 1997). Many researchers demonstrated the suitable of chitin and its derivatives as enzyme supports. Many enzymes can be immobilized on partially deacetylate chitin by chemical linking or by adsorption, such as glucoamylase (Denise, 1990), lipase (Fu, 1990), peptidase (Jun, 1996) and so on.

Table 1.4 Minimal inhibitory concentrations (MIC, PPM) of chitosan and derivatives for different bacterial cultures (Chen, 1998).

Bacterial culture	DD_{69}	SC_1	SC_2	SBC
Gram positive				
Staphylococcus aureus	100	100	>2000	200
Listeria monocytogenes	100	100	>2000	100
Bacillus cereus	1000	500	NT	>2000
Gram negative				
Escherichia coli	100	100	NT	100
Vibrio parahaemolyticus	100	100	>2000	100
Pseudomonas aeruginosa	200	200	>2000	2000
Shigella dysenteriae	200	100	>2000	100
Vibrio cholerae	200	>2000	>2000	2000
Aeromonas hydrophila YMI	500	200	>2000	200
Aeromonas hydrophila $CCRC_{13881}$	2000	200	>2000	500
Salmonella typhimurium	>2000	200	>2000	2000

DD_{69} = 69% deacetylated chitosan SCI = 0.63%Sulphonated chitosan

SC_2 = 13.03%Sulphonated chitosan SBC=Sulphobenzoyl chitosan

NT = Not tested

Environment and pollution control applications

Cell wall containing chitin, chitosan and chitosan derivatives were used as the removal of many dyes such as methyl orange, phenolic dyes, polycyclic dyes, acid dyes and azo dyes by hydrophobic interaction or sorption via their backbone or amino group. Chitin had been reported to adsorb cadmium and lead on its surface from seawater. Chitosan was used for the removal of hexavalent chromium. Highly porous gelled chitosan beads crosslinked with glutaraldehyde were effective for the removal of cadmium ions from waste water. Moreover, chitosan was used for the sorption of uranium from nuclear effluents. A process scheme that describes the treatment procedure indicates that the carboxylic sites and the amino sites of chitosan act directly by ion exchange and sorption of uranyl, ions respectively (P. A. Felse, 1999).

VII The chitin modifying system.

Different organisms produce a wide variety of enzymes that exhibit different substrate specificity and properties useful for various functions. The complete enzymatic modification of chitin to free GlcNAc and GlcN are preformed by a chitin modifying system, the action of which is known to be synergistic and consecutive as shown in Figure 1.5. Chitin in the environment is depolymerized to chitooligosaccharides by secreted chitinase. Once degraded, chitooligosaccharides are transported and degraded to chitobiose and GlcNAc by chitodextrinase and N-acetyl-β-D-glucosaminidase, respectively. On the other hand, chitin is deacetylated to chitosan by chitin deacetylase and futher hydrolyzed to chitosan oligosaccharides by chitosanase. The further degradation yields D-glucosamine by β-D-glucosaminidase. GlcNAc and GlcN may be shunted to cell wall biogenesis or modified and isomerized to from fructose-6-phosphate, which is metabolized via the glycolysis.

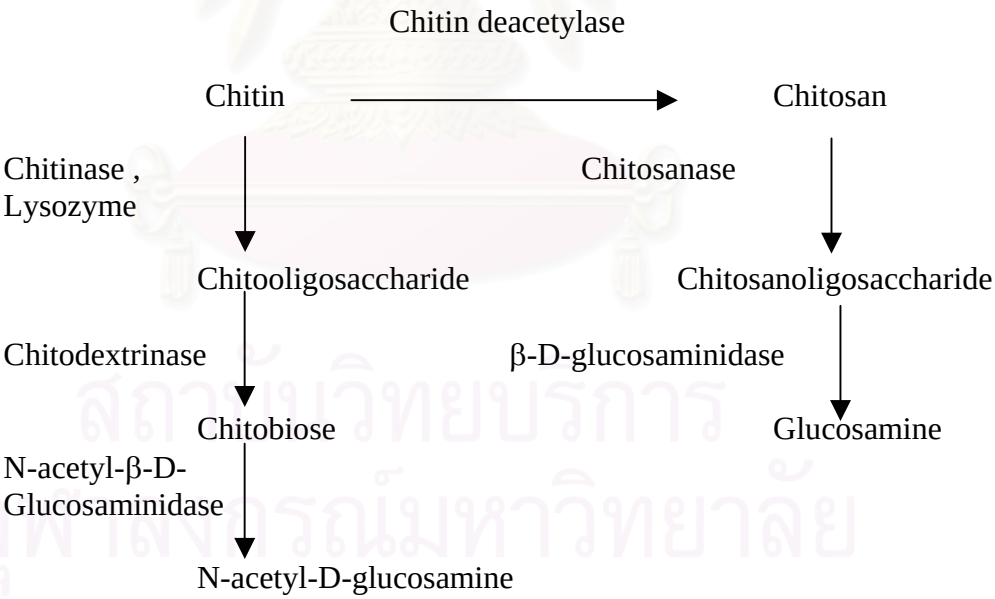


Figure 1.5 Chitin modifying system

Predicted pathway for chitin modifying system (Goodsen,1997).

VII Chitinases

Chitinases are glycosyl hydrolases that hydrolyse the (1-4)-β-linkage between GlaNAc in the chitin or chitodextrins to $(GlcNAc)_n$. The classification of chitinolytic enzyme in not yet very clear. Chitinases are broadly classified by their type of reaction that the enzymes catalyze and their substrate specificity, which is recommended by IUBMB. (IUBMB; Nomenclature Committee of International Union of Biochemistry and Molecular Biology). However, the IUB classification is not helpful for understand evolutionary relationships among these enzymes. In 1991, Henrissat and his co-workers purposed a systematic comparison of the primary sequences of glycosyl hydrolases and grouped these enzymes into 35 families and presently updated to 80 families.

Classification of chitinases according to their mode of action.

Chitinases are classified as endochitinases and exochitinases. The endochitinase activity is defined as the random cleavage at the internal points in the chitin chain. The exochitinase activity is defined as the progressive hydrolysis starting at the non-reducing ends of chitin with the release continuously of $(GlcNAc)_2$ or GlcNAc. For glycosyl hydrolase (EC3.2.1.X), the first three digits indicate the enzymes hydrolyzing O-glycosyl linkages whereas the last number (X) indicated the substrate and sometimes reflects molecular mechanism as shown in Table 1.5.

Table 1.5 Nomenclature of chitinolytic enzymes.

Mode of action	Chitinolytic enzymes
Random hydrolysis of the chain	Chitinase, 1,4-β-*N*-acetylglucosaminidase(EC3.2.1.14)
Hydrolysis of terminal non-reducing sugar	[a,b]Early classification; Chitobiase (EC3.2.1.29), β-D-Acetylglucosaminidase (EC3.2.1.30)
Successive removal of sugar unit from the non-reducing end	[c]Present classification; β-*N*-Acetylhexosaminidase (EC3.2.1.52)

[a]Recommendations of the Nomenclature Committee of the IUBMB, 1961 Chitobiase (chitobiose acetylaminodeoxyglucohydrolase(EC3.3.1.29) β-N-acetylglucosaminidase (β-2-acetylamino-2-deoxy-D-glucoside acetylaminodeoxyglucohydrolase (EC3.2.1.30)

[b]Recommendations of the Nomenclature Committee of the IUBMB, 1978 EC3.2.1.29 deleted and the enzyme was included with β-N-acetylglucosaminidase

[c]Recommendations of the Nomenclature Committee of the IUBMB, 1992 Entry EC3.2.1.30 deleted and the enzyme β-N-acetylglucosaminidase was included with β-*N*-Acetylhexosaminidase (EC3.2.1.52)

Classification of chitinases based on amino acid sequences.

Henrissat *et. al.* chitinase and N-acetylhexosaminidase into three families 18, 19 and 20. Generally, chitinases are found in family 18 and 19. Family 18 contains several conserved amino acids, as shown in Figure 1.6a., which the enzymes of *Bacillus circulans, Serratia marcessens, Pyrococcus kadakaraensis* and *Trichoderma harzianum.* These sequences are found in the active site, and include a glutamic acid(E) residue (residue in bold) which played a crucial role in catalytic mechanism. Almost all of plant chitinases are grouped in family 19. The conserved amino acids from these family are represented by barley chitinase (*Hordeum valgare*), potato chitinase (*Solanum tuberosum*), pea chitinase (*Pisum sativum*) and *Arabidopsis thaliana*, as shown in Figure 1.6b. Two glutamic acid residues (residue in blue) are important in the mechanism of action of family 19. (Henrissat, 1991)

```
a)    S_marcessens        LPSIGGWTLSDPF--FDGVDIDWEFPGG
      T_harzanum          ILSIGGWTWSTNF--FDGVDIDWEYPAD
      B_circulans         IISVGGWTYSNRF--FDGVDIDWETPVS
      P_kodakaraensis     LISVGGWTLSKYF--FDGVDLDWEYPVS
                          : *:**** *  *  *****:***** * .
b)
P_sativum   KREVAAFFGQTSHETTGGWATAPADGPYSWGYCFKQEQNPASDYCEPSATWPCASG
A_thaliana  KREIAAFLGQTSHETTGGWPTAPADGPYAWGYCFLREQNP-SDYCQASSEFPCASG
H_valgare   KREVAAFLAQTSHETTGGWATAPADGAFAWGYCFKQERGASSDYCTPSAQWPCAPG
S_tuberosum KREIAAFFAQTSHETTGGWASAPADGPYAWGYCFLRERGNPGDYCPPSSQWPCAPG
            ***:***:.**********.:*****.::***** :*:.  .*** .*: :***.*
```

Figure 1.6 The local alignment of several chitinases from

a). Glycosylhydrolase family 18 b). Glycosylhydrolase family 19

Structure and function of catalytic and noncatalytic domains.

Chitin degradation by numerous chitinases involves the synergistic action of multiple proteins. These proteins typically contain conserved modules that function as catalytic domains, chitin binding domains, and may also contain accessory domain with unknown function, such as fibronectin type III like domain. An arrangement of these domains is different for each chitinase, which is shown in Figure 1.7.

Catalytic domains

The three dimensional (3D) structures of some members of family 18 chitinase have been determined such as *Serratia marcescens* chiA and *Bacillus circulans* WL-12 chiA. The 3D structure reveal that they share a similar $(\beta/\alpha)_8$ barrel (as shown in Figure 1.8a.). That is eight strands of parallel β sheet are laid down with the helices as the return stroke. The eight strands of the sheet bend into barrel with the helices forming a ring toward the outside. However, the structure of the only structure of the member of family 19 chitinase has been solved from *Hordeum valgare* (barley) as shown in Figure 1.8b). The 3D structure of the protein revealing a mixture of secondary structures, including 10 α-helical segments, and one three-stranded β sheet.

Chitin Binding Domain (ChBD)

There are three families of chitin binding domains. ChBD family 1 is found of chitinases from *Bombyx mori* and *Penaeus japonicus*. ChBD family 2 is found in chitinases from *Arabidopsis thaliana, Hordeum vulgare* and *Oryza sativa*. ChBD family 3 is found in chitinases from *Aeromonas caviae*, *Bacillus circulans* and *Vibrio harveyi*. However, only 3D structure of ChBD from *B. circulans* WL-12 is completely solved using NMR technique. This domain contained two antiparallel β-sheets. One sheet is composed of three strands (β_2, β_3, and β_5) and the other is composed of two strands (β_1, and β_4). No region characteristic of an α-helix exists. These five β-strands form the hydrogen bond networks. The two antiparallel β-sheets formed by these hydrogen bond networks fold into the topology of a twisted β-sandwich with an angle of about 45° between the sheets. The β-sheet formed by β_2, β_3, and β_5 makes a flat surface on the molecule, and the loop connecting β_4, and β_5 run on the opposite side to the surface. The core region formed by the hydrophobic and aromatic residues makes the overall structure rigid and compact as shown in Figure 1.9. ChBD of this

chitinase is required for the enzyme to bind specifically to insoluble chitin and to hydrolyze it efficiently(Takahisa, 2000). In contrast, chitinaseA from *S. marcescens* contains a putative ChBD in its N-terminal region (ChiN) but no sequence similarity exists between ChiN and ChBD from *B. circulans* WL-12. Moreover, important differences are found in the surface residues of these two domains. ChiN has adjacently arranged tryptophans exposed on a continuous surface with the conserved aromatic residues of catalytic domain. These residues play important roles in guiding a chitin chain into the catalytic site (Uchiyama, 2001).

Fibronectin Type III like Domain (FnIIID)

FnIIIDs have been identified in a set of depolymerase from bacterial and an animal fibronectin. It is likely that the bacterial modules were initially acquired from an animal source and were then spread further between distantly related bacteria by horizontal transfers (P. Bork, R. F. Doolittle, 1992). 3D Structure of FnIIID of chitinaseA1 *from B. circulans*WL-12 was solved by NMR technique. Structure of $FnIIID_{chiA1}$ is a β-sandwich fold with two antiparallel β-sheet that are packed face to face. One sheet is composed of three β-strands and the other of four β-strands. There are three loops in the each direction of N-terminus and C-terminus connects to the seven β-strands, as shown in Figure 1.10. FnIIID comprises domain-intrinsic and domain-specific regions. The former, made up of relatively conserved residues, are responsible for forming the FnIIID scaffold, which comprises a hydrogen-bond network and hydrophobic core. The scaffold is common to all FnIIID structures and endows the domain with its the mechanical extensibility against tension and its high refolding speed. In contrast, exposed residues that are not well conserved across the FnIIID family form the domain-specific regions. These residues often form the recognition site for the FnIIID of an interacting partner protein. It has been reported that the deletion of $FnIIID_{chiA1}$ has no impact on the chitin binding activity of chiA1, but causes a significant decrease in the colloidal chitin hydrolyzing activity. The natural function of the FnIIID remains unclear(Goo, 2002).

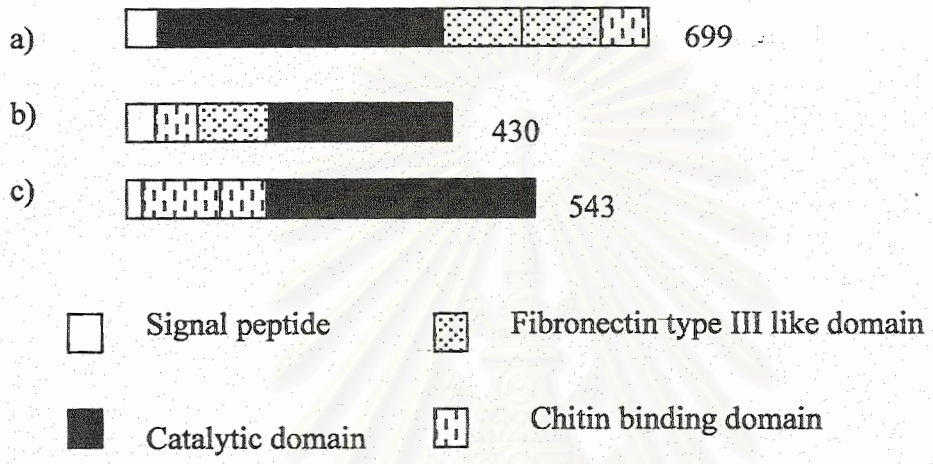


Figure 1.7 An arrangement of accessory domains of family 18 chitinase

An arrangement the different domains is differed for each chitinases;

a) ChitinaseA1 from *B. circulans*WL-12

b) ChitinaseC from *Alteromonas* sp.O-7

c) ChitinaseA from *Microbulbifer degradans* 2-40

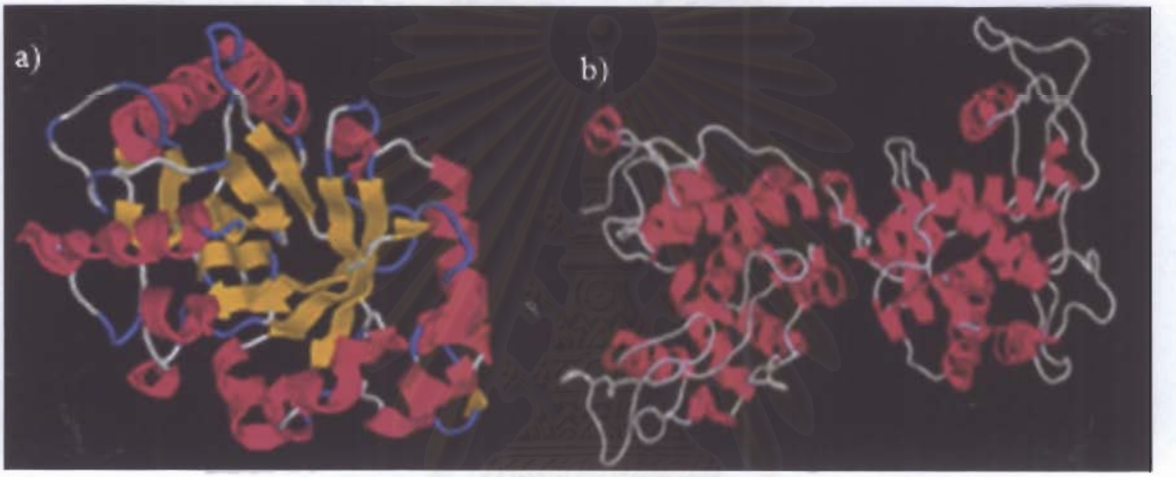


Figure 1.8 The 3D structures of some members of family 18 and 19 chitinases

a) The $(\beta/\alpha)_8$ barrel of family 18 chitinases.

b) The mixture of secondary structure of family 19 chitinases

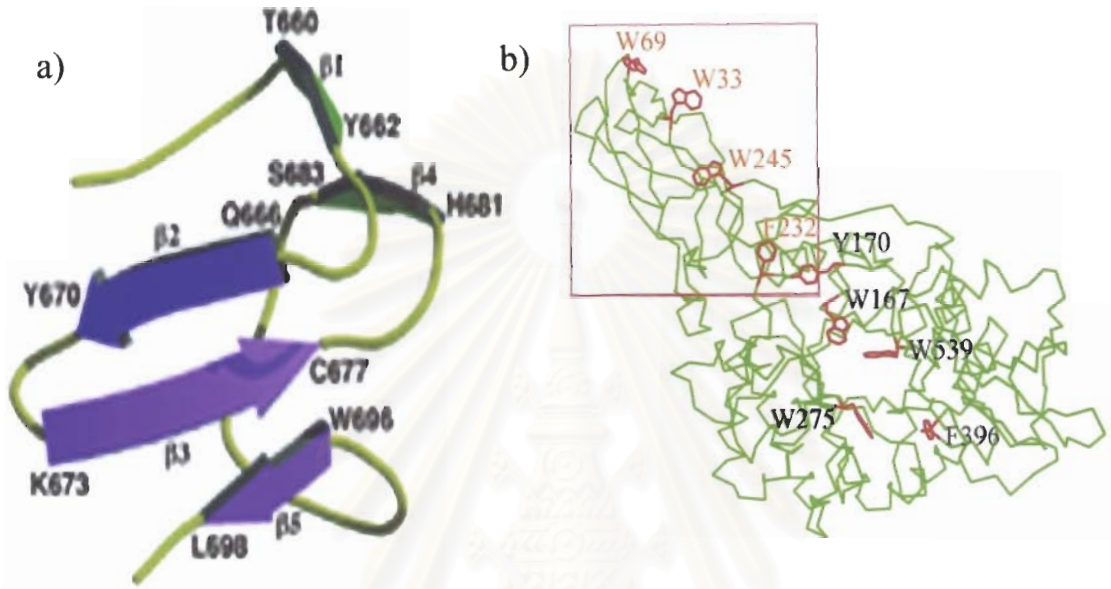


Figure 1.9 Chitin binding domain

a). A twisted β-sandwich of chitin binding domain of chitinaseA1 from *Bacillus circulans* WL-12. The secondary structure elements and both end residues of each β-strand are shown.

b). α-carbon chain (green) of *Serratia* ChiA with the side chains of aromatic residues (red). Four linearly aligned aromatic residues outside of the catalytic cleft are labeled with *orange* letters.

Figure 1.10 Fibronectin Type III likes Domain of chitinaseA1

A β-sandwich of Fibronectin Type III likes Domain of chitinaseA1 from *Bacillus circulans* WL-12

Mechanism of chitinase

There are two general mechanistic pathways for acid-catalyzed glycosyl hydrolysis which result in the following: 1) retaintion of the stereochemistry of the anomeric oxygen at C1' relative to the initial configuration or 2) inversion of the stereochemistry.

The substrate binding cleft of barley chitinase has been studied extensively. It has been hypothesized to contain at least six sugar binding subsites labeled A-F, from the nonreducing end. The hydrolytic profile for hexasaccharides by barley chitinase suggests the preferred binding of substrates may be at site B-G, that is, hexasaccharides are cleaved into two trisaccharides. This binding mode, together with the catalytic residues, is shown in Figure1.10. Two carboxylates are hypothesized to be responsible for the catalysis, Glu 67 as the catalytic acid and Glu 89 as a base. Hydrolysis would occur between sugar in sites D and E. The importance of these two residues to catalysis has since been confirmed by site-directed mutagenesis. Conversion of either of these acids to the corresponding amide eliminates measurable activity. The mechanism is hypothesized to be inverting, because the space between the second carboxylate, Glu 89 and the susceptible glycosidic bond demands that attacking water be interposed. This inverting mechanism is confirmed using nuclear magnetic resonance (NMR) to follow the anemic configuration of the sugar product, which is a α form. As indicated in Figure 1.11a), the inverting mechanism proceeds through a positively charged oxocarbenium intermediate which has a distorted geometry. It assumes a roughly half-chair configuration compared with the chair conformation of the other sugars. The single displacement mechanism involved Glu 89 acts as a base to polarize the attacking water molecule, whereas Glu 67 acts as an acid to protonate O4 of the leaving sugar.

The example of the retaining mechanism is from family 18 chitinases. The glycoside hydrolysis has been requires two acid residues one of which involves in protonation of the β-1,4-glycosidic oxygen atom, leading to a positively charged intermediate, as shown in Figure 1.11b. The secondary carboxylate group (either through covalent or electrostatic interactions) stabilizes the intermediate and then attacked by a solvent molecule, which replaces the leaving sugar group. Distortion of the D sugar by interactions between the enzyme and substrate is through to play a major role in transition state stabilization. This nucleophilic attack by water yields the

hydrolysis product, which necessarily retains the initial anomeric configuration. This is commonly referred to as the double displacement mechanism of hydrolysis (Brameld,1997).

It has also been suggested that the retaining mechanism of family 18 chitinases may involve substrate assistance. That is, the N-acetyl group at position 2 of the scissile sugar may itself facilitate the reaction via formation of a transient oxazolinium intermediate as shown in Figure 11c. This view is supported by the crystal structure of a complex between the chitinase called hevamine and inhibitor allosamidin which contains an oxazoline moiety which mimics the transition intermediate of the reaction (Scheltinga, 1995).

Application of chitinase

Chitinases in biocontrol of plant pathogenic fungi and insects

Insects and pathogenic fungi contain chitin in their protective membrane, induction of chitinases in plants is the main defense response. Most of these chitinases are induced in vegetative plant organs by infection but some are also present in seeds. Hadwiger and Beckman demonstrated that extracts of the pea endocarp contain chitinase and chitobiase activities. In fact, there is no other better proof for the contribution of plant chitinases in self defense than the formation of chitosan in the cell wall of a bean rust fungus, *Uromyces viciae-fabae,* to combat with the chitinase activity (Hadwiger and Beckman, 1980). Furthermore, Shapira et al. cloned *S. marcescens* chitinase gene in *E. coli* and the chitinase preparation obtained was found to be effective in reducing disease incidence caused by *S. rolfsii* in beans and *R. solani* in cotton under greenhouse conditions (Shapira *et al.*, 1989). The mycoparasitic and entomopathogenic fungi produce chitinases for invasion and as one of the host killing components. A *Fusarium chlamydosporum* strain, a mycoparasite of groundnut rust, *Puccinia arachidis* produces an endo-chitinase that inhibits germination of uredospores of the rust fungus. This indicates the significant contribution of chitinase in the biocontrol of groundnut rust (Mathivanan, Kabilan, and Murugesan, 1998). Chitinolytic enzymes of *T. harzianum,* a most studied mycoparasitic fungus, were found to be inhibitory to a wider range of deleterious fungi than similar enzymes from other sources (Lorito, 1993). To control pests such as longhorn beetles and aphids, the enzymatic treatment before or simultaneously

along with the entomopathogenic fungus, itself was successfully tried. The insect-pathogenic fungi, *Beauveria bassiana, B.*

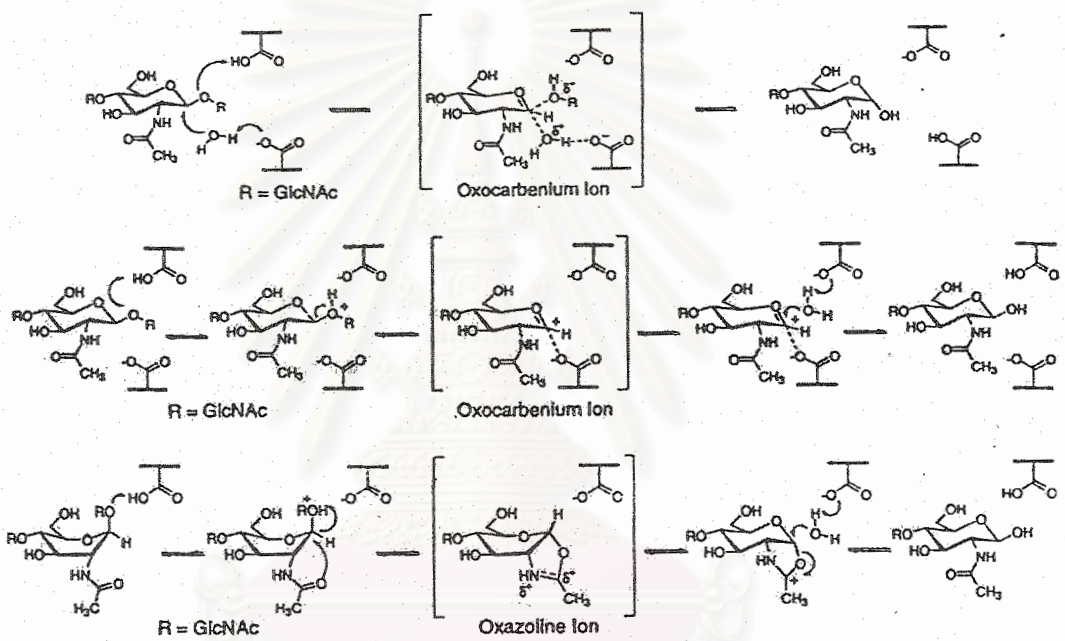


Figure 1.11. Chitinase mechanism

a). Double displacement hydroysis mechanism, which requires two acid residues in the active site and leads to retainion of the anomeric configuration.

b). Single displacement mechanism which requires only one acid residue in the acitve site and results in inversion of the anomeric configuration.

c). Anchimeric stabilization hydrolysis mechanism of family 18 chitinases where the substrate is distorted to a boat conformation and the oxazoline ion intermediate is stabilized through anchimeric assistance from the neighboring C2' acetyl group.

brongniartii, and *Verticillium lecanii* produced cuticle degrading enzymes when grown on chitin containing medium. The pretreatment of insects with the enzyme solution was reported to be useful.

Mosquito control

Entomopthogenic fungus like *B. bassiana* could not infect eggs of mosquitoes (*Aedes aegypti*), a vector of yellow fever and dengue, and related species may be due to aquatic environment. The scarabaeid eggs laid in the soil found to be susceptible to *B. bassiana*. *M. Verrucaria* is a fungus, which produces a total complex of an insect cuticle degrading enzymes. It has been seen that both first (I) and fourth (IV) instar larvae of a mosquito, which can be killed within 48 h with the help of the crude preparation from *M. verrucaria*. Though 100% mortality was observed with the crude enzyme (170-mg protein per liter) preparation within 48 h, with purified endo-chitinase lethal time (LT50) was 48 h and 120 h for I and IV instar larvae, respectively(Mendonsa, 1996).

Production of chitooligosaccharides

A chitinase from *Vibrio alginolyticus* was used to prepare chitopentaose and chitotriose, which showed anti-tumor activity from colloidal chitin (Murao *et al.*, 1992) Indeed, specific combination of chitinolytic enzymes with high levels of endo-chitinases and low activity of *N*-acetylglucosaminidase and exo-chitinases would be necessary to obtain the desired chain length of the oligomers. Whereas to obtain GlcNAc the higher proportion of exochitinase and *N*-acetylglucosaminidase was necessary. Alternatively, transglycosylation activity of variety of endo-chitinases will also be useful to generate desired chitooligomers or sometimes oligomers with changed glycosidic linkages. Nanjo *et. al.* observed accumulation of hexamer when tetramer or pentamer were incubated with *Nocardia orientalis* chitinase (Nanjo *et al.*, 1989). A chitinase from *T. Reesei* also exhibited similar type of efficient transglycosylation reaction (Usui, Matsui and Isobe, 1990). Stoyachenko, Varlamov and Davankov reported four major chitinases from *S. kurssanovii*. One of them (Chi-26) showed accumulation of hexamer in the reaction medium containing tetramer and

pentamer (Stoyachenko,Varlamov and Davankov, 1994). The purified *N*-acetylglucosaminidase of *N. orientalis* also showed transglycosylation activity. The β-1,6-linked disaccharide of GlcNAc and trisaccharide were synthesized during the hydrolysis of chitobiose (Nanjo *et al.*, 1990).

Single cell protein production

For the effective utilization of chitinous waste, Revah–Moiseev and Carrod used chitinase from *S. marcescens* to hydrolyze chitinous material and yeast, *Pichia kudriavzevii* for single cell protein (SCP) that was acceptable as aquaculture. The criteria used to evaluate SCP production are growth yield, total protein and nucleic acid contents. The protein contents in the organisms used were between 39 to 73% whereas the nucleic acid contents were 1–11%. *M. Verrucaria* chitinase was used for chitin hydrolysis and *S. cerevisiae* for SCP. The high *N*-acetylglucosaminidase activity in the culture filtrate of *M. verrucaria* yielded high levels of GlcNAc. The total protein contents were reported to be 61% with very low contents of nucleic acids (3.1%)(Vyas and Deshpande, 1991).

Miscellaneous applications

Fungal protoplasts have gained importance in the mycological research as a strain improvement for biotechnological applications. Chitinase/chitosanase is one of the major components of the fungal cell wall lysing enzyme complex. It has been seen using various mycolytic enzyme preparations singly or in combination for protoplast isolation that high chitinase levels permit effective fungal mycelia degradation (H. S. Kelkar, Shankar and Deshpande,1990). The chitinase-gold complex can be used for locate sugar residues in thin sections of plants and fungi that have chitinous cell wall (Benhamou and Asselin, 1989). A wide spread ability to produce *N*-acetylglucosaminidase has been observed in fungi. Therefore, *N*-acetylglucosaminidase activity of the soil samples was significantly correlated with the estimate of fungal biomass. Similarly, using chitinase and chitin-binding protein a method has been suggested for the detection of fungal infection in humans (Laine, 1998). The enzyme like tannase is used in the food industry to remove unwanted tannins and for producing gallic acid that is used as preservative. The enzyme produced by *Aspergillus niger*, is strongly bound to the mycelium and its release by

chemical and physical means is not efficient. Enzymatic hydrolysis of the cell walls using chitinase preparation was found to be effective in the recovery of tannase enzyme (Barthomeuf, Regerat and Pourrat, 1994).

VIII Chitosanase

Chitosanases have been generally recognized as enzymes that attack chitosan but not chitin. The Enzyme Commission defines chitosanase (EC 3.2.1.13) as an enzyme catalyzing the endo hydrolysis of β-1,4-glycosidic linkage between GlcNAc and GlcN residues in the partially acetylated chitosan or between GlcNs in the 100% deacetylation(DD) chitosan.

Classification of chitosanase according to their mode of action.

The specificity of linkage recognition for chitosanase was usually investigated using 20-30% acetylated chitosan as substrate. Chitosanases were classified into three groups. The first group hydrolyzed only GlcN-GlcN, the second group hydrolyzed GlcNAc-GlcN or GlcN-GlcN, and the third group hydrolyzed GlcN-GlcNAc or GlcN-GlcN, as shown in Table 6 (Fukamizo *et al.*, 1994).

Classification of chitosanases based on amino acid sequence

Henrissat *et. al.* divided chitosanases into four families 8, 46, 75 and 80 based on their primary structures. The most studied chitosanases belong to the family 46 of glycosyl hydrolases such as chitosanases from *B. circulans* MH-K1, *B. ehimensis* and *Streptomyces* sp.174. In 1996 Shimosaka *et al.* reported another chitosanase sequence, which was produced by *Fusarium solani*. It had no detectable similarity with family 46 enzymes. The same reserch group has determined recently the sequence of a new fungal chitosanase from *Aspergillus oryzae*. These two chitosanases are also similar to the enzyme from *A. fumigatus* and *Metarhizium anisopliae*. These four enzymes are now members of the family 75. A new chitosanase from *Mastsuebacter chitosanotabidus* had been characterized. Its sequence is highly similar to that from *Spingobacterium mnltivorum*. These two chitosanases have been classified in the family 80. Family 8 includes enzymes with more diversified substrate specificcities than the other above listed families. Besides chitosanases, it includes cellulases, lincheninases and endoxylanases. The best characterized chitosanase from this family

is the enzyme from *Bacillus* sp. No.7M, the only known chitosanase which cleavage specifictity is restricted to the GlcN-GlcN linkage. Another extensively studied chitosanases belonging to this family are produced by *B. circulans* WL-12 and *Paenibacillus fukuinensis* D2.

Table 1.6 Three groups of chitosanases are classified on the basis of the degradation products of partially acetylated chitosan substrate (Fukamizo *et al.*, 1994).

	Group I	Group II	Group III
Degradation products	●◙ ●●◙ ●◘ ●●◘ ●○◘ ●●○◘ ●○○◘ ●○○○◘	●◙ ●●◙ ●●●◙ ●○●●◙ ●●○●◙ ●○●●●◙ ●●○●●◙	●◙ ●●◙ ●○●◙ ●●○●◙ ○●●◙
Cleavage sites	GlcN-GlcN, GlcNAc-GlcN	GlcN-GlcN	GlcN-GlcN GlcN-GlcNAc

● GlcN, ○ GlcNAc, ◙ GlcN at reducing end and ◘ GlcNAc at the reducing end

a).

```
BAC_CIRC        KPEQDDLNWIKYYGYCEDIEDEDERGYTIGLFGATTGGSRDTHPDGPDLF
BAC_EHIM        KPEQDDLNWIKYYGYCEDIND-DERGYSIGIFGATTGGPRDTHPDGPELF
BUR_GLAD        KPEQDSLDWTKFYGYCEDIGD--DRGYTMGIFGATTGGPNDGGPDGPALF
STR_N174        SAENSSLDWKAQYKYIEDIGD--GRGYTGGIIGFCSG----TG-DMLELV
NOC_N106        SAENSSLDWKSQYKYIEDIKD--GRGYTAGIIGFCSG----TG-DMLDLV
CVK2            LPENSTTQWWKNYNYASCLKD--GRGWTVTIYGACSG----TG-DLLMVL
                 .*:.  :*   * * . : *   **::  : *  :*       *   :.
```

b).

```
A_FUMIGA     DLDANIHPYVVFGNE----DHSPKFKPQSHGMQPLSVMAVVCNGQLHYGIWGDTNG
M_ANISOP     DLDANLHPYVVFGNE----GASPSFNPQSKGMKPLSVMAVVCNNQVFYGVWGDTNG
A_ORYZAE     DLDANIHPYVVFGNEN-DDGDDPEFDPRKHGMEPLSVMAVVCNKKLFYGIWGDTNG
F_SOLANI     DLDANIHPYIVFGNEGGTKKGWKTFDPEKHGIKPLSVMAVVCGNKMFYGIWGDENG
             *****:***:*****         *.*..:*::*********. ::.**:*** **
```

c).

```
MAT_CHIT        YPENGTTNYQEVGPWRYCEVDYEAAQGISDYRGDTFGPVGVTTV
SPH_MULT        YPENGTTNYQDPEPWRYCEVDYEASEGISDYRGNTFGPVGVTTV
                **********:  ***********::*******:**********
```

Figure 1.12 Alignment of several chitosanases

The local alignment of several chitosanases from

a). Glycosyl hydrolase family 46 b). Glycosyl hydrolase family 75

c). Glycosyl hydrolase family 80

Three dimensional structure of chitosanases.

The structure information of chitosanses is available only from crystal structure of *B. circulans* MH-K1 and *Streptomyces* sp.174, which belongs to family 46 chitosanase. From overall structures comparison of these two enzymes (see Figure1.13), although the sequence identity is only 20% but the superimposed C-α atoms of each domain show a high degree of similarity in both secondary and tertiary structures. Only four marked differences are found in the molecular structure. First, the N-terminal region of MH-K1 chitosanase is 16 residues longer than the N174 chitosanase. So, there are two additional helices. Second, MH-K1 chitosanase has two β-strands following α-helices in the top region of an updomain, where N174 chitosanase has only a α-helix in the updomain of the enzyme. Third, MH-K1 chitosanase has and additional protruding loop of the cleft, whereas there is only a short loop in N174 chitosanase. Forth, the chitosanase structures are completely different at the C-terminal region of the chitosanases, e.g.helical structure in MH-K1 chitosanase and two β-sheets in N174 chitosanase(Marcotte *et al.*,1996 and Saito *et al.*, 1999).

Mechanism of chitosanase

The binding model was constructed on the basic of the structure of human lysozyme complexed with tetrasaccharides. The interaction between the cleft and the substrate analogue was specified only at three subsite C, D and E among the six sugar, where E was a reducing end. (as shown in Figure 1.14) The substrate was cleaved between D and E sugars by the two catalytic residues, glutamic acid and aspartic acid. Like chitinase and other glycosyl hydrolases, chitosanases are classified into two mechanisms of action duing to the substrate binding site structure; the average distance between the two catalytic residues is ~ 9.5 Å in inverting enzyme, whereas it is 5.3 Å in retaining enzyme. MH-K1 chitosanase and N174 chitosanase are the inverting enzyme because of the distance between oxygen atom of two catalytic residues was 13.8 and 10.9, respectively. When binding to chitosan hexamer, these two catalytic residues make close contact with the glycosidic bond between D and E sugars. Glutamic acid in the central helix acts as a proton donor, which donated a proton to the aspartic acid in the upper domain. Aspartic acid activates a water

molecule, which then attacks the C1 carbon of the sugar located in the catalytic site (Fukamizo *et al.*, 2001 and Saito *et al.*, 1999).

Biological role of chitosanases

Chitosanases can be used for nutritional purposes by microbs growing on chitosan or chitin containing sustrates. Chitosanases of certain fungi probably function in the solftening or lysis of cell walls for the initiation of hyphal branches and for cell seperation during growth. In the autolytic phase of growth of *Mucor rouxii*, chitosanases are involved in the cell wall degradation (Alfonso, Martinez and Reyes, 1992). In various plants species, chitosanases are secreted as pathogenesis related proteins. Chitosanase activity was detected in VAM colonized leek (*Allium porrum*) and onion (*Allium cepa*) roots. VAM fungi could trigger several host defense responses. Chitosanases have antimicrobial activity which could be active during VAM symbiosis, but their significance in symbiotic interactions is unknown (Dumas-Gaudot. *et al.*, 1992). In peanut, the chitosanase is involved in the defense mechanism against pathogenic and toxigenic fungi (Cureo *et al.*,1992). It is assumed that chitosanases could act as defense enzymes in plants against pathogenic fungi containing chitosan in their cell walls.

The constitutive hydrolase with dual activity was detected in tomato stems (Pegg and Young, 1982)) and in bacterial (Watanabe *et al.*, 1992) . Recently, acidic hydrolases with chitinase and chitosanase activities were detected in a sweet orange callus tissue (*Citrus sinensis*)(Osswald *et al.*, 1994). From this evidence suggested that enzymes possessing both chitinase and chitosanase activity would be advantageous to plant defensive mechanism as a wider variety of plant pests could be attacked. Also, oligosaccharides produced as a result of chitinase/chitosanase action on fungal pathogens may act as elicitors to induce or trigger host defense reactions. They also consider that from the standpoint of energy conservation in the biological system, it is much more efficient to have multifunctional enzymes were though to the responsible for the release of the chitosan oligomer from the fungal cell wall, which have antimicrobial activities.(Kendra, Christian and Hadwiger, 1989) Thus, chitosanses are involved in various functions in different organisms, and these may be nutritional, physiological or defensive.

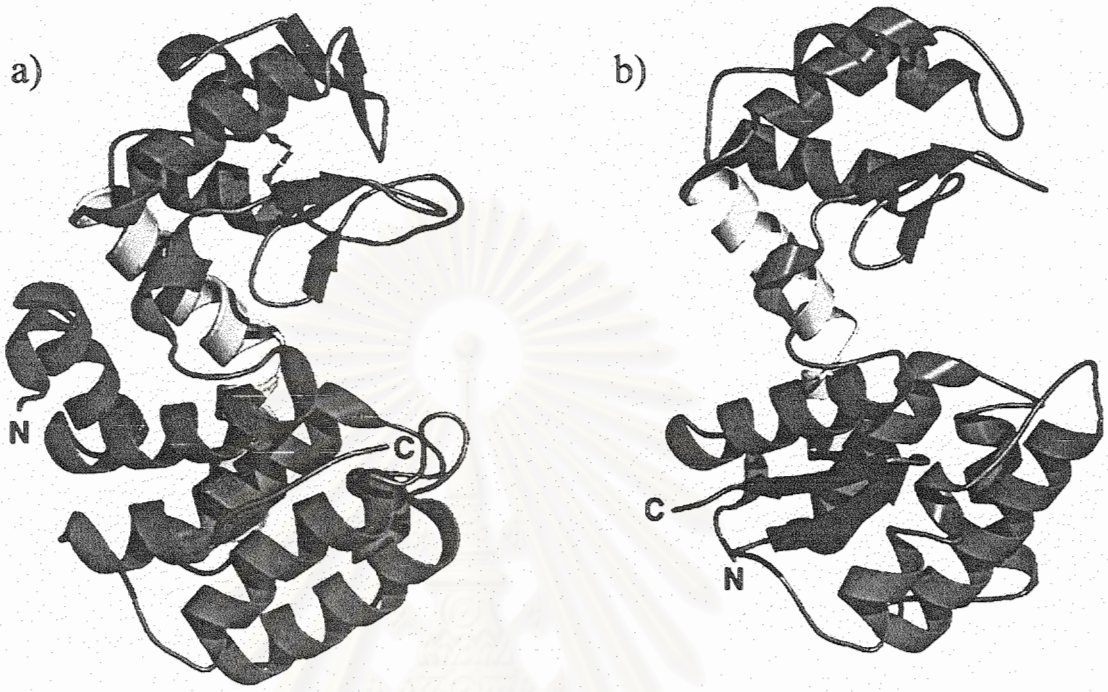


Figure 1.13 The structure comparison of chitosanases from *Bacillus* and *Streptomyces*

The overall structures of MH-K1(left) and N174 (right) chitosanases are illustrated as ribbon diagrams. The backbone helices are shown in yellow. The protruding roof ofthe dleft in MH-K1 chitosanase is shown in pink.

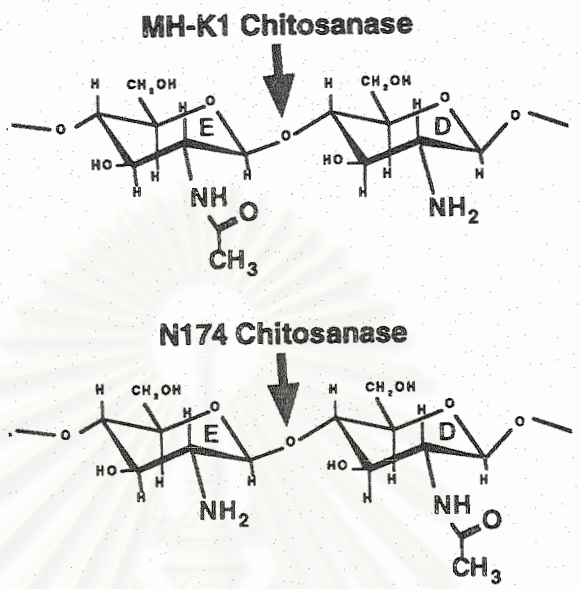


Figure 1.14 Substrate cleavage position of MH-K1 and N174 chitosanase

The substrate cleavage position for the partially acetylated chitosan in MH-K1 and N174 chitosanase.

Applications of chitosanases

Chitosanase have been used for the generation of fungal protoplasts, especially of the membranes of Mucorales that is used as a model for somatic hybridization in genetic studies (Fenton *et.al.*, 1978). Chitosanase has been used for the preparation of chitosan oligomers which possess biological properties, such as induction of callose (Conrath et al., 1989; Cheng and Lim, 2000), immunopotentiation (Suzuki *et al.*, 1984; Papineau *et al.*, 1989) and inhibition of growth of plant pathogens (Price and Storck, 1975; Linthorst, 1991)

In previously work, a chitinolytic bacterium was screened from soil in Thailand. A bacterium from Krabi province able to hydrolyze crystalline chitin. This strain was identified as a *Bacillus circulans* PP8. Strain PP8 produced chitinase and chitosanase when it is grown in medium containing 0.02% colloidal chitin. In addition, chitinase gene from this bacterium was cloned by shot gun cloning. There were 3 colonies produced a clear zone around the colony and were designated pST24, pST847 and pST1691. pST847 with the highest chitinase activity was chosen for this study.

In this study, a chitinase gene from pST847 was sequenced and the chitinase gene was expressed by using two expression systems. Chitinolytic enzyme from the original strain and the clone were characterized. A recombinant chitinase expressed by pET expression system was purified and characterized.

CHAPTER II

MATERIALS & METHODS

1. Equipments

Autoclave : Model HA-30, Hirayama Manufacturing Corperation, Japan.

Autopipette : Pipetman, Gilson, France.

Centrifuge : Refrigerated centrifuge : Model J-21C, Beckman Instrument Inc., U.S.A.

Centrifuge : Microcentrifuge High Speed : Model 1110 Mikro 22R, Hettich Zentrifugen, Germany.

Electrophoresis Unit : Model Mini-protein II Cell, BioRad, U.S.A.

Incubator : Model OB-28L Fisher Scientific Inc., U.S.A.

Magnetic stirrer and heater : Model IKAMA®GRH, Janke & Kunkel Gmbh & Co.KG, Japan.

pH meter : PHM 83 Autocal pH meter, Radiometer, Denmark.

Membrane filter : cellulose nitrate, pore size 0.2 μ, Whatman, Japan.

Spectrophotometer : Jenway 6400, England.

Vortex : Model K 550-GE, Scientific Industries, U.S.A.

Water bath : Charles Hearson Co. Ltd., England.

U.V. transilluminator : 2011 MA Crovue, San Gabrial, U.S.A.

Transformation apparatus : Gene pulser™ : BioRad, U.S.A.

High performance liquid chromatography : Shimadzu, Japan.

Orbital shaker : Gallenkamp, Germany.

Power supply : Model EC 135-90, E-C Apparatus Corperation.

Microcentrifuge tubes 1.5 ml : Bioactive, Thailand.

Sequencer : Model CEQ™8000 Genetic Analysis system, Beckman Coulter, U.S.A.

2. Chemicals

Acetonitrile (HPLC grade), Merck, Germany.

Acetone, Mallinekrodit, U.S.A.

Acrylamide, Merck, Germany.

Agarose, SEAKEM LE Agarose, FMC Bioproducts, U.S.A.

Ammonium hydroxide, Merck, Germany.

Ammonium persulfate, Sigma, U.S.A.

Ammonium sulfate, Sigma, U.S.A.

Ampicillin, Biobasic Inc, Thailand.

Bacto-Agar, DIFCO, U.S.A.

Boric acid, Merck, Germany.

Bovine Serum Albumin (BSA), Sigma, U.S.A.

Bromophenol blue, Merck, Germany.

β-mercaptoethanol, Fluka, Swithzerland.

5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), Sigma, U.S.A.

Calcium chloride, Merck, Germany.

Chloroform, Sigma, U.S.A.

Citric acid, Sigma, U.S.A.

Coomassie® brilliant blue R, Acros organics, Belgium.

Coomassie® brilliant blue G 250, Fluka, Switzerland.

CM-cellulose, Sigma, U.S.A.

DEAE-cellolose, Sigma, U.S.A.

Deoxyribonucleic acid, Promega, Co. Ltd., U.S

Diethylbarbituric acid, Sigma U.S.A.

D(+) glucosamine hydrochloride, Sigma, U.S.A.

Di-potassium hydrogen phosphate anhydrous, Carlo Erba Reagenti, Italy.

Di-Sodium hydrogen phosphate, Fluka, Switzerland.

Dislysis tubing, Sigma, U.S.A.

Ethidium bromide, Sigma, U.S.A.

Ethylenediamine tetraacetic acid (EDTA), Fluka, Switzerland.

Ethyl alcohol absolute, Carlo Erba Reagenti, Italy.

Ethylene glycol chitin, Seikagrku Corporation, Japan.

Ethylene glycol chitosan, Seikagrku Corporation, Japan.

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

Flaked chitin, Seafresh, Thailand.

Flaked chitosan, Seafresh, Thailand.

Fluorescent brightener 28, Sigma U.S.A.

Formaldehyde, Sigma, U.S.A.

Glacial acetic acid, BDH, England.

Glucose, Sigma, U.S.A.

Glycerol, Scharlau, Spain.

Glycine, Sigma, U.S.A.

Hydrochloric acid, Lab Scan, Ireland.

Isopropyl-β-D-thiogalactopyranoside (IPTG), Serva, Heidelberg, Germany.

Isoamyl alcohol, Merck, Germany.

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

Magnesium sulfate 7-hydrate, BDH, England.

Methanol, Scharlau, Spain.

N-acetyl-D-glucosamine, Sigma, U.S.A.

N, N'-dimethylformamid, Merck, Germany.

N, N'-methyl-bis-acrylamide, Sigma, U.S.A.

NNN'N'-tetramethyl-1,2-diaminoethane, Carlo Erba Reagenti, Italy.

Phenol, BDH, England.

85%Phosphoric acid, Lab Scan, Ireland.

Potassium acetate, Merck, Germany.

Potassism cholride, Sigma, U.S.A.

Potassium ferricyanide, BDH, England.

Potassium phosphate monobasic, Carlo Erba Reagenti, Itly.

Qiaquick Gel Extraction Kit, Qiagen, Germany.

Qiaquick Miniprep Kit, Qiagen, Germany.

Sodium azide, BDH, England.

Sodium carbonate, BDH, England.

Sodium chloride, Univar, Australia.

Sodium dihydrogen orthophosphate, Carlo Erba, Italy.

Sodium dodecyl sulfate, Sigma, U.S.A.

Sodium hydroxide, Carlo Erba, Italy.

Silver nitrate, Baker Analyed, Germany.

Tris-base, USB, U.S.A.

Tri-Sodium citrate dihydrate, Carlo Erba, Italy.

TritonX, Merck, Germany.

Tryptone, Scharlau, Spain.

Xylene cyanol FF, Sigma, U.S.A.

Yeast extract, Scharlau, Spain.

3. Bacterial strains

Escherichia coli BL21(DE3) *(*F$^-$ *ompT hsdS(*$r_B m_B$*) gal dcm(*DE3*)* was used for *chi66* expression.

Escherichia coli BL21(DE3)pLysS *(*F$^-$ *ompT hsdS(*$r_B m_B$*) gal dcm*(DE3) pLysS(Cm^R) was used as high–stringency expression host.

Escherichia coli JM109 (*F$^-$ traD36 proA$^+$ proB$^+$ lacI^q lacZ ΔM15/recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB, mcrA)*) was used for DNA manipulation.

Escherichia coli Rosetta(DE3)pLysS *(F$^-$ ompT hsdS(*$r_B m_B$*) gal dcm lacY1*(DE3) pLysSRARE(Cm^R) , which provieded rare codon tRNAs was used as high–stringency expression host.

Escherichia coli TOP10 (F$^-$mcrA, *Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697galU galK rpsL (Str^R) endA1 nupG* was used as expression host for overexpreesion vector, pTrcHis2C.

4. Plasmid vectors

4.1 pBlueScript/SK$^-$ was used as subcloning vector of pST847 for chitinase gene localization and DNA sequencing

4.2 pET19b was used as overexpression vector for chitinase gene.

4.3 pGEM T-easy was used as an alternative vector for TA cloning.

4.4 pTrcHis2C was used as the same purpose as pET19b

4.5 pST847, which has chitinase activity was used as DNA template for subcloning and chitinase gene localization.

5. Enzymes

5.1. Restriction endonuclease

- *Bam*HI, *Cla*I, *Pst*I, *Nco*I, N*otI* , *Sac*I and *Xho*I were purchased from New England Biolabs Inc., U.S.A.

- *Eco*RI, *Hind*III were purchased from GIBCOBRL, U.S.A.

5.2. Calf intestine alkaline phosphatase was purchased from GIBCOBRL, U.S.A.

5.3. Lysozyme was purchased from Sigma, U.S.A.

5.4. Pfu polymerase was purchased from Promega, Co. Ltd., U.S.

5.5. Proteinase K was purchased from GIBCOBRL, U.S.A.

5.6. RNase A was purchased from Sigma, U.S.A.

5.7. T_4 DNA ligase was purchased from Promega, Co. Ltd., U.S.A.

6. Media preparation

Luria-Bertani broth (LB medium)

LB broth consists of 1%(w/v) Bacto tryptone, 0.5%(w/v) yeast extract and 0.5%(w/v) NaCl. For solid medium, 1.5%(w/v)agar was added, supplemented with 100 μg/ml ampicillin when needed.

LB-Coloidal chitin agar

LB-starch agar consists of 1%(w/v) Bacto tryptone, 0.5%(w/v) yeast extract, 0.5%(w/v) NaCl and 0.05% (w/v, dry weight) colloidal chitin. For solid medium, 1.5%(w/v)agar was added, supplemented with 100 μg/ml ampicillin when needed.

Colloidal Chitin Minimum Medium (CCMM)

Colloidal chitin minimum medium was used for enzyme production. The medium consists of 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)$MgSO_47H_2O$, 0.6%(w/v)KH_2PO_4 and 1%(w/v)K_2HPO_4 with pH 7.5. For solid medium, 1.5% agar was added, supplemented with 100 μg/ml ampicillin when needed.

Chitosanase Detection Medium (CDM)

Chitosanase detection medium is a selective media for screening of chitosanase producing colony. The medium consists of 0.1%(w/v, dry weight)colloidal chitosan, 0.05%(w/v)yeast extract, 0.1%(w/v)$(NH_4)_2SO_4$, 0.1%(w/v)NaCl, 0.03%(w/v) and 0.03%(w/v)K_2HPO_4 with pH 7.0. For solid medium, 1.5% agar was added, supplemented with 100 μg/ml ampicillin when needed.

7. Identification of bacterial

Biochemical characteristics

Culture of *Bacillus.* sp.PP8 was first chacterized by Thailand Institute of Scientific and Technological Research, Ministry of Science and Technology and

recharacterized by The National Institute of Health, Department of Medical Sciences, Ministry of Public Health the data from the two organizations were compared.

Characterization of a gene coding for 16S ribosomal RNA

The partial 16S ribosomal RNA gene of *Bacillus.* sp.PP8 was *in vitro* amplified via the Polymerase Chain Reaction by modified method of U. Edwards *et. al*.(U. Edwards, 1989) Using the thermal profile involved 30 cycles of denaturation at 94^{o}C for 1 min., primer annealing at 60^{o}C for 2 min., and extension at 72^{o}C for 3 min. The following oligonucleotides were used in *in vitro* amplification and for sequencing

pA 5'-AGAGTTTGATCCTGGCTCAG-3'
pD 5'-CAGCAGCCGCGGTAATAC-3'
pE 5'-AAACTCAAAGGAATTGACGG-3'
pH' 5'-AAGGAGGTGATCCAGCCGCA-3'.

The amplified PCR product was purified by using QIAgen quick Gel Extraction kit (QIAgen, Germany), cloned into pGEM T-easy and transformed to *E. coli* Top-10. The recombinant colonies were selected by blue-white colony screening. The plasmid containing 16S ribosomal RNA gene was sequenced using the dideoxy-chain termination method with CEQ^{TM} 8000 Genetic Analysis system. The sequence of 16S ribosomal RNA gene was aligned with others obtained from GenBank using Basic Local Alignment Search tool (BLAST)(S. F. Altschul *et. al*., 1990)

8. Chitinase and chitosanase assay

Chitinase and chitosanase activities were determined by modification of Schales' method. The assay was based on the increase of reducing sugar. In a total volume of 1.5 ml consisting of an appropriate amount of the enzyme solution, 1.0 mg/ml colloidal chitin (10 mg/ml powdered chitin) or 1.0 mg/ml colloidal chitosan, 0.15 ml of 1.0 M Tris-HCl at pH 8.0 or 7.0 for chitinase and chitosanase assay, respectively. Reaction mixture was incubated at 50^{o}C for an hour. Two ml of color reagent (color reagent was made by dissolving 0.5 g potassium ferricyanide in 1 litter of 0.5 M sodium carbonate and stored in a brown bottle) were mixed with reaction mixture in a test tube stoppered with glass ball. The hydrolysis reaction was stopped by boiling for 15 min. After rapid cooling, an excess colloidal substrate was removed

by centrifugation at 5000 g for 10 min. The adsorbance of the supernatant (A_1) was measured at 420 nm by a spectrophotometer against deionized water. A blank value (A_0) was obtained by using pre-heat enzyme (heating in boiling water for 20 min) instead of the native enzyme. The enzyme activity was determined from the difference between A_0 and A_1. One unit (U) of enzyme activity was defined as the amount of the enzyme capable of liberating 1 μmole *N*-acetylglucosamine or D-glucosamine per min (appendix B and C). Specific activity was defined as units per mg protein of the enzyme.

9. Detection of chitinase activity after SDS-PAGE.

After electrophoresis, gel was divided into two panels. The first one was stained with coomassie blue R-250. The second one was incubated overnight at 37^{o}C in 0.1 M Tris-HCl pH 7.0-8.0 containing 1%(v/v) Triton X-100. A residue of Triton X-100 was removed by washing with deionized water. Gel was then stained with 0.01% (w/v) fluorescent brightener for 15-30 min and destained with deionized water. The hydrolysis zones were visualized through a transilluminator.

10. Cultivation of *Bacillus.* sp.PP8 and chitin modifying enzyme production of *Bacillus.* sp.PP8

To study the relation between enzyme production and cultivation time, the culture mediums at time interval of cultivation were determined for enzyme activity. A single colony of *Bacillus* sp.PP8, which produced clear zone around the colony, was grown in 2 ml of LB medium at 37^{o}C overnight with 250 rpm rotation sharking as an innoculum. 0.5 ml of the innoculum was innoculated to 50 ml of 0.02% colloidal chitin minimum medium in 250 ml Erlenmeyer flask and incubated with 250 rpm shaking at 37^{o}C. 1 ml of culture medium was collected during 108 hours at 12 hours interval for enzyme activity assay.

To study the effect of various carbon sources on enzyme production, *Bacillus* sp.PP8 was grown in 50 ml of minimum medium containing 0.02% colloidal chitin, 0.2% powdered chitin, 2% flake chitin, 0.02% colloidal chitosan, 2% flake chitosan, 0.2% GlcNAc and 0.2%GlcN in 250 ml Erlenmeyer flask. The culture were incubated with 250 rpm shaking at 37^{o}C, and 1 ml of culture medium was collected everyday for 5 days. Activity of enzymes in culture medium was assay.

11. Characterization of chitinase and chitosanase

Chitinase and chitosanase activities were assayed by the colorimetric method as previously described for their properties as follows:

Optimum pH of chitinase and chitosanase

The optimum pH of chitinase and chitosanase was determined by using different pHs buffer as reaction buffer. In pH range of 2-6, citrate buffer was used. In the next range, phosphate buffer was used in pH range of 6-7. In pH range of 7-10, Tris-HCl buffer was used. To reduced an ionic strength effect of various salts, an universal buffer (pH 3-11) was used to correct this effect. The reaction mixtures at different pH were incubated as previously described in chitinase assay.

Optimum temperature of chitinase and chitosanase.

The optimum temperature for chitinase and chitosanase activity was determined by incubating the reaction mixture at different temperature range of 20-90^{o}C. The, increase of the reducing end was measured using standard assay.

Enzyme stability

The stability of crude enzyme in the 50 mM buffer at various pH values (pH 2-10) at 4^{o}C was investigated. The remaining enzymes activity was measured everyday for 5 days by using 1.0mg/ml colloidal chitin or 1.0mg/ml colloidal chitosan as substrate.

The stability of crude enzyme on temperature was determined by storing the enzyme in 50 mM Tris-HCl, pH 7.0 and 8.0 at 4^{o}C, 30^{o}C, 37^{o}C and 50^{o}C. The crude enzyme was collected at 2 hours interval for 12 hour and the remaining activity was determination.

Substrate specificity

The crude and the partially purified enzyme were incubated with each of following substrates; 1.0 mg/ml colloidal chitin, 1.0mg/ml powdered chitin, 1.0mg/ml regenerated chitin, 0.1 mg/ml partially *N*-acetyl chitin (PNAC), 0.1 mg/ml glycol chitin, 1.0 mg/ml flake chitin, 0.1 mg/ml glycol chitosan, 1.0 mg/ml 80%DD colloidal chitosan 1.0 mg/ml 100%DD colloidal chitosan and 1.0 mg/ml flake chitosan at 50^{o}C for an hour. The increase of reducing end was determined using standard assay.

12. Determination of hydrolytic products by chitinolytic enzyme from *Bacillus.* sp.PP8

To study a hydrolysis product of the crude enzyme of strain PP8, 50 mU/ml of crude chitinase was incubated under optimum conditions for an overnight. The completely hydrolysis was boiled for 15 min and centrifuged at 10,000 rpm for 10 min. Then 0.3 ml of a supernatant was withdrawn and mixed with 0.7 ml of acetronitrile, and filtrated through a 0.45 micron filter. The hydrolysis product was analyzed by HPLC with the following conditions; Shode Asahipak NH2P-50 colum, 30:70 water:aetronitrile and flow rate 1.0 ml/min, 20 μl injection and 210 nm UV detector.

13. General techniques in genetic engineering.

Preparation of competent cells

A single colony of *E. coli* was cultured as a starter in 2 ml of LB-broth and incubated at 37 °C with 250 rpm shaking for an overnight. The starter was diluted in 200 ml of LB-broth, and the culture was incubated at 37 °C with 250 rpm shaking until the optical density at 600 nm of the cells reached 0.5-0.6 (~3-4 hours).The culture was chilled on ice for 15 minutes and the cells were harvested by centrifugation at 5,000 rpm for 15 minutes at 4 °C. The supernatant was discarded. The cell pellet was washed twice with 1 volume and 0.5 volume of cold sterile ultrapure water, respectively. The cells were resuspended and centrifuged at 6,000 rpm for 15 minutes at 4°C. The supernatant was discarded. The pellet was washed with 10 ml of ice cold sterile 10% (v/v) glycerol, and finally resuspended in a final volume of 1-2 ml of ice cold sterile 10% glycerol. The cell suspension was divided into 40 μl aliquots and store at -80°C until used.

Electroporation

The competent cells were thawed on ice. Forty microlitres of the cell suspension were mixed with 1-2 μL of the ligation mixture. Then mixed its thoroughly and placed on ice for 1 minute. The mixture was electroporated in a cold 0.2 cm cuvette with the apparatus setting as follows ; 2.5 μF, 200 Ω of the pulse controller unit and 2.50 kV.

After one pulse was applied, the cells were resuspended in 1 mL of LB broth and incubated at 37 °C for 1 hour with shaking at 250 rpm. The cell culture were spread on the LB agar, CCMM agar or CDA containing 100 μg/ml and appropriated amount of IPTG and X-gal for the blue-white colony screening.

Plasmid preparation

Plasmid harboring cells were cultured in LB broth(1.5 ml) and harvested by centrifugation at 5000 x g, 4°C for 3 minutes. The packed cells were resuspended in 100 μl of Solution I (25 mM Tris-HCl, pH 8.0, 10 mM Na_2EDTA and 50 mM glucose), mixed by vortexing well and kept on ice for 15 minutes. A 200 μl of Solution II (1% SDS, 0.2 N NaOH) was added, mixed by inversion and kept on ice for 5 minutes. The mixture was neutralized by adding 150 μl of Solution III (3 M sodium acetate, pH 4.8), mixed by inversion and kept on ice for 30 minutes. After centrifugation at 12,000 rpm for 10 minutes, and aqueous phase was collected. The supernatant was extracted with one volume of phenol:chloroform:isoamyl alcohol (25:24:1). Two volumes of absolute alcohol were added, mixed and stored at –20 °C for 30 minutes. The plasmid was pelleted by centrifugation at 12000 rpm for 10 minutes, washed with 70% ethanol and air dried for 10 minutes. The pellet was dissolved in TE buffer containing 20 μg/ml DNase-free RNaseA.

Agarose gel electrophoresis

To determined the size of DNA using 0.7-1.5% agarose gel in TAE buffer (40 mM Tris-HCl, 20 mM acetic acid and 2 mM EDTA, pH 8.0), DNA samples with 1x tracking dye were loaded into the wells. The gels were run at 100 volts for 1 hour, or until bromophenol blue reached the bottom of the gel. After electrophoresis, the gels were stained with ethidium bromide solution(2.5 μg/ml)for 2-5 minutes, and the DNA bands were visualized under UV light from UV transluminator. The gels were photographed through Syngene gel documentation. The sizes of DNA fragments were determined by comparing the relative mobility with those of the standard DNA fragments(λ/*Hin*dIII and pBR322/*Msp*I marker).

DNA fragment extraction from agarose gel

QIAquick gel extraction kit was used for extracting DNA fragment from agarose gel, and performed according to the kit protocol. Briefly, DNA fragment was excised from an agarose gel, added 3 volume of buffer QG and incubated for 10 minutes at 50°C. After the gel slice has dissolved completely, the sample was applied to the QIAquick column, and centrifuged for 1 minute. The flow-through was discarded. Buffer QG was added and centrifuged for 1 minute. The column was washed twice with buffer PE and centrifuged for 1 minute. Finally, the elution buffer was added to the center of the QIAquick membrane to elute the DNA, the column was left stand for 1 minute, and then centrifuged for 1 minute.

14. Restriction mapping of pST847 to determine the restriction site on the plasmid.

The pST847 was digested with *BamH*I, *Eco*RI, *Hin*dIII, *Pst*I, *Xho*I, *BamH*I + *Eco*RI, *BamH*I + *Hin*dIII, *BamH*I + *Pst*I , *BamH*I + *Xho*I , *Eco*RI + *Hin*dIII, *Eco*RI + *Pst*I, *Eco*RI + *Xho*I, *Hin*dIII + *Pst*I, *Hin*dIII + *Xho*I and *Pst*I + *Xho*I.

15. Deletion of pST847 for chitinase gene location and DNA sequencing

A pST847 with chitinase activity was digested with *Xho*I to two fragments, a 6 and a 3 kb in size. The former (pSN*XP*-6.3), a pBluescripSK$^-$ and a part of pST847 closely combined, was religated. The latter (pSN*XX*-3.0), *Xho*I- *Xho*I fragment was ligated to *Xho*I site of the pBluescripSK$^-$. A religated plasmid was digested with *BamH*I and *Pst*I to two fragments, a 4.2 and a 1.8 kb in size. The smaller fragment, which ligated to a same site of the pBluescrip/SK$^-$ was named pSN*PB*-1.8 and a religated plasmid was designated to pSN*BB*-4.2. All process was shown in figure 15.The ligation reactions were incubated for 16-24 hours at 12^{o}C in an ice bath.

The ligation products were transformed into *E. coli* Top-10. The transformed cells were grown on LB-IPTG-X-gal agar containing100 μg/ml of ampicillin at 37°C for overnight. The recombinant plasmids were prepared from white colony. All mutants plasmids were sequenced by Bio Service Unit, Thailand.

16. The chitinase gene analysis

The nucleotide sequence was analyzed by Genetyx-win version 3.1 for promotor and open reading frame prediction, translation to deduced amino acid

sequence and molecular weight and isoelectic point calculation. A signal peptide were examined by signalIP. A restriction map of chitinase gene were analyzed by Strider.

To compare chitinase gene of *Bacillus* sp.PP8 with other chitinases, deposited in the GenBank, the nucleotide sequence and deduced amino acid sequence were analyzed by BLAST search and the closest sequence was aligned by CLUSTAL W. Homology modeling of the structure of this chitinase was accomplished by SWISS MODEL version 36.002.

17. Expression of chitinase gene using pET system

Construction of the recombinant plasmid**.**

Two primers were designed according to the available chitinase gene from *Bacillus* sp. PP8. The cloning site of the *Nco*I and *Bam*HI were incorporated into forward and reverse primer, respectively. The primer sequences are as follow;

Fchi66*Nco*I	5’-CATGCCATGGAAATCGTGTTGATCAAC-3’
Rchi66*Bam*HI	5’-CGGGATCCGTAGCGATACATTTCAC-3’

Thirty cycles of *in vitro* amplified were performed with a following temperature profile. The first step; denaturation at 94^{o}C for 30 sec, primer annealing at 46^{o}C for 30 sec extension at 72^{o}C for 4.40 min all of these were repeated for 5 times. In addition, to decrease non-specific amplification products, the second step was added. The second step; denaturation at 94^{o}C for 30 sec, primer annealing was gradient at 50, 55 and 60^{o}C for 30 sec, extension at 72^{o}C for 4.40 min excepted for the final cycle where extension proceeded for 10 min. An amplification gene product was recovered and purified from gel through the QIAquick gel extraction kit following the manufacture’s recommended procedure. The restriction enzymes *Nco*I and *Bam*HI were used to generated the in-frame open reading frame recombinant plasmid after the ligating the insert to these two cloning sites of pET19b (+).

Identification of positive clones

The recombinant plasmids were transformed into *E. coli* Top-10 and the transformants were screened onto a LB/ampicillin (100μg/ml) plate. 12 clones were selected for plasmid extraction and restriction enzyme analysisanalyzed. The clones, which contained the appropriate insert, were plating in LB-colloidal chitin/ampicillin

(100 μg/ml) containing 1 mM IPTG for phenotype analysis. The recombinant plasmids which obtained from the same clones as those produced clear zones around the colony were sequenced by using CEQ^{TM}8000 Genetic Analysis system to confirm the DNA sequence surround the cloning site by using T7 promoter as a sequence primer.

Optimized expression of chitinase gene

After target plasmid was estabished in *E. coli* expression host i.e. BL21(DE3), BL21(DE3)pLysS and Rosetta(DE3), the single colony was picked from a freshly streaked plate and inoculate 50 ml LB, 2xLB and terific broth containing the appropriate antibiotic (100 μg/ml ampicillin for BL21(DE3) and 100 μg/ml ampicillin and 34 μg/ml chloramphenicol for) BL21(DE3)pLysS and Rosetta(DE3))in 250 ml Erlenmeyer flask. Incubated with shaking at 37^{o}C and 30^{o}C until OD_{600} reached 0.8. Removed samples for the uninduced control. To the remainder, add IPTG from 0.5 M stock to final concentration of 1 mM and continue the incubation for 60 hour, 0.5 ml of culture medium was collected at 0, 4, 6, 8, 12, 16, 24, 36, 48 and 60 hour. Then harvested the cells and medium fraction by centrifugation at 5000 rpm for 10 min at 4^{o}C. To verify the target protein, the cell pellet and medium fraction were analyzed by SDS-PAGE followed by activity staining and chitinase acitivity assay. The cells pellet was completely resuspened by mixing 50 μl of 1x phosphate-buffered saline (PBS) and 50 μl of 5x sample buffer. The mixture was heated for 5 min in boiling water. Then, stroed at –20^{o}C until SDS-PAGE analysis. 20 μl of the medium fraction was measured for the chitinase activity by colorimetric method under standard conditions. 1/10 volume of 100% TCA (w/v) was added to the remainder and vortexed for 15 sec. Placed on ice for minimum of 15 min. Then the mixture was centrifuged at 12000 rpm for 10 min. The supernanant was discarded. Protein pellet was washed twice with 100 μl of acetone. Removed and discarded the acetone from the loose pellet by centrifugation at 12000 rpm for 5 min. Final pellet was allowed to air dry throughly. The pellet was completely resuspened by mixing 50 μl of 1x phosphate-buffered saline (PBS) and 50 μl of 5x sample buffer. The mixture was heated for 5 min in boiling water. Then, strored at –20^{o}C until SDS-PAGE was analysis.

18. Expression of chitinase gene using pTrc system

Construction of recombinant plasmid

Two primers were designed according to the available chitinase gene from *Bacillus* sp. PP8. The cloning site of the *Bam*HI and *Sal*I were incorporated into forward and reverse primer, respectively. The primer sequences are as follow;

Fchi66 *Nco*I 5'-CATGCCATGGAAATCGTGTTGATCAAC-3'
Rchi66 *Sal*I 5'-ACGCGTCGACTTATTCGCAGCCTCCG-3'

Thirty cycles of *in vitro* amplified were performed with a following temperature profile. The first step; denaturation at 94^{o}C for 30 sec, primer annealing at 47^{o}C for 30 sec extension at 72^{o}C for 4.40 min all of these were repeated for 5 times. In addition, to decrease non-specific amplification products, the second step was added. The second step; denaturation at 94^{o}C for 30 sec, primer annealing at 81^{o}C for 30 sec, extension at 72^{o}C for 4.40 min excepted for the final cycle where extension proceeded for 10 min. An amplification gene product was recovered and purified from gel through the QIAquick gel extraction kit following the manufacture's recommended procedure. The restriction enzymes *Nco*I and *Sal*I were used to generated the in-frame open reading frame recombinant plasmid after the ligating the insert to these two cloning sites of pTrcHis2-C.

Identification of positive clones

The recombinant plasmids were transformed in to *E. coli* Top-10 and the transformants were screened onto a LB/ampicillin (100μg/ml) plate. 12 clones were selected for plasmid extraction and restriction enzyme analysis. The clones, which presented the appropriate insert, were plating in LB-colloidal chitin/ampicillin (100 μg/ml) containing 1 mM IPTG for phenotype analysis. The recombinant plasmids which obtained from the same clone with those produced clear zones around the colony were sequenced by using CEQ^{TM}8000 Genetic Analysis system to confirm the DNA sequence surround the cloning site by using pTrcHis forward as a sequencing primer.

Optimized expression of chitinase gene

After target plasmid was estabished in *E. coli* Top-10, picked the single from a freshly streaked plate and inoculate 50 ml LB, 2xLB and terific broth containing the appropriate antibiotic (100 μg/ml in 250 ml erlenmeyer flask. Incubated with shaking at 37°C and 30°C until OD_{600} reached 0.8. Removed samples for the uninduced control. To the remainder, add IPTG from 0.5 M stock to final concentration of 0.25, 0.5, 0.75 and 1 mM and continue the incubation for 60 hour, 0.5 ml of culture medium was collected at 0, 4, 6, 8, 12, 16, 24, 36, 48 and 60 hour. Then, harvested the cells and medium fraction by centrifugation at 5000 rpm for 10 min at 4°C. To verify the target protein, the cell pellet and medium fraction were analyzed by SDS-PAGE followed by activity staining and chitinase acitivity assay. The cells pellet was completely resuspened by mixing 50 μl of 1x phosphate-buffered saline (PBS) and 50 μl of 5x sample buffer. The mixture was heated for 5 min in boiling water. Then, stroed at –20°C until SDS-PAGE analysis. 20 μl of the medium fraction was measured for the chitinase activity by colorimetic method under standard conditions. 1/10 volume of 100% TCA (w/v) was added to the remainder and vortexed for 15 sec. Placed on ice for minimum of 15 min. Then, centrifuged at 12000 rpm for 10 min. The supernanant was discarded. Protein pellet was washed twice with 100 μl of acetone. Removed and discarded the acetone from the loose pellet by centrifugation at 12000 rpm for 5 min. Final pellet was allowed to air dry throughly. The pellet was completely resuspened by mixing 50 μl of 1x phosphate-buffered saline (PBS) and 50 μl of 5x sample buffer. The mixture was heated for 5 min in boiling water. Then, stroed at –20°C until SDS-PAGE analysis.

19. Purification of chitinase from *E. coli* BL21(DE3) harboring the pETchi66

E. coli BL21(DE3) harboring the pETchi66 was grown at 30°C in LB/ampicilin (100 μg/ml) to an OD_{600} of about 0.7. IPTG was then added to the final concentration of 1 mM. For preparative 1 L culture was used and after 60 hour of induction the supernatant of culture medium was collected by centrifugation.

Purification by using column chromatography

The secreted chitinase was concentrated through VIVA(50) flow (10 kDa cut off). The recombinant chitinase was purified by using a classical way, column

chromatography. The concentrate was applied onto DEAE-cellulose column which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.0). The bound fractions were eluted with a gradient of increasing concentration of NaCl (0-1 M, 400 ml). The eluate with chitinase activity was concentrated by VIVA (2) flow and a saturated ammonium sulfate solution was added to the ion exchange fraction with chitnase activity to give a final concentration of 1.5 M. This solution was applied to a phenyl-sepharose column, previously equilibrated with 50 mM Tris-HCl pH 7.0 containing 1.5 M $(NH_4)_2SO_4$. The column was washed with a 10-bed volume of the same buffer and eluted with a 10-bed volume of decreasing $(NH_4)_2SO_4$ gradient (1.5-0 M) at a flow rate of 1.0 ml/min. The fractions with chitinase activity were collected and analyzed by SDS-PAGE followed by activity staining.

Single step purification, chitin adsorption.

Colloidal chitin was added to the concentrated filtrate in the amount of 1 mg /10mg protein. The suspension was allowed to stand for 6 hours at 4-8^{o}C and centrifuged at 10,000 rpm for 15 min. The pellet was washed with twice the original volume of 0.5 M Tris-HCl, pH 7.0 , then suspended up to the original volume in the same buffer and incubated at 50^{o}C for an overnight. A clear solution was centrifuged at 10,000 rpm for 15 min at 4^{o}C. The residual colloidal chitin was washed twice with 0.5 M Tris-HCl, pH 7.0. The supernatant was concentrated and simultaneously dialyzed in 100 mM Tris-HCl, pH 7.0 by VIVA (50) flow. The partially purified chitinase was analyzed by SDS-PAGE followed by activity staining and characterization.

CHAPTER III

RESULT

Identification of bacterial strain PP8

Physiological and biochemical identification

Bacterial strain PP8 was first sent to identify at The Thailand Institute of Scientific and Technological Research, Ministry of Science and Technology. This strain was gram positive bacterial, which formed ellipsoidal, subterminal spores. PP8 was a rod shape bacillus, which grew both aerobically and anaerobically on LB agar. The colony on nutrient agar was white in color and had a rough surface. The interior of its colony had a circular motion. This bacterium did not grow at 55^{o}C and was negative for Voges-Proskauer (VP) reaction. This bacterium was fermentative and produced acids from glycogen and almost sugars, except adonitol, sorbitol, xylitol, D-arabitol, L-sorbose, L-xylose, D-lyxose, D-tagatose and D-fucose as shown in appendix A. It produced catalase and capable of starch hydrolysis. Thus it was identified as *Bacillus circulans.*

Strain PP8 was reidentified by The National Institute of Health, Department of Medical Sciences, Ministry of Public Health. This strain was gram-positive bacterial, which formed spores as subterminal, ellipsoidal and having approximately the same width as a rod. It grew both aerobically and anaerobically on LB agar. This bacterium grew at 55^{o}C and was a positive for Voges-Proskauer (VP) reaction. It produced catalase and capable of starch hydrolysis. This bacterium was fermentative and produced acids from most sugars, except adonitol, sorbitol, xylitol, D-arabitol, L-sorbose, L-xylose, D-lyxose, D-tagatose and D-fucose as shown in appendix B. Thus it was identified as *Bacillus coagulans.*

Characterization of a gene coding for 16S ribosomal RNA

The partial 16S ribosomal RNA gene of *Bacillus* sp.PP8 was *in vitro* amplified via the PCR. The amplified PCR product of the 16S rRNA of *Bacillus* sp.PP8 was ligated to pGEM T-easy and transformed to *E. coli* Top-10. The recombinant colonies were selected by blue-white screening. The plasmid containing a 1.5 kb of 16S ribosomal RNA gene (p16SPP8) was digested with *Eco*RI to confirm the presence of the insert, as shown in Figure3.1. p16SPP8 was sequenced using the dideoxy-chain termination method with CEQ^{TM} 8000 Genetic Analysis system. The nucleotide

sequence of the 16S rRNA gene of *Bacillus* sp.PP8 spanning 1557 bp (is shown in figure 3.2. The sequence of 16S ribosomal RNA gene was compared with others obtained from the GenBank using BLAST. The highest similarity for 16S rRNA gene of a strain PP8 is the 16S rRNA gene from *Bacillus lautus*, which was later renamed to *Paenibacillus lautus*. Linear alignment of the two sequences, *Bacillus* sp.PP8 and *Paenibacillus lautus*, showes marked similarity between the two sequences. The overall sequence similarity is 97%, which was shown in Figure 3.3.

Strain PP8 was biochemically identified twice, which classified this bacterium, as *Bacillus circulans* and *Bacillus coagulans.* Whereas, a gene coding for 16S ribosomal RNA of this strain was more similarity to *Paenibacillus lautus*. Because of endlessly confusion, so we called this bacterial strain *Bacillus* sp. PP8.

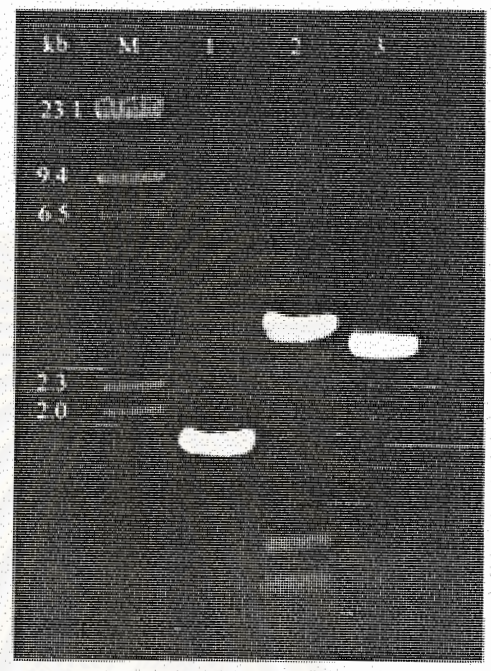


Figure 3.1 PCR product and restriction digested of 16S rRNA clone

0.7% agarose gel electrophoresis pattern of PCR amplified product and p16S digestion product.

Where Lane M: λ/*Hind*III Marker

Lane 1: 16S rRNA gene of *Bacillus* sp. PP8 from *in vitro* amplification

Lane 2: p16S/*Eco*RI

Lane 3: undigested p16S

AGAGTTTGAT CCTGGCTCAG GACGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAGC 60
GGACTTGATG GAGTGCTTGC ACTCCTGATG GTTAGCGGCG GACGGGTGAG TAACACGTAG 120
GCAACCTGCC CTCAAGACTG GGATAACTAC CGGAAACGGT AGCTAATACC GGATAATTTA 180
TTTTGCAGCA TTGTGGAATA ATGAAAGGCG GAGCAATCTG TCACTTGAGG ATGGGCCTGC 240
GGCGCATTAG CTAGTTGGTG GGGTAACGGC CCACCAAGGC GACGATGCGT AGCCGACCTG 300
AGAGGGTGAA CGGCCACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG GAGGCAGCAG 360
TAGGGAATCT TCCGTAATGG GCGAAAGCCT GACGGAGCAA CGCCGCGTGA GTGATGAAGG 420
TTTTCGGATC GTAAAGCTCT GTTGCCAAGG AAGAACGTCT TCTAGAGTAA CTGCTAGGAG 480
AGTGACGGTA CTTGAGAAGA AAGCCCCGGC TAACTACGTG CCAGCAGCCG CGGTAATACG 540
TAGGGGGCAA GCGTAGTCCG GAATTATAGG GCGTAAAGCG CGCGCAGGCG GTTACATAAA 600
AGTCTAGGTG TTATAAAACC CGAGGCTCAA CTTACAGGGT CGCACTGGAA ACTGGAGAAC 660
TAGAGTGCAG AAGAGGAGAG TGGAATACCA CGTGTAGCGG TGAAATGCGT AGATATGTGG 720
AGGAACACCA GTGGCGAAGG CGACTCTCTG GGCTGTAACT GACGCTGAGG CGCGAAAGCG 780
TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG AATGCTAGGT 840
GTTAGGGGTT ACGATACCCT AGGTGCCGAA GTTAACACAT AAAGCATACC GCCTGGGGAG 900
TACGGTCGTC GCAAGACTGA AACTCAAAGG AATTGACGGG GACCCGCACA AGCAGTGGAG 960
TATGTGGTTT AATTCGAAGC AACGCGAAGA ACCTTACCAA GTCTTGACAT CCCTCTGAAT 1020
CCTCTAGAGA TAGAGGCGGC CTTCGGGACA GAGGTGACAG GTGGTGCATG GTTGTCGTCA 1080
GCTCGTGTCG TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCTTG ATTTTAGTTG 1140
CCAGCACTTC GGGTGGGCAC TCTAGAATGA CTGCCGGTGA CAAACCGGAG GAAGGCGGGG 1200
ATGACGTCAA ATCATCATGC CCCTTATGAC TTGGGCTACA CACGTACTAC AATGGCTGGT 1260
ACAACGGGAA GCGAAGCCGC GAGGTGGAGC CAATCCTATA AAAGCCAGTC TCAGTTCGGA 1320
TTGCAGGCTG CAACTCGCCT GCATGAAGTC GGAATTGCTA GTAATCGCGG ATCAGCATGC 1380
CGCGGTGAAT ACGTTCCCGG GTCTTGTACA CACCGCCCGT CACACCACGA GAGTTTACAA 1440
CACCCGAAGT CGGTGGGGTA ACCCGCAAGG GAGCCAGCCG CCGAAGGTGG GGTAGATGAT 1550
TGGGGTGAAG TCGTAACAAG GTAGCCGTAT CGGAAGGTGC GGCTGGATCA CCTCCTT 1557

Figure 3.2 Nucleotide sequence of the 16S rRNA gene of *Bacillus* sp.PP8

The nucleotide sequence of the 16S rRNA gene of *Bacillus* sp. PP8 comprised 1557 bp. The positions of amplification primers and sequencing primers are underlined and double underlined, respectively.

```
Bacillus_strain_PP8       AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGC
Paenibacillus_lautus      ----------------------CGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGC
                                                ************************************

Bacillus_strain_PP8       GGACTTGATGGAGTGCTTGCACTCCTGATGGTTAGCGGCGGACGGGTGAGTAACACGTAG
Paenibacillus_lautus      GGACTTGATGGAGTGCTTGCACTCCTGAAGGTTAGCGGCGGACGGGTGAGTAACACGTAG
                          ************************** ******************************

Bacillus_strain_PP8       GCAACCTGCCCTCAAGACTGGGATAACTACCGGAAACGGTAGCTAATACCGGATAATTTA
Paenibacillus_lautus      GCAACCTGCCCTCAAGACTGGGATAACTACCGGAAACGGTAGCTAATACCGGATAATTTA
                          ************************************************************

Bacillus_strain_PP8       TTTTGCAGCATTGTGGAATAATGAAAGGCGGAGCAATCTGTCACTTGAGGATGGGCCTGC
Paenibacillus_lautus      TTTCACAGCATTGTGGAATAATGAAAGACGGAGCAATCTGTCACTTGGGGATGGGCCTGC
                          ***  ********************** ****************** ************

Bacillus_strain_PP8       GGCGCATTAGCTAGTTGGTGGGGTAACGGCCCACCAAGGCGACGATGCGTAGCCGACCTG
Paenibacillus_lautus      GGCGCATTAGCTAGTTGGTGGGGTAACGGCCCACCAAGGCGACGATGCGTAGCCGACCTG
                          ************************************************************

Bacillus_strain_PP8       AGAGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
Paenibacillus_lautus      AGAGGGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
                          ************************************************************

Bacillus_strain_PP8       TAGGGAATCTTCCGTAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGG
Paenibacillus_lautus      TAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGG
                          ************** ***********************************************

Bacillus_strain_PP8       TTTTCGGATCGTAAAGCTCTGTTGCCAAGGAAGAACGTCTTCTAGAGTAACTGCTAGGAG
Paenibacillus_lautus      TTTTCGGATCGTAAAGCTCTGTTGCCAAGGAAGAACGTCTTCTAGAGTAACTGCTAGGAG
                          ************************************************************

Bacillus_strain_PP8       AGTGACGGTACTTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
Paenibacillus_lautus      AGTGACGGTACTTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
                          ************************************************************

Bacillus_strain_PP8       TAGGGGGCAAGCGTAGTCCGGAATTATAGGGCGTAAAGCGCGCGCAGGCGGTTACATAAA
Paenibacillus_lautus      TAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTT-C-TTTA
                          ************** ************ ************************ * *  *

Bacillus_strain_PP8       AGTCTAGGTGTTATAAAACCCGAGGCTCAACTTACAGGGTCGCACTGGAAACTGGAGAAC
Paenibacillus_lautus      AGTCT-GGTGTTT--AAACCCGAGGCTCAACTT-C-GGGTCGCACTGGAAACTGGGGGAC
                          ***** ******   ****************** * ****************** * **

Bacillus_strain_PP8       TAGAGTGCAGAAGAGGAGAGTGGAATACCACGTGTAGCGGTGAAATGCGTAGATATGTGG
Paenibacillus_lautus      TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGG
                          * ************************ **********************************

Bacillus_strain_PP8       AGGAACACCAGTGGCGAAGGCGACTCTCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCG
Paenibacillus_lautus      AGGAACACCAGTGGCGAAGGCGACTCTCTGGGCTGTAACTGACGCTGAGGCGCGAAAGCG
                          ************************************************************

Bacillus_strain_PP8       TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGT
Paenibacillus_lautus      TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGT
                          ************************************************************

Bacillus_strain_PP8       GTTAGGGGTTACGATACCCTAGGTGCCGAAGTTAACACATAAAGCATACCGCCTGGGGAG
Paenibacillus_lautus      GTTAGGGGTTTCGATACCCTTGGTGCCGAAGTTAACACATTAAGCATTCCGCCTGGGGAG
                          ********** ********* ****************** ****** ************

Bacillus_strain_PP8       TACGGTCGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAG
Paenibacillus_lautus      TACGGTCG---CAAGACTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAG
                          ********   ************************************************

Bacillus_strain_PP8       TATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAGTCTTGACATCCCTCTGAAT
Paenibacillus_lautus      TATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAGTCTTGACATCCCTCTGAAT
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Bacillus_strain_PP8       CCTCTAGAGATAGAGGCGGCCTTCGGGACAGAGGTGACAGGTGGTGCATGGTTGTCGTCA
Paenibacillus_lautus      CCTCTAGAGATAGAGGCGGCCTTCGGGACAGAGGTGACAGGTGGTGCATGGTTGTCGTCA
                          ************************************************************

Bacillus_strain_PP8       GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATTTTAGTTG
Paenibacillus_lautus      GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATTTTAGTTG
                          ************************************************************

Bacillus_strain_PP8       CCAGCACTTCGGGTGGGCACTCTAGAATGACTGCCGGTGACAAACCGGAGGAAGGCGGGG
Paenibacillus_lautus      CCAGCACTTNGGGTGGGCACTCTAGAATGACTGCCGGTGACAAACCGGAGGAAGGCGGGG
                          ********* **************************************************

Bacillus_strain_PP8       ATGACGTCAAATCATCATGCCCCTTATGACTTGGGCTACACACGTACTACAATGGCTGGT
Paenibacillus_lautus      ATGACGTCAAATCATCATGCCCCTTATGACTTGGGCTACACACGTACTACAATGGCTGGT
                          ************************************************************

Bacillus_strain_PP8       ACAACGGGAAGCGAAGCCGCGAGGTGGAGCCAATCCTATAAAAGCCAGTCTCAGTTCGGA
Paenibacillus_lautus      ACAACGGGAAGCGAAGCCGCGAGGTGGAGCCAATCCTATAAAAGCCAGTCTCAGTTCGGA
                          ************************************************************

Bacillus_strain_PP8       TTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCTAGTAATCGCGGATCAGCATGC
Paenibacillus_lautus      TTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCTAGTAATCGCGGATCAGCATGC
                          ************************************************************

Bacillus_strain_PP8       CGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTACAA
Paenibacillus_lautus      CGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTACAA
                          ************************************************************

Bacillus_strain_PP8       CACCCGAAGTCGGTGGGGTAACCCGCAAGGGAGCCAGCCGCCGAAGGTGGGGTAGATGAT
Paenibacillus_lautus      CACCCGAAGTCGGTGGGGTAACCCGCAAGGGAGCCAGCCGCCGAAGGTGGGGTAGATGAT
                          ************************************************************

Bacillus_strain_PP8       TGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTT
Paenibacillus_lautus      TGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGG--------------------
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Figure 3.3 Nucleotide sequence alignment of 16S rRNA gene

Nucleotide sequence of 16S rRNA gene of *Bacillus* sp.PP8 was aligned with a 16S rRNA gene of *Paenibacillus lautus*. Where * denotes identical nucleotides.

Chitinase and chitosanase production by *Bacillus* sp.PP8

Bacillus sp.PP8 was grown in the medium containing 0.02% colloidal chitin, and the culture medium was collected at 12 hours interval for 108 hours. Each collected culture medium was examined for chitinase and chitosanase activities using crystalline chitin (powdered chitin), amorphous chitin (colloidal chitin) and 100%DD chitosan as substrates. Strain PP8 produced crystalline chitin hydrolyzing enzyme at first, it reached its maximum activity (13.14 mU/ml) at 36 hours of cultivation. The amorphous chitin hydrolyzing enzyme was produced secondly. The amorphous chitin hydrolyzing enzyme reached its maximum activity (30.21 mU/ml) at 60 hours of cultivation and gradually decreased after futhur cultivation. Chitosanase was produced next after chitinases, it reached its maximum activity (88.35 mU/ml) at 84 hours of cultivation (Figure 3.4).

When various types of chitinous substrates were added in the culture medium (0.02% colloidal chitin, 0.2% powdered chitin, 2% flaked chitin, 0.02% colloidal chitosan, 2% flaked chitosan, 0.2% GlcNAc or 0.2%GlcN), the enzyme producing profile of *Bacillus* sp.PP8 significantly differed for each chitinous substrates. The highest chitinase activity was detected in the medium containing colloidal chitin. Flaked chitin, powdered chitin and GlcNAc also induced significant levels of chitinase but it was much lower than observed in the medium containing colloidal chitin. When *Bacillus* sp.PP8 was grown in the medium containing chitosan and GlcN, essentially no chitinase activity was observed in culture supernatant, as shown in Figure 3.5.

On the other hand, the highest chitosanase activity was detected in the medium containing colloidal chitosan. Flaked chitosan, colloidal chitin, flaked chitin and powdered chitin also induced significant levels of chitinase, but it was much lower than that observed in the medium containing colloidal chitosan. When *Bacillus* sp.PP8 was grown in the medium containing chitin, GlcNAc and GlcN, essentially no chitosanase activity was observed in culture supernatant, as shown in Figure 3.6.

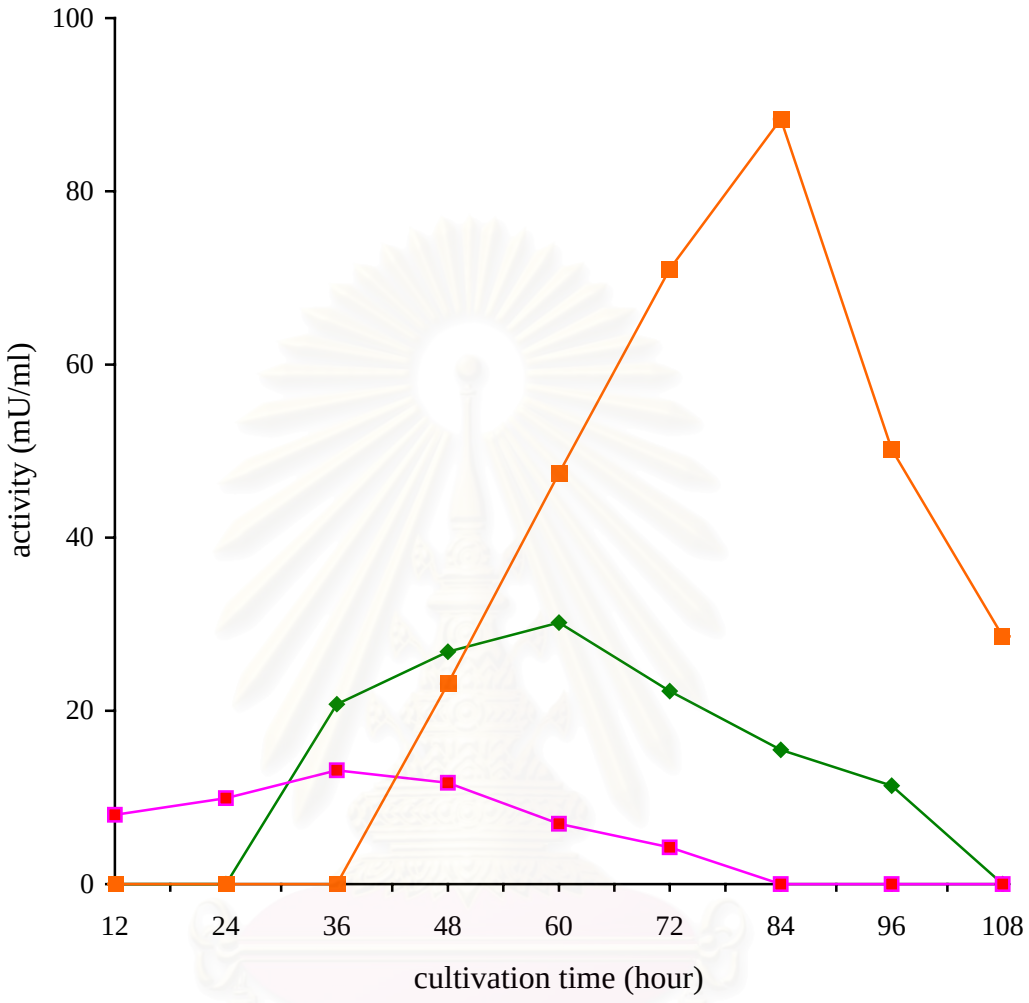

Figure 3.4 Chitinolytic enzyme production profile.

Bacillus sp.PP8 Strain PP8 was grown in the medium containing 0.02% colloidal chitin at 37°C. Colloidal chitin hydrolyzing enzyme (◆), powdered chitin hydrolyzing enzyme (■) and chitosanase (■). Enzyme activity in the culture supernatant at each time point was measured using colloidal chitin, powdered chitin and chitosan as substrates. The reactions were incubated with 0.15 M Tris-HCl, pH 7.0 at 50°C for 1 hour.

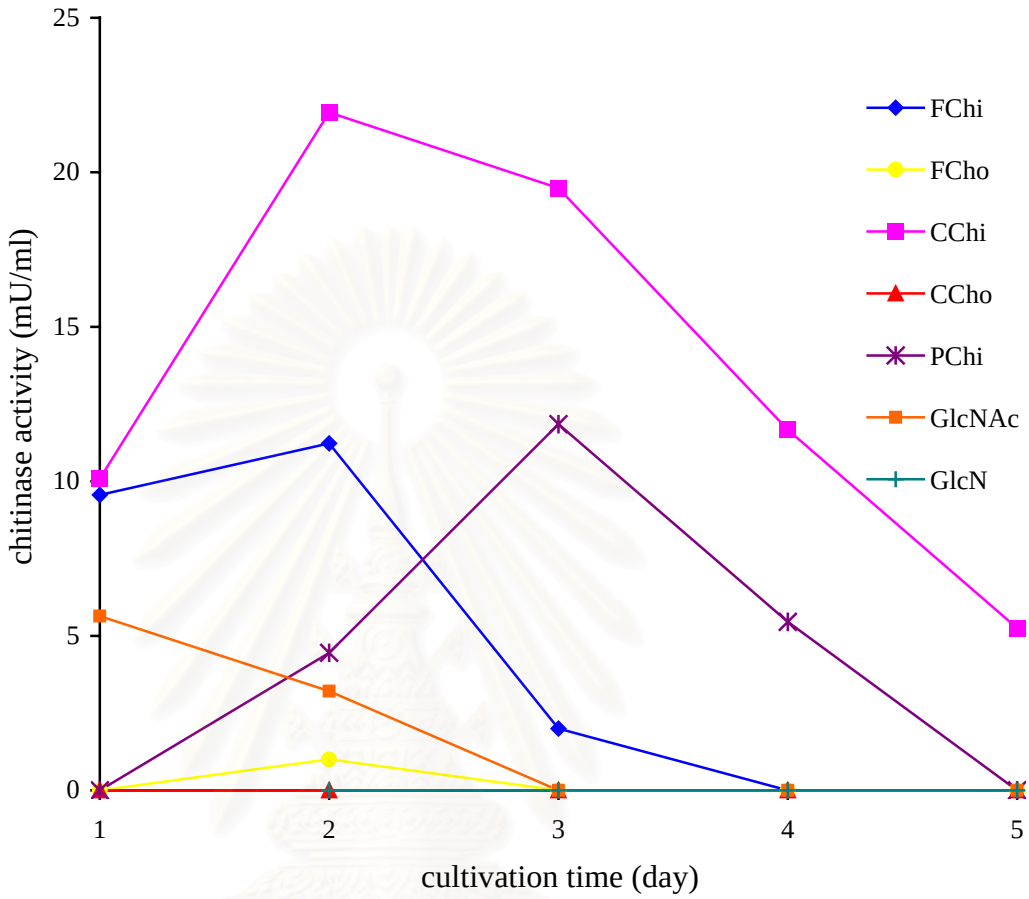

Figure 3.5 Chitinase production profile in medium containing various chitinous substrates

Bacillus sp.PP8 was grown in the medium containing 0.02% colloidal chitin;CChi (■), 0.2% powdered chitin; PChi(*), 2% flake chitin;FChi (◆), 2% flake chitosan;FCho (●), 0.02% colloidal chitosan;CCho (▲), 0.2% *N*-acetylglucosamine; GlcNAc (■) and 0.2% GlcN; glucosamine (|) as a carbon source. Chitinase activity in the culture supernatant was measured using colloidal chitin as the assay substrate. The reactions were incubated with 0.15 M Tris-HCl, pH 7.0 for 1 hour.

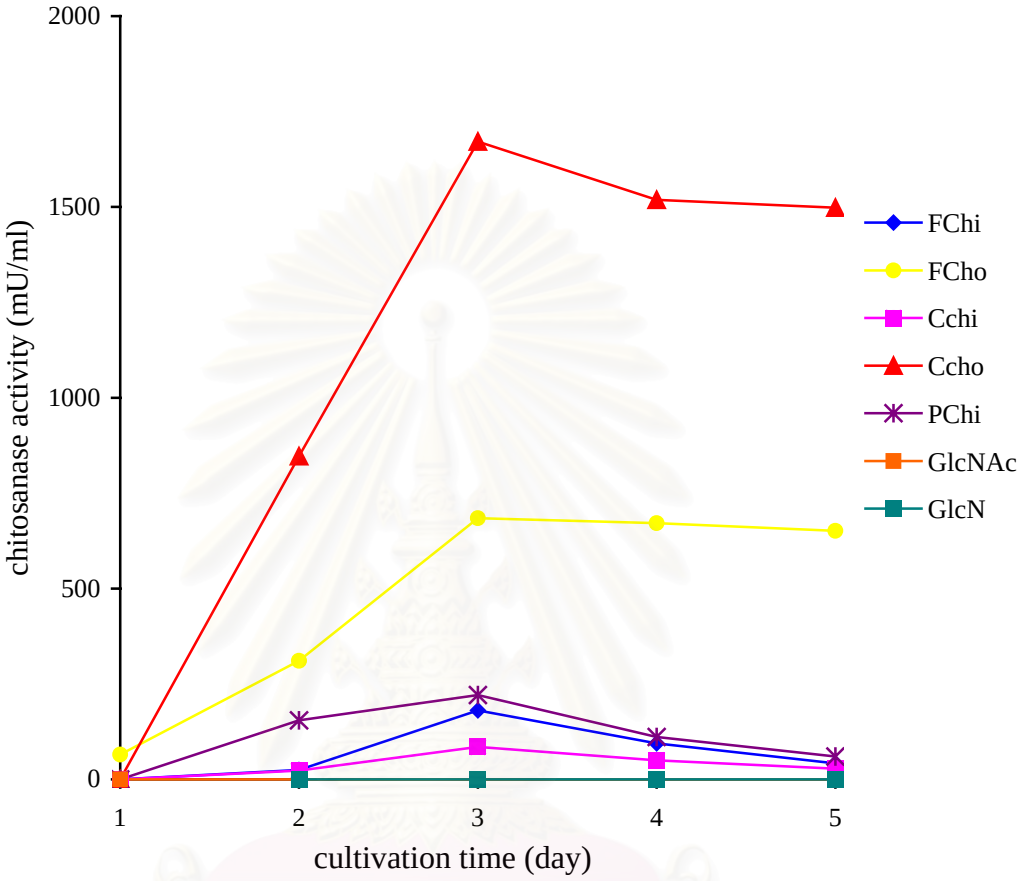

Figure 3.6 Chitosanase production profile in medium containing various chitinous substrates

Bacillus sp.PP8 was grown in the medium containing 0.02% colloidal chitin;CChi (■), 0.2% powdered chitin; PChi(*), 2% flake chitin;FChi (◆), 2% flake chitosan;FCho (●), 0.02% colloidal chitosan;CCho (▲), 0.2% *N*-aectylglucosamine; GlcNAc (■) and 0.2% GlcN; glucosamine (|) as a carbon source. Chitosanase activity in the culture supernatant was measured using colloidal chitosan as the assay substrate. The reactions were incubated with 0.15 M Tris-HCl, pH 7.0 for 1 hour.

Determination of chititolytic enzyme and chitosanase produced by *Bacillus* sp.PP8 using SDS-PAGE followed activity staining

To determine, how many chitinolytic enzymes were produced and secreted into the culture medium by *Bacillus* sp. PP8. Strain PP8 was grown in medium containing colloidal chitin and the culture medium was collected every 24 hours. Crude enzyme from PP8 was analyzed by SDS-PAGE followed by activity staining. We observed five bands of 145, 66, 55, 40 and less than 15 kDa in size containing chitinase activity. As shown in Figure 3.7, a 40 kDa protein with chitinolytic activity was produced in the first day of cultivation and reached its maximum activity in the second day of cultivation. Whereas, a smaller protein (less than 15 kDa) and a 55 kDa protein that contained chitinolytic activity were detected in the second day of cultivation. While, a 66 kDa protein contained chitinolytic activity was detected in the third day of cultivation.

When *Bacillus* sp. PP8. Strain PP8 was grown in medium containing colloidal chitosan and the culture medium was collected for SDS-PAGE followed activity staining analysis. Only a single band of 47.5 kDa with chitosanase activity was observed in culture medium of the first day and second day of cultivation, which was shown in Figure 3.8.

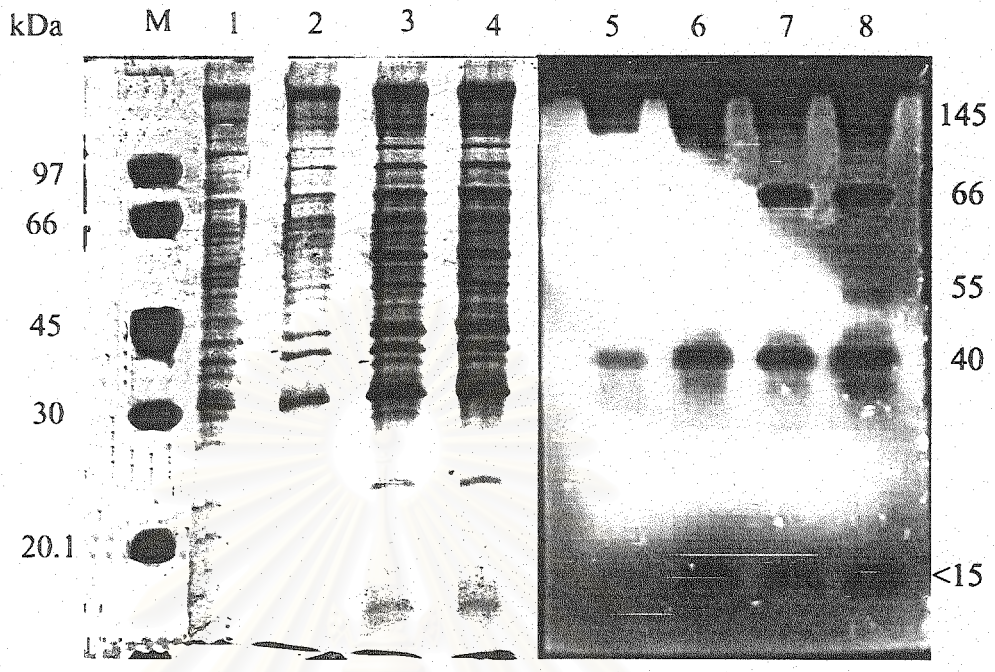

Figure 3.7 Chitinolytic enzyme production by *Bacillus* sp. PP8

Crude chitinase of strain PP8 was produced in 0.02% colloidal chitin minimal medium at 37°C for 4 days. The culture supernatant was analyzed using 12.5% SDS-PAGE followed by activity staining.

Lane 1-4: protein staining and Lane 5-8: activity staining

Lane M: standard molecular weight Marker

Lane 1 and 5: supernatant of *Bacillus* sp.PP8 culture medium at the first day of cultivation.

Lane 2 and 6: supernatant of *Bacillus* sp.PP8 culture medium at the second day of cultivation.

Lane 3 and 7: supernatant of *Bacillus* sp.PP8 culture medium at the third day of cultivation.

Lane 4 and 8: supernatant of *Bacillus* sp.PP8 culture medium at the forth day of cultivation.

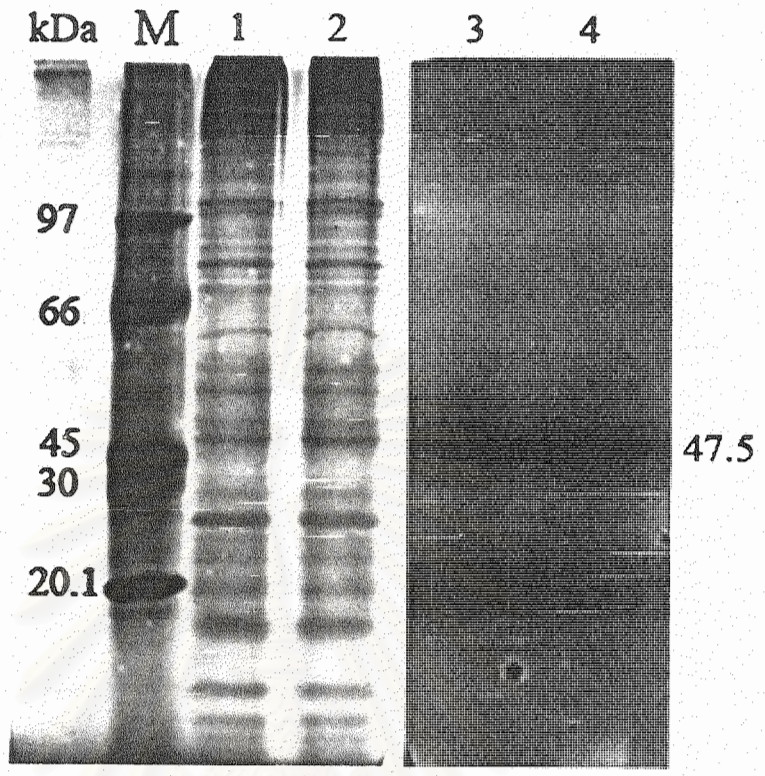

Figure 3.8 Chitosanase production by *Bacillus* sp. PP8

Crude chitosanase from PP8 was produced in 0.02% colloidal chitosan minimal medium at 37°C for 2 days. The culture supernatant was analyzed by 12.5% SDS-PAGE followed by activity staining.

Lane 1-2: protein staining and Lane 3-4 were a activity staining

Lane M: standard molecular weight marker

Lane 1 and 3: supernatant of *Bacillus* sp.PP8 culture medium at the first day of cultivation

Lane 2 and 4: supernatant of *Bacillus* sp.PP8 culture medium at the second day of cultivation

Characterization of crude chitinase and chitosanase from *Bacillus* sp.PP8

The crude enzyme was characterized for its physicochemical properties by colorimetic method, which has been previously described in Materials and Methods.

The optimum temperature of crude chitinase under standard assay condition was 50^{o}C, retaining 56.88% and 64.38% of the maximum activity at 30^{o}C and 70^{o}C, respectively, as showed in Figure 3.9. Crude chitinase from strain PP8 showed two optimum pH in acid and basic range. The optimum pH of acid range was pH 6.0. While, the optimum pH of basic range was pH 8.0 in Tris-HCl. (Figure 3.10)

The stability of crude chitinase against various pHs was determined by comparative measuring the residual activity after incubation at various pHs from 2.0-10.0 at 4^{o}C for 5 days. After incubation of the enzyme at pH 5.0-9.0, the residual activity was 100-80% when compared to the non-incubation control. The enzyme activity was completely lost after incubated at pH 2.0-3.0 and 11.0-12.0 for 1-3 days, However, the enzyme retained 50% of its activity after incubation at pH 4.0 and 10.0 for 2 days, shown in Figure 3.13. In addition, temperature stability was also determined by measuring the residual activity after incubation the crude chitinase in 50 mM Tris-HCl, pH 8.0 at 4, 30, 37, and 50^{o}C for 12 hours. After incubation of the enzyme at various temperatures, the crude chitinase retained its full activity.

Whereas, the optimum temperature of crude chitosanase was assayed in 0.15 M Tris-HCl, pH 7.0 or 0.15 M acetate buffer, pH 4.5 for heterogeneous and homogenous reaction, respectively. The optimum temperature of the heterogeneous reaction was 50^{o}C, while the optimum temperature of the homogeneous reaction was ranging from 40-70^{o}C, retaining 90% of the maximum activity at 40^{o}C, as showed in Figure 3.11. The optimum pH of crude chitosanase was 7.0 of Tris-HCl buffer and retaining 89.84% of the maximum activity when phosphate buffer at pH 7.0 was used as reaction buffer, as showed in Figure 3.12.

The stability of crude chitosanase against various pHs was determined by comparative measuring the residual activity after incubation at various from pHs 2.0-10.0 at 4^{o}C for 5 days. After incubation of the enzyme in the pH 5.0-9.0, the residual activity was in the range of 100-80% of the non-incubation control. Although the enzyme activity was completely lost after incubated at pH 2.0-3.0 and 11.0-12.0 for 3 days, the enzyme lost 50% activity of full activity after incubation at pH 4.0 and 10.0 for 1 day, which was shown in Figure 3.14. In addition, temperature stability was also determined by measuring the residual activity after incubation the crude chitosanase

in 50 mM Tris-HCl, pH 7.0 at 4, 30, 37, and 50^{o}C for 12 hours. After incubation of the crude chitosanase in various temperatires, the enzyme retained its full activity.

Substrate specificity

Crude enzyme of *Bacillus* sp.PP8, which was grown on medium containing 0.02% colloidal chitin at 37^{o}C for 60 hours was incubated with various substrates at 50^{o}C, in 0.15 M Tris-HCl, pH 8.0 for chitinase assay. As shown in Table 3.1 crude chitinase from strain PP8 effectively hydrolyzed amorphous chitin i.e. colloidal chitin was about 80% relative to the value of regenerated chitin. Crude PP8 also hydrolyzed crystalline chitin, but the relative degradation for powdered chitin and flaked chitin was about 30% and 5% of degradation of regenerated chitin, respectively. Only about 20% of soluble chitin, glycol chitin was hydrolyzed by this crude enzyme. In addition, the hydrolysis of many kinds of chitosan at pH/temperature 7.0/50^{o}C was carried out using crude enzyme of *Bacillus* sp.PP8 which, was grown on the medium containing 0.02% colloidal chitosan. From the result, crude chitosanase can hydrolyze soluble chitosan more effectively than amorphous chitosan, However, it did not hydrolyze flaked chitosan.

Table 3.1 Degradation of some carbohydrates by crude enzyme of *Bacillus* sp.PP8

Carbohydrates	Relative degradation (%)	
	Chitinase (pH 8.0)	Chitosanase(pH 7.0)
Glycolchitin	17.37	0
Colloidal chitin	77.89	0
Regenerated chitin	100.00	0
Powdered chitin	27.56	0
Flaked chitin	4.67	0
Soluble chitosan (80%DD)	ND	93.17
Soluble chitosan (100%DD)	ND	100.00
Colloidal chitosan	ND	83.63
Glycolchitosan	ND	44.78
Flaked chitosan	ND	0

Where %DD was % deacetylation ND was not determined

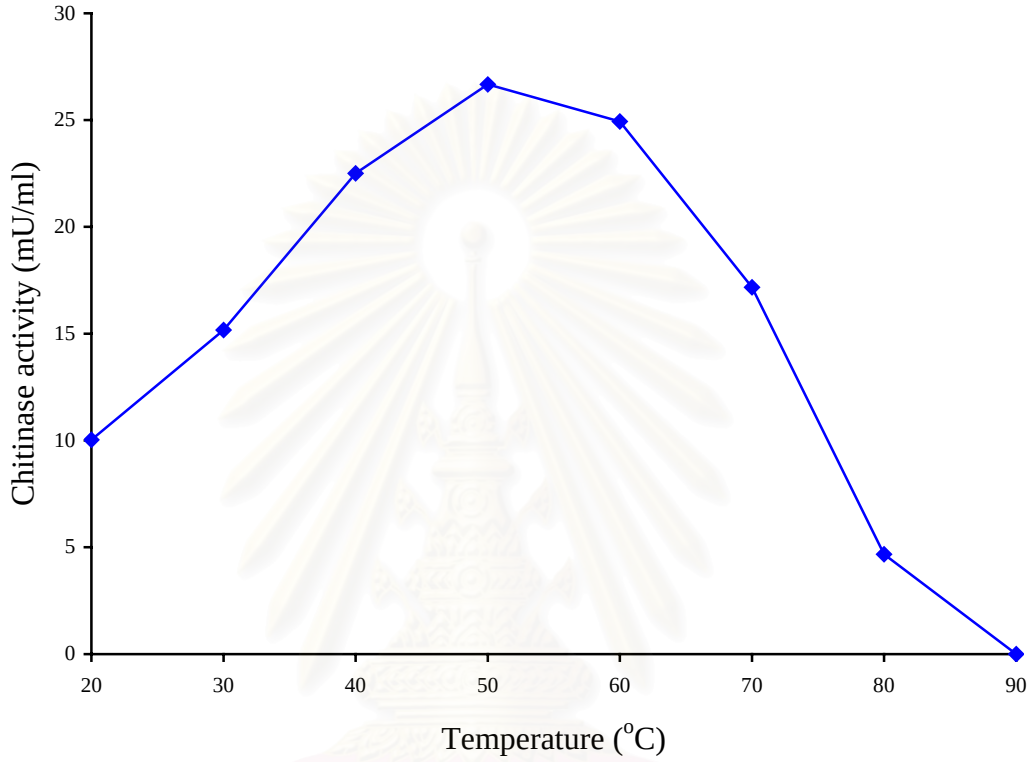

Figure 3.9 Optimum temperature of crude chitinase from *Bacillus* sp. PP8

Effect of temperature on chitinase activity was determined. Crude enzymes was incubated at 20-90°C in 0.15 M Tris-HCl, pH 7.0 for 1 hour, when colloidal chitin was as substrate.

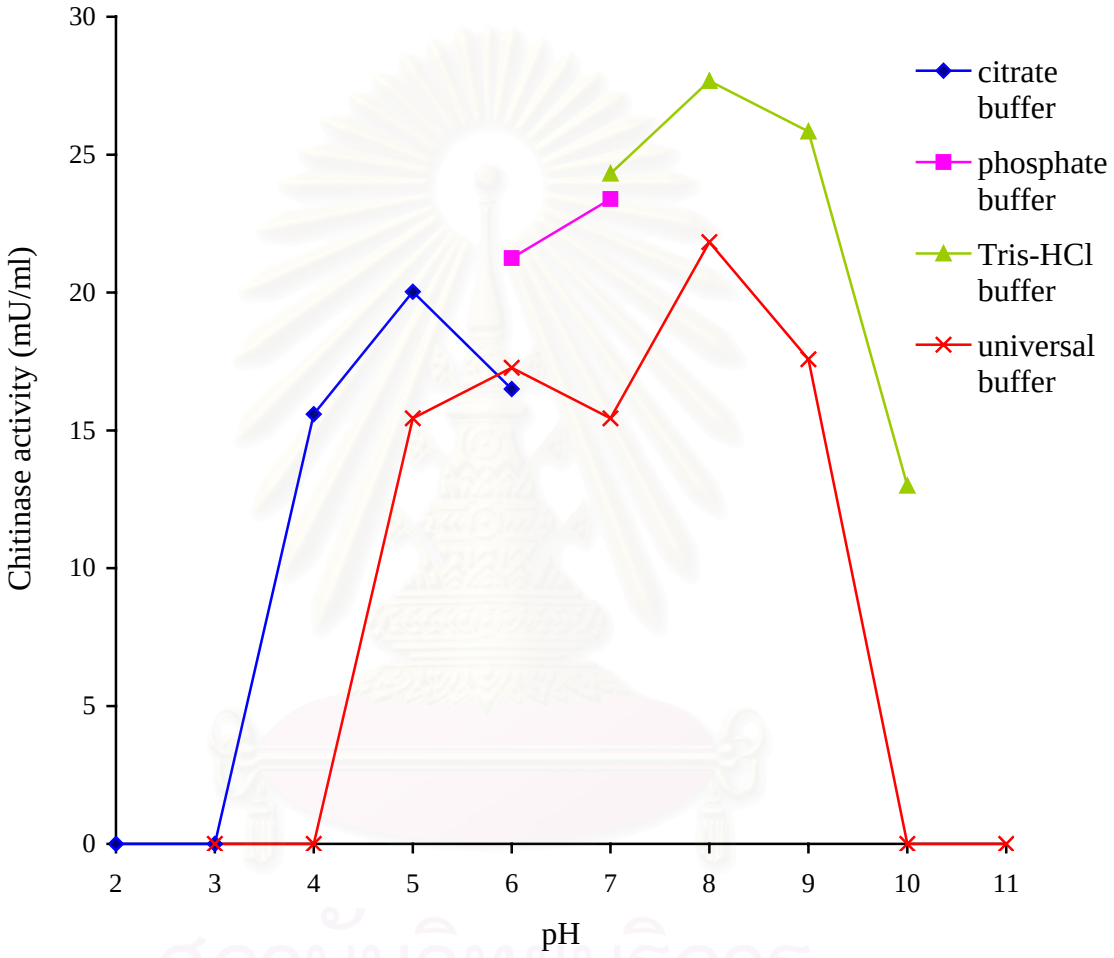

Figure 3.10 Optimum pH of crude chitinase from *Bacillus* sp. PP8

Effect of pH on chitinase activity was determined. Crude enzyme was incubated at pH 2.0-11.0 at 50^{o}C for 1 hour, using colloidal chitin as substrate

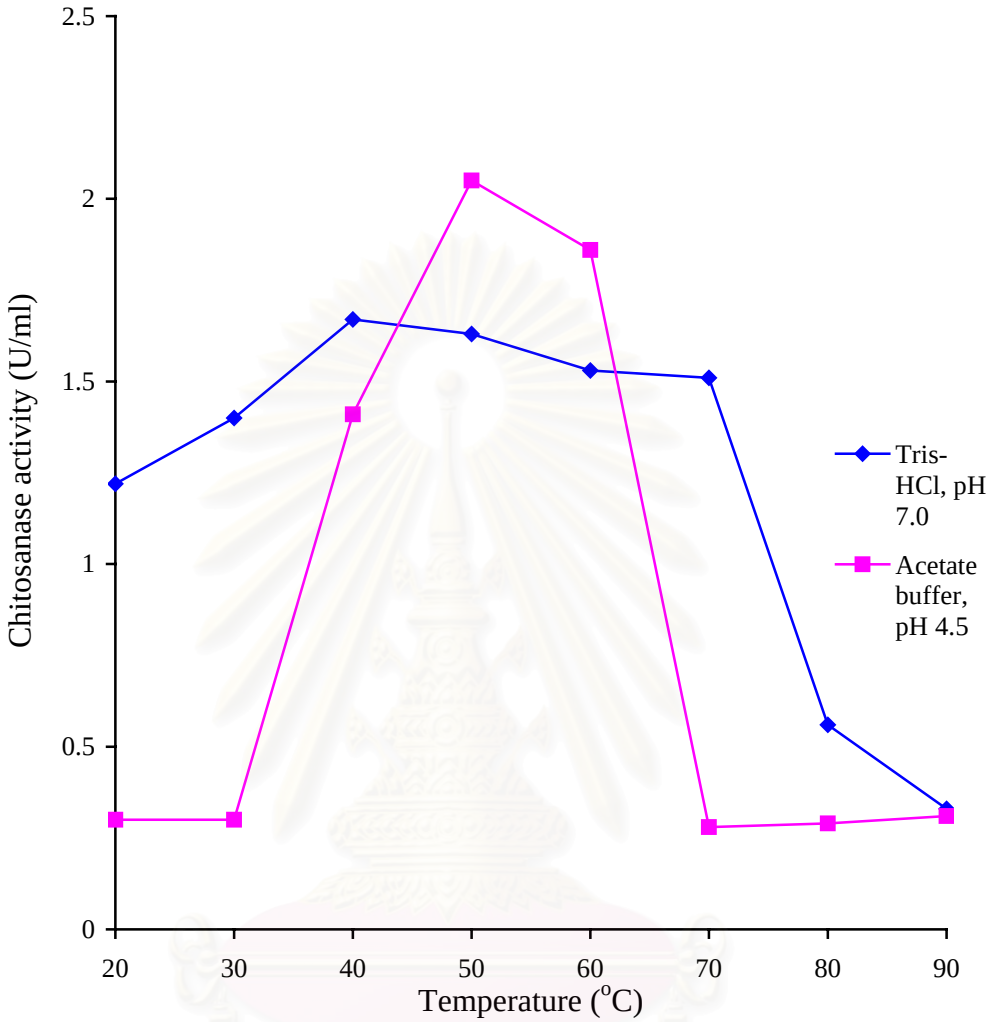

Figure 3.11 Optimum temperature of crude chitosanase from *Bacillus* sp. PP8

Effect of temperature on chitosanase activity was determined. Crude enzyme was incubated at 20-90°C in Tris-HCl pH 7.0 and acetate buffer pH 4.5. Enzyme activities were determined by using 100%DD soluble chitosan as substrate.

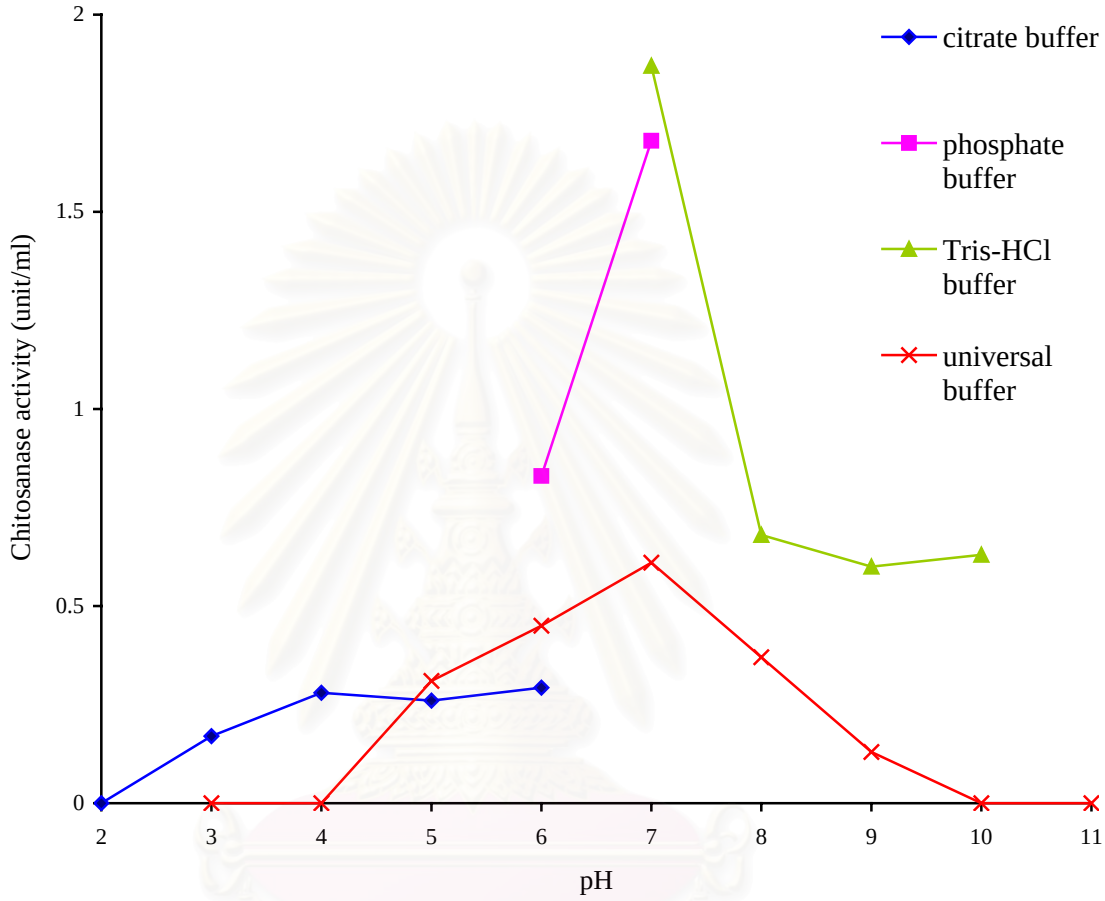

Figure 3.12 Optimum pH of crude chitosanase from *Bacillus* sp. PP8

Effect of pH on chitosanase activity was determined. Crude enzyme was incubated at 50°C in various pHs. Chitosanase activitie was determined by using 100%DD soluble chitosan as substrate.

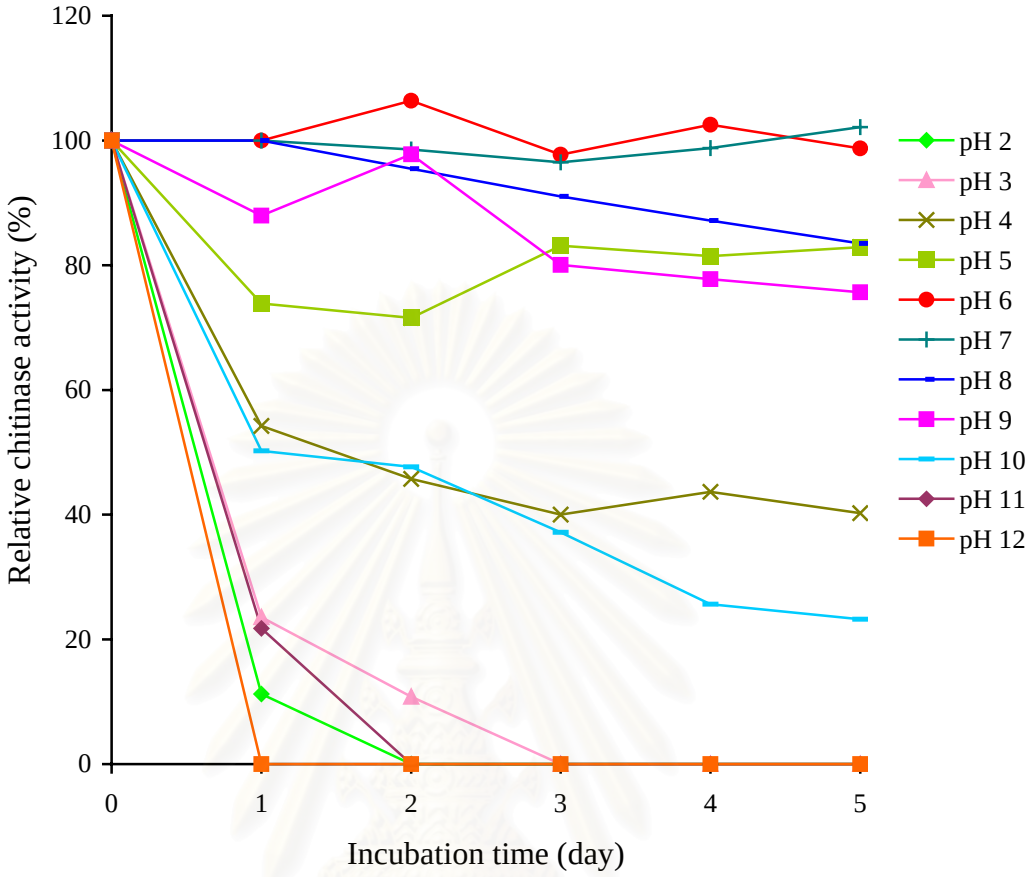

Figure 3.13 Effects of pH on the stability of crude chitinase of *Bacillus* sp.PP8

Crude chitinase was incubated at 4°C in various pHs for 5 days. The residual activity was assayed in 0.15 M Tris-HCl, pH 7.0 at 50°C. Colloidal chitin was used as substrate.

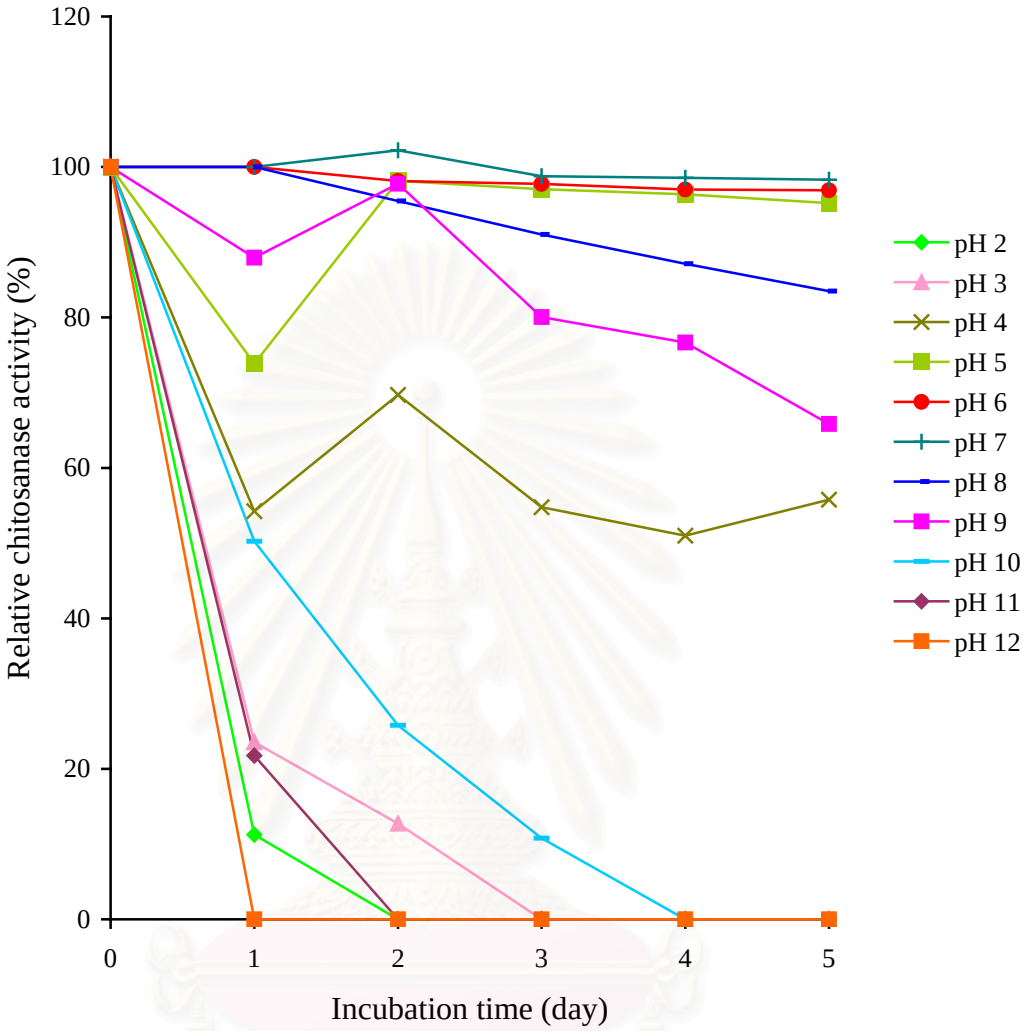

Figure 3.14 Effects of pH on the stability of crude chitosanase of *Bacillus* sp.PP8

Crude chitosanase was incubated at 4°C at various pH for 5 days. The residual chitosanase activity was assayed in 0.15 M Tris-HCl, pH 7.0 at 50°C using 100%DD soluble chitosan as substrate.

Analysis of hydrolysis products

Hydrolysis product of chitinase was examined using colloidal chitin as the substrate. As shown in Figure 3.15, colloidal chitin was hydrolyzed to GlcNAc and $(GlcNAc)_2$ as a major product (95% of total product) while small amount of $(GlcNAc)_3$ was also detected. The ratio between GlcNAc and $(GlcNAc)_2$ was 2:1.

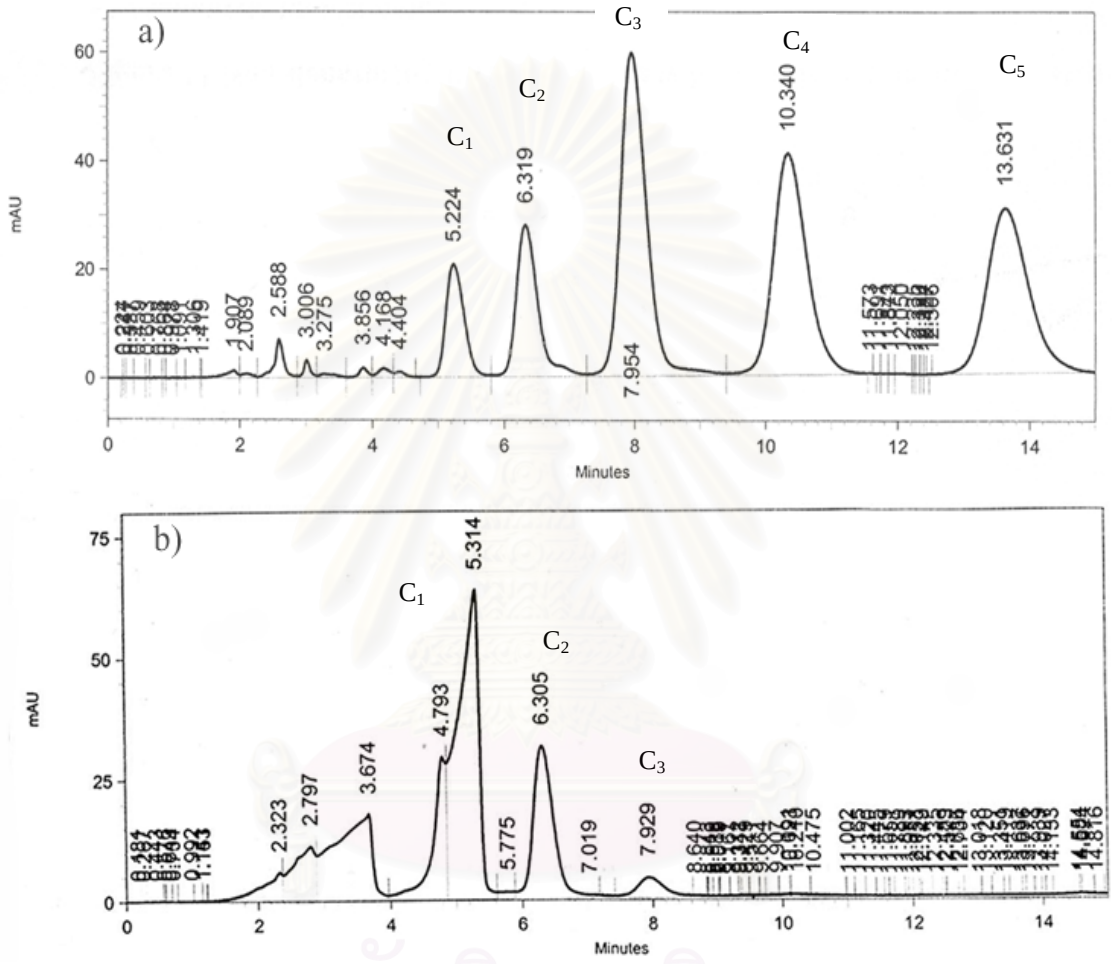

Figure 3.15 Hydrolysis product of crude chitinase from *Bacillus* sp. PP8

HPLC chromatogram of hydrolytic products of colloidal chitin hydrolysis by crude chitinase from *Bacillus* sp. PP8 at 50°C for an overnight.

a) The standard GlcNAc-$(GlcNAc)_4$ C_1:GlcNAc, C_2:$(GlcNAc)_2$, C_3:$(GlcNAc)_3$, C_4:$(GlcNAc)_4$ and C_5:$(GlcNAc)_5$

b) HPLC chromatogram of hydrolytic products of colloidal chitin hydrolyzed by crude chitinase from *Bacillus* sp. PP8.

Deletion of pST847 for chitinase gene localization and nucleotide sequencing.

To locate the chitinase gene, pST847 was subjected to deletion by restriction enzyme digest. The DNA fragments were subcloned as shown in Figure 3.16. For construction of pSN*XP*-6.3 and pSN*XX*-3.0, the pST847 was digested with *Xho*I. The linearized DNA fragments plus the cloning vector, 6.3 kb was religated, and 3.0 kb fragment was ligated to *Xho*I digested pBluescripSK$^-$, using T4 DNA ligase, to produced transformant with 3.3 and 3.0 kb inserted fragment, respectively (Figure 3.17). The pSN*XP*-6.3 was subsequently double digestion with *Bam*HI and *Pst*I into two fragments, a 4.5 kb and a 1.8 kb in size. The smaller fragment, which was ligated to pBluescripSK$^-$ was named pSN*PB*-1.8 and the religated plasmid was designated the pSN*BB*-4.5. All deletion derivatives were introduced into *E. coli* Top-10, and transformants were selected by overnight incubation at 37^{o}C on LB/ampicillin plate. The positive clones for chitinase activity were nucleotide sequenced using the dideoxy-chain termination method with CEQ^{TM} 8000 Genetic Analysis system. All of the constructs were plated on LB/colloidal chitin agar. Only pSN*XX*-3.0 produced clear zone around the colony. Therefore, pSN*XX*-3.0, which was the chitinase producing clone was designated pSNchi66, and the chitinase produced by *E. coli* harboring pSNchi66 was called CHI66.

Nucleotide sequence of chi66 gene revealed a single open reading frame containing 1797 bp, from a ATG start codon at position 186 to the TAA stop codon at nucleotide 1982. The deduced amino acid sequence of the chitinase gene contained 599 amino acids with signal peptide corresponding to 66.2 kDa. A region upstream of the putative ATG start codon contained the sequence GTGAGG at nucleotide 129 to 134, similar to the -35 consensus sequence, and the sequence TAGCTT at nucleotide 156 to 161, similar to the -10 consensus sequence. A putative Shine Dargano sequence, AGGAG was found, without spacer, upstream from the ATG start codon (as shown in Figure 3.18.). The sequence was aligned with other chitinases obtained from GenBank using BLAST. The chitinase gene most similarity with chi66 gene of a strain PP8 is from *Bacillus licheniformis*. Linear alignment of the two sequences, *Bacillus* sp.PP8 and *B. licheniformis,* showed marked similarity between the two sequences. The overall sequence similarity was calculated as 99% and E value was 0.00. Linear alignment of the two sequences was shown in Figure 3.20.

Homology modeling of the 3Dstructure of chi66 was accomplished by SWISS Model Protein Modeling, which was showed in Figure 3.21. The CHI66 was

classified into family 18 glycosylhydrolase, which showed a α/β_8 barrel catalytic domain. The whole structure comprised of three main domains. First, the catalytic domain with two conserved regions, Kxxx(S/A)xGG and (F/L)DGxDxDxE, where x was any amino acid, spanned 391 amino acid residues from residue 41 to 431 of CHI66. Second, the fibronectin type III like domain spanned 69 amino acid residues from residue 458-526 of CHI66. Third, the chitin binding domain with a conserved region GxxVxxxGxxYxAxWWTxGxxPxxxxxW, where x was any amino acid, spanned 43 amino acid residues from residue 548-590 of CHI66.

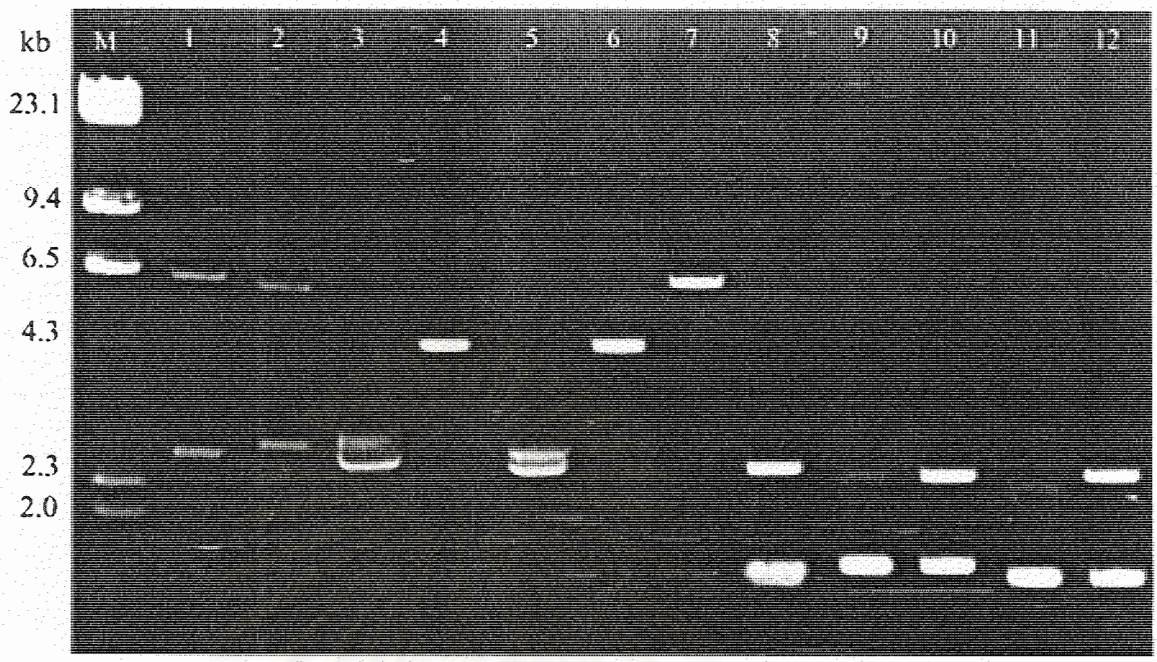

Figure 3.16 Restriction mapping of pST 847 derivatives

The derivatives of pST847 were digested and analyzed in 0.7%agarose electrophoresis

Where Lane M: a λ/*Hind*III,

Lane 1: pST847/*Pst*I Lane 2: pST847/*Xho*I

Lane 3: pST847/*Pst*I+*Xho*I Lane 4: pSNchi66

Lane 5: pSNchi66/*Xho*I Lane 6: pSN*XP*-6.3

Lane 7: pSN*XP*-6.3/ *Xho*I Lane 8: pSN*XP*-6.3/*Bam*HI+ *Pst*I

Lane 9: pSN*PB*-1.8 Lane 10:pSN*PB*-1.8/*Bam*HI+ *Pst*I

Lane 11: pSN*BB*-4.5 Lane 12:pSN*BB*-4.5/*Bam*HI+ *Pst*I

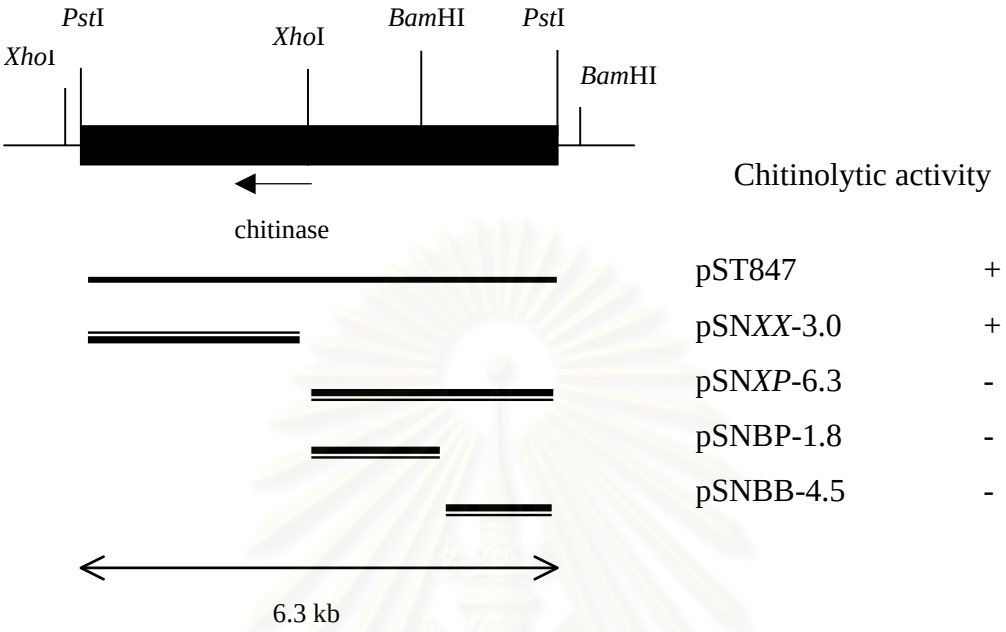

Figure 3.17 **Derivative of pST**847

All derivatives were introduced into E. coli Top-10 by transformation. The sizes of deletion fragments were estimated by 0.7 % agarose gel electrophoresis. The black box indicated the 6.3 kb inserted fragment of pST847. The arrow indicated the direction of transcription. The chitinolytic phenotype of each derivative is indicated by +, positive activity; -, negative activity.

```
TATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGGGTACAGCTCCCGCCGGTCAGGAT

CCTTGTTGTCTTCAATGTATCTGCTGCTATTAGATGACAAGGAAAAATAAAAACCAGCAA
  -35          -10
AAAAGGCGGTGAGGAAAAAGAGAGTTCTAGTTTCATAGCTTGCCAAAAAATTGCTTGTAA
 SD
AGGAGATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTATCTTTCATTT
      M  K  I  V  L  I  N  K  S  K  K  F  F  V  L  S  F  I  F

TTGTTATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAA
  V  M  M  L  S  L  S  F  V  N  G  E  V  A  K  A  D  S  G  K

AGAACTATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAG
  N  Y  K  I  I  G  Y  Y  P  S  W  G  A  Y  G  R  D  F  Q  V
TTTGGGATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCT
  W  D  M  D  V  S  K  V  S  H  I  N  Y  A  F  A  D  I  C  W
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  Q  L  L  K  L  K  K  S  H  P  H  L  K  T  F  I  S  V  G  G
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 E  Y  P  V  S  G  G  L  P  G  N  S  T  R  P  E  D  K  R  N
ACTACACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTGACGCTGCAGAAGCAAAAGACG
  Y  T  L  L  L  Q  E  V  R  K  K  L  D  A  A  E  A  K  D  G
GCAAGGAATACTTGCTGACGATCGCATCCGGCGCAAGTCCCGATTATGTAAGCAACACTG
  K  E  Y  L  L  T  I  A  S  G  A  S  P  D  Y  V  S  N  T  E
AGCTCGATAAAATCGCTCAAACCGTGGATTGGATTAACATTATGACCTATGACTTTAATG
  L  D  K  I  A  Q  T  V  D  W  I  N  I  M  T  Y  D  F  N  G
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  G  W  Q  S  I  S  A  H  N  A  P  L  F  Y  D  P  K  A  K  E
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  G  Y  K  R  Y  W  N  D  Q  A  K  V  P  F  L  Y  N  A  E  N
ATGGCAATTTCATCACTTATGATGATGAACAATCATTCGGCCACAAAACGGATTTTATTA
  G  N  F  I  T  Y  D  D  E  Q  S  F  G  H  K  T  D  F  I  K
AAGCAAACGGATTAAGCGGAGCAATGTTCTGGGATTTCAGCGGCGATTCCAATCGGACGC
  A  N  G  L  S  G  A  M  F  W  D  F  S  G  D  S  N  R  T  L
TTCTCAATAAATTGGCAGCCGATTTAGATTTTGCACCGGACGGAGGCAATCCGGAGCCGC
  L  N  K  L  A  A  D  L  D  F  A  P  D  G  G  N  P  E  P  P
CTTCATCGGCACCTGTGAATGTGCGTGTAACCGGAAAAACTGCTACAAGTGTCAGCCTGG
```

```
  S  S  A  P  V  N  V  R  V  T  G  K  T  A  T  S  V  S  L  A
CGTGGGATGCGCCGAGCAGCGGAGCAAACATTGCGGAATATGTCGTGTCATTTGAAAACC
  W  D  A  P  S  S  G  A  N  I  A  E  Y  V  V  S  F  E  N  R
GGTCGATATCTGTAAAAGAAACATCAGCGGAAATAGGCGGCTTGAAGCCGGGTACGGCCT
  S  I  S  V  K  E  T  S  A  E  I  G  G  L  K  P  G  T  A  Y
ACTCATTTACTGTTTCAGCAAAGGATGCGGATGGAAAGCTCCATGCCGGACCAACGGTAG
  S  F  T  V  S  A  K  D  A  D  G  K  L  H  A  G  P  T  V  E
AGGTCACGACGAATTCTGACCAAGCCTGTTCATATGACGAATGGAAAGAGACGAGCGCAT
  V  T  T  N  S  D  Q  A  C  S  Y  D  E  W  K  E  T  S  A  Y
ACACAGGCGGAGAGCGGGTTGCATTTAACGGAAAAGTGTATGAAGCGAAATGGTGGACGA
  T  G  G  E  R  V  A  F  N  G  K  V  Y  E  A  K  W  W  T  K
AAGGCGACCGGCCTGATCAATCCGGTGAATGGGGCGTATGGCGGCTGATCGGAGGCTGCG
  G  D  R  P  D  Q  S  G  E  W  G  V  W  R  L  I  G  G  C  E
AATAATACCATCGTGCTGATGAACAAAGGGGTCATTCAGCAGGAGGCGCCGCCCGAAGTG
*

GTGTACCGCAAGCCCAGCAATCTGTTTGCGGCCCAGTTCATCGGGGTTCCTCCCATGAAC

ATTGCGCCGCTGGGGGACATCGTATGTAAAGTGCGCGACGTTCGGGTTCCGTCCGGAAAG

CGTTGTGATCGGCACGGAACCGGAATCGTTTCACTACACGGCCAAGGGCGAGATCATCAC
```

Figure 3.18 **Nucleotide sequence and deduced amino acid sequence of CHI**66 **from *Bacillus* sp. PP8**

Nucleotide sequence and deduced amino acid sequence of CHI66 from *Bacillus* sp. PP8. Coding region started at position 186 and end at 1982. The -35 and -10 region of the putative promoter sequence and possible Shine-Dalgarno (SD) sequence for the ribosome binding site were underlined. The signal sequence was underlined with a broken line. The asterisk represented a stop codon

```
Bacillus_licheniformis    CTGCAGAACCCTTTTCAAACGCCAAAATTTTAATGAAACCATGCT-----
Bacillus_sp.              -------------TATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGG
                                      *  *  ** **     **  *   **   **

Bacillus_licheniformis    -GTCGATCTCACTTTTGTAAGCGTTTTCCCTTGTTGTCTTCAATGTATCT
Bacillus_sp.              GGTACAGCTCCCGCCGGTCAGGAT----CCTTGTTGTCTTCAATGTATCT
                           **  * *** *    ** **  *    **********************

Bacillus_licheniformis    GCTGCTATTAGATGACAAGGAAAAATATAAAACCAGCAAAAAAGGCGGTG
Bacillus_sp.              GCTGCTATTAGATGACAAGGAAAAATA-AAAACCAGCAAAAAAGGCGGTG
                          ************************** **********************

Bacillus_licheniformis    AGGAAAAAGAGAGTTCTAGTTTCATAGCTTGCCAAAAAATTGCTTGTAAA
Bacillus_sp.              AGGAAAAAGAGAGTTCTAGTTTCATAGCTTGCCAAAAAATTGCTTGTAAA
                          ************************************************

Bacillus_licheniformis    GGAGATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTT
Bacillus_sp.              GGAGATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTAT
```

```
                          ********************************************** *

Bacillus_licheniformis    CTTTCATTTTTGTTATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTT
Bacillus_sp.              CTTTCATTTTTGTTATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTT
                          **************************************************

Bacillus_licheniformis    GCAAAAGCCGATTCCGGAAAAAACTATAAAATCATCGGCTACTATCCATC
Bacillus_sp.              GCAAAAGCCGATTCCGGAAAGAACTATAAAATCATCGGCTACTATCCATC
                          ******************** ****************************

Bacillus_licheniformis    ATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGGGATATGGACGTTTCGA
Bacillus_sp.              ATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGGGATATGGACGTTTCGA
                          **************************************************

Bacillus_licheniformis    AAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAGGGAAGG
Bacillus_sp.              AAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAGGGAAGG
                          **************************************************

Bacillus_licheniformis    CATGGGAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCA
Bacillus_sp.              CATGGGAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCA
                          **************************************************

Bacillus_licheniformis    GGATGAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCG
Bacillus_sp.              GGATGAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCG
                          **************************************************

Bacillus_licheniformis    ATCCCTGGATTGACGCACAAAAGGCAAATCCCGGGGATGTCTGGGATGAA
Bacillus_sp.              ATCCCTGGATTGACGCACAAAAGGCAAATCCCGGGGATGTCTGGGATGAA
                          **************************************************

Bacillus_licheniformis    CCGATCCGCGGCAACTTTAAACAATTGTTGAAGCTGAAAAAGAGCCACCC
Bacillus_sp.              CCGATCCGCGGCAACTTTAAACAATTGTTGAAGCTGAAAAAGAGCCACCC
                          **************************************************
Bacillus_licheniformis    TCATTTGAAAACGTTCATATCGGTCGGGGGGTGGACTTGGTCTAACCGCT
Bacillus_sp.              TCATTTGAAAACGTTCATATCGGTCGGGGGGTGGACTTGGTCTAACCGCT
                          **************************************************

Bacillus_licheniformis    TTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTCCGCCGCTTCG
Bacillus_sp.              TTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCCGCTTCG
                          ****************************************** **********

Bacillus_licheniformis    CCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTG
Bacillus_sp.              GCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTG
                           *************************************************

Bacillus_licheniformis    GGAATATCCGGTCAGCGGAGGATTGCCGGGGAACAGCACACGTCCGGAAG
Bacillus_sp.              GGAATATCCGGTCAGCGGAGGATTGCCGGGGAACAGCACACGTCCGGAAG
                          **************************************************

Bacillus_licheniformis    ATAAAAGAAACTACACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTGAC
Bacillus_sp.              ATAAAAGAAACTACACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTGAC
                          **************************************************

Bacillus_licheniformis    GCTGCAGAAGCAAAAGACGGCAAGGAATACTTGCTGACGATCGCATCCGG
Bacillus_sp.              GCTGCAGAAGCAAAAGACGGCAAGGAATACTTGCTGACGATCGCATCCGG
                          **************************************************


Bacillus_licheniformis    CGCAAGTCCCGATTATGTAAGCAACACTGAGCTCGATAAAATCGCTCAAA
Bacillus_sp.              CGCAAGTCCCGATTATGTAAGCAACACTGAGCTCGATAAAATCGCTCAAA
                          **************************************************

Bacillus_licheniformis    CCGTGGATTGGATTAACATTATGACCTATGACTTTAATGGCGGATGGCAA
Bacillus_sp.              CCGTGGATTGGATTAACATTATGACCTATGACTTTAATGGCGGATGGCAA
                          **************************************************

Bacillus_licheniformis    AGCATAAGCGCCCATAATGCACCGCTGTTCTATGATCCAAAAGCGAAAGA
Bacillus_sp.              AGCATAAGCGCCCATAATGCACCGCTGTTCTATGATCCAAAAGCGAAAGA
                          **************************************************

Bacillus_licheniformis    AGCAGGCGTTCCAAACGCTGAGACCTACAATATTGAAAACACTGTGAAAC
Bacillus_sp.              AGCAGGCGTTCCAAACGCTGAGACCTACAATATTGAAAACACTGTGAAAC
                          **************************************************

Bacillus_licheniformis    GCTACAAGGAAGCCGGTGTCAAGGGTGACAAATTAGTGCTTGGAACACCG
Bacillus_sp.              GCTACAAGGAAGCCGGTGTCAAGGGTGACAAATTAGTGCTTGGAACACCG
```

```
                          **************************************************

Bacillus_licheniformis    TTCTACGGAAGGG-CTGGAGCGGTTGTGAATCCAGGGGGCACGGAGAATA
Bacillus_sp.              TTCTACGGAAGGGGCTGGAGCGGTTGTGAACCAGGGGGGCACGGAGAATA
                          ************* *************** *  ****************

Bacillus_licheniformis    TCAGAAATGCGGACCGGCTAAAGAAGGGACATGG--GA-AAAGGGCGTAT
Bacillus_sp.              TCAGAAATGCGGACCGGCTAAAGAAGGGACATGGTTGATAAAGGGCGTAT
                          ***********************************  ** ***********
Bacillus_licheniformis    TCGATTTTTCAGATCTTGAAAGGAACCTATGTGAATCAAAACGGCTATAA
Bacillus_sp.              TCGATTTTTCAGATCTTGAAAGGAAC-TATGTGAATCAAAACGGCTATAA
                          ************************** ***********************

Bacillus_licheniformis    AAGGTATTGGAACGATCAAGCAAAAGTGCCGTTTTTGTATAATGCGGAAA
Bacillus_sp.              AAGGTATTGGAACGATCAAGCAAAAGTGCCGTTTTTGTATAATGCGGAAA
                          **************************************************

Bacillus_licheniformis    ATGGCAATTTCATCACTTATGATGATGAACAATCATTCGGCCACAAAACG
Bacillus_sp.              ATGGCAATTTCATCACTTATGATGATGAACAATCATTCGGCCACAAAACG
                          **************************************************

Bacillus_licheniformis    GATTTTATTAAAGCAAACGGATTAAGCGGAGCAATGTTCTGGGATTTCAG
Bacillus_sp.              GATTTTATTAAAGCAAACGGATTAAGCGGAGCAATGTTCTGGGATTTCAG
                          **************************************************

Bacillus_licheniformis    CGGCGATTCCAATCGGACGCTTCTCAATAAATTGGCAGCCGATTTAGATT
Bacillus_sp.              CGGCGATTCCAATCGGACGCTTCTCAATAAATTGGCAGCCGATTTAGATT
                          **************************************************

Bacillus_licheniformis    TTGCACCGGACGGAGGCAATCCGGAGCCGCCTTCATCGGCACCTGTGAAT
Bacillus_sp.              TTGCACCGGACGGAGGCAATCCGGAGCCGCCTTCATCGGCACCTGTGAAT
                          **************************************************

Bacillus_licheniformis    GTGCGTGTAACCGGAAAAACTGCTACAAGTGTCAGCCTGGCGTGGGATGC
Bacillus_sp.              GTGCGTGTAACCGGAAAAACTGCTACAAGTGTCAGCCTGGCGTGGGATGC
                          **************************************************

Bacillus_licheniformis    GCCGAGCAGCGGAGCAAACATTGCGGAATATGTCGTGTCATTTGAAAACC
Bacillus_sp.              GCCGAGCAGCGGAGCAAACATTGCGGAATATGTCGTGTCATTTGAAAACC
                          **************************************************

Bacillus_licheniformis    GGTCGATATCTGTAAAAGAAACATCAGCGGAAATAGGCGGCTTGAAGCCG
Bacillus_sp.              GGTCGATATCTGTAAAAGAAACATCAGCGGAAATAGGCGGCTTGAAGCCG
                          **************************************************

Bacillus_licheniformis    GGTACGGCCTACTCATTTACTGTTTCAGCAAAGGATGCGGATGGAAAGCT
Bacillus_sp.              GGTACGGCCTACTCATTTACTGTTTCAGCAAAGGATGCGGATGGAAAGCT
                          **************************************************

Bacillus_licheniformis    CCATGCCGGACCAACGGTAGAGGTCACGACGAATTCTGACCAAGCCTGTT
Bacillus_sp.              CCATGCCGGACCAACGGTAGAGGTCACGACGAATTCTGACCAAGCCTGTT
                          **************************************************

Bacillus_licheniformis    CATATGACGAATGGAAAGAGACGAGCGCATACACAGGCGGAGAGCGGGTT
Bacillus_sp.              CATATGACGAATGGAAAGAGACGAGCGCATACACAGGCGGAGAGCGGGTT
                          **************************************************

Bacillus_licheniformis    GCATTTAACGGAAAAGTGTATGAAGCGAAATGGTGGACGAAAGGCGACCG
Bacillus_sp.              GCATTTAACGGAAAAGTGTATGAAGCGAAATGGTGGACGAAAGGCGACCG
                          **************************************************
Bacillus_licheniformis    GC-TGATCAATCCGGTGAATGGGGCGTATGGCGGCTGATCGGAGGCTGCG
Bacillus_sp.              GCCTGATCAATCCGGTGAATGGGGCGTATGGCGGCTGATCGGAGGCTGCG
                          ** **********************************************

Bacillus_licheniformis    AATAAGA----------GAAAGTCAAATGGATAGAAAACGATAAAGAGA-
Bacillus_sp.              AATAATACCATCGTGCTGATGAACAAAGGGGTCATTCAGCAGGAGGCGCC
                          ***** *          **    **** ** *     *  *  * * *

Bacillus_licheniformis    ----GATTTGGGGAACAGCTTCTCACGTCTTCTCTTTATGGACAAAGGAG
Bacillus_sp.              GCCCGAAGTGGTGTACCGCAAGCCCAGCAATCTGTTTGCGGCCCAGTTCA
                              **  *** * ** **    *  *   *** ***  ** * *

Bacillus_licheniformis    TCTGAGTAAA---CATGAAGAAAGCCGCTTCATCTTTTTTATCTTGTATG
Bacillus_sp.              TCGGGGTTCCTCCCATGAACATTGCGCCGCTGGGGGACATCGTATGTAAA
                          ** * **      ****** *  **  *          *    ****
```

```
Bacillus_licheniformis      CTGCTCCTCG--CGCTTTTTATCCCGAACCGGCAGATTTCCGCAGAGA-C
Bacillus_sp.                GTGCGCGACGTTCGGGTTCCGTCCGGAAAGCGTTGTGATCGGCACGGAAC
                             *** *  **  **  **   *** ***   *  *   ** ***  ** *

Bacillus_licheniformis      TTCAAGCGAAAGACCGGACTGCCGTCCGGAAGGGCTATGGGACTCGGGCG
Bacillus_sp.                CGGAATCGTTTCACTACACGGCC------AAGGGCGA-GATCATCACGCG
                               ** **    **   ** ***      ****** * *    **  ***

Bacillus_licheniformis      TTGAACA-TGTGCCGTATTGCGATGTATACGACAAGGACGGCCGTGAAAA
Bacillus_sp.                AGAAATGCTGGGCTCCGAGACGATTTATCAGGTGAAGACGACCG------
                               **   ** **       **** ***  *   * **** ***

Bacillus_licheniformis      GCTGGCCAATCAGTTAGACCGGAGAATCATCGG--TTACTTTACGAGCTG
Bacillus_sp.                -ATGGTCATGCATTCA---TGGTGAAATGTCCGGATGACA----------
                              *** **  ** * *    ** ***   ** *  * **

Bacillus_licheniformis      GCGTACGGGAAAAGGAAATCAAGATCGTTATTTAGTAAGCGATATCCCCT
Bacillus_sp.                --------------------------------------------------

Bacillus_licheniformis      GGAAGTACTTGAGCC
Bacillus_sp.                ---------------
```

Figure 3.19 Nucleotide alignment of chitinase gene from *Bacillus* sp.PP8 and *B. licheniformis*

The nucleotide sequence of chi66 from *Bacillus* sp.PP8 and chi65 of *B. licheniformis* PR-1 were aligned using CLUSWAL X (1.64b)), Where * denotes the identical sequence.


```
Bacillus_licheniformis      MKIVLINKSKKFFVFSFIFVMMLSLSFVNGEVAKADSGKNYKIIGYYPSW
Bacillus_sp3PP8             MKIVLINKSKKFFVLSFIFVMMLSLSFVNGEVAKADSGKNYKIIGYYPSW
                            **************:**********************************

Bacillus_licheniformis      GAYGRDFQVWDMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQD
Bacillus_sp3PP8             GAYGRDFQVWDMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQD
                            **************************************************

Bacillus_licheniformis      ENGVIDAPNGTIVMGDPWIDAQKANPGDVWDEPIRGNFKQLLKLKKSHPH
Bacillus_sp3PP8             ENGVIDAPNGTIVMGDPWIDAQKANPGDVWDEPIRGNFKQLLKLKKSHPH
                            **************************************************

Bacillus_licheniformis      LKTFISVGGWTWSNRFSDVAADPAARENSAASPVEFLRKYGFDGVDLDWE
Bacillus_sp3PP8             LKTFISVGGWTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWE
                            *************************** ***.****************
```

```
Bacillus_licheniformis      YPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGKEYLLTIASGA
Bacillus_sp3PP8             YPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGKEYLLTIASGA
                            **************************************************

Bacillus_licheniformis      SPDYVSNTELDKIAQTVDWINIMTYDFNGGWQSISAHNAPLFYDPKAKEA
Bacillus_sp3PP8             SPDYVSNTELDKIAQTVDWINIMTYDFNGGWQSISAHNAPLFYDPKAKEA
                            **************************************************

Bacillus_licheniformis      GVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRAGAVVNPGGTENIR
Bacillus_sp3PP8             GVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSGCEPGGHGEYQ
                            ***********************************. :  :***  : :

Bacillus_licheniformis      NADRLKKG-HGKRAYSIFQILKGTYVNQNGYKRYWNDQAKVPFLYNAENG
Bacillus_sp3PP8             KCGPAKEGTWLIKGVFDFSDLERNYVNQNGYKRYWNDQAKVPFLYNAENG
                            :..  *:*    :.   *. *: .*************************

Bacillus_licheniformis      NFITYDDEQSFGHKTDFIKANGLSGAMFWDFSGDSNRTLLNKLAADLDFA
Bacillus_sp3PP8             NFITYDDEQSFGHKTDFIKANGLSGAMFWDFSGDSNRTLLNKLAADLDFA
                            **************************************************

Bacillus_licheniformis      PDGGNPEPPSSAPVNVRVTGKTATSVSLAWDAPSSGANIAEYVVSFENRS
Bacillus_sp3PP8             PDGGNPEPPSSAPVNVRVTGKTATSVSLAWDAPSSGANIAEYVVSFENRS
                            **************************************************

Bacillus_licheniformis      ISVKETSAEIGGLKPGTAYSFTVSAKDADGKLHAGPTVEVTTNSDQACSY
Bacillus_sp3PP8             ISVKETSAEIGGLKPGTAYSFTVSAKDADGKLHAGPTVEVTTNSDQACSY
                            **************************************************

Bacillus_licheniformis      DEWKETSAYTGGERVAFNGKVYEAKWWTKGDRLINP----VNGAYGG--
Bacillus_sp3PP8             DEWKETSAYTGGERVAFNGKVYEAKWWTKGDRPDQSGEWGVWRLIGGCE

                            ******************************  :.    *    **
```

Figure 3.20 Alignment of deduced amino acid sequence of CHI66 from *Bacillus* sp. PP8 with CHI65 of *B. licheniformis*

A deduced amino acid sequence of CHI66 were aligned with CHI65 from *B. licheniformis* by CLUSTAL X (1.64b). Where * denotes the identical sequence, : and . denotes the amino acid in the same group.

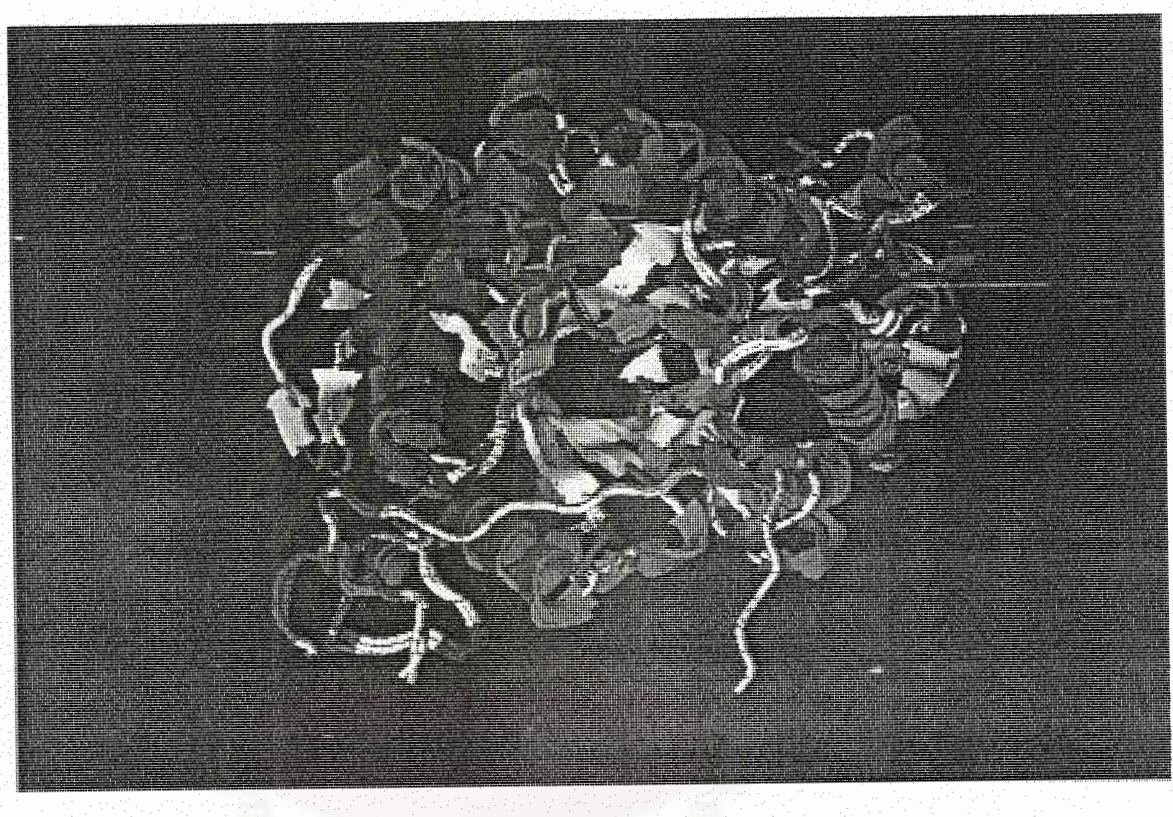

Figure 3.21 Theoretical Structure of Chi66

Theoretical Structure of CHI66 was accomplished by SWISS Model Protein Modeling (SWISS-MODEL version 36.0002)

respectively. Optimum pH was 8.0 of Tris-HCl was an optimum buffer, which was shown in Figure 3.22 and 3.23.

The stability of crude recombinant enzyme against various pH was determined by comparative measuring the residual activity after incubation at various pHs 2.0-10.0. After incubation of the enzyme in the pH 6.0-8.0 at 4^{o}C for 2 months, the residual activity was in the range of 90-80% of the non-incubation control. Although the enzyme activity was completely lost after incubated at pH 2.0-3.0 and 11.0-12.0, the enzyme retained more over 50% of the full activity after incubated at pH 5.0 and 10.0 for a week, which was shown in Figure 3.24. In additional, temperature stability was also determined by measuring the residual activity after incubation at various temperatures. After incubation of the enzyme in the 50 mM Tris-HCl, pH 7.0 and 8.0 at 4, 30, 37, and 50^{o}C for 12 hour, the enzyme retained its full activity.

To determine, what chitinase was produced and secreted to culture medium of the cell harboring the pSNchi66, the crude enzyme was comparative analyzed with crude enzyme from *Bacillus* sp. PP8 by SDS-PAGE followed by activity staining. Five bands of crude enzyme of PP8 contained chitinase activity. On the other hand, only a single band of 66 kDa with chitinase activity was observed in the crude recombinant enzyme, as showed in Figure 3.25.

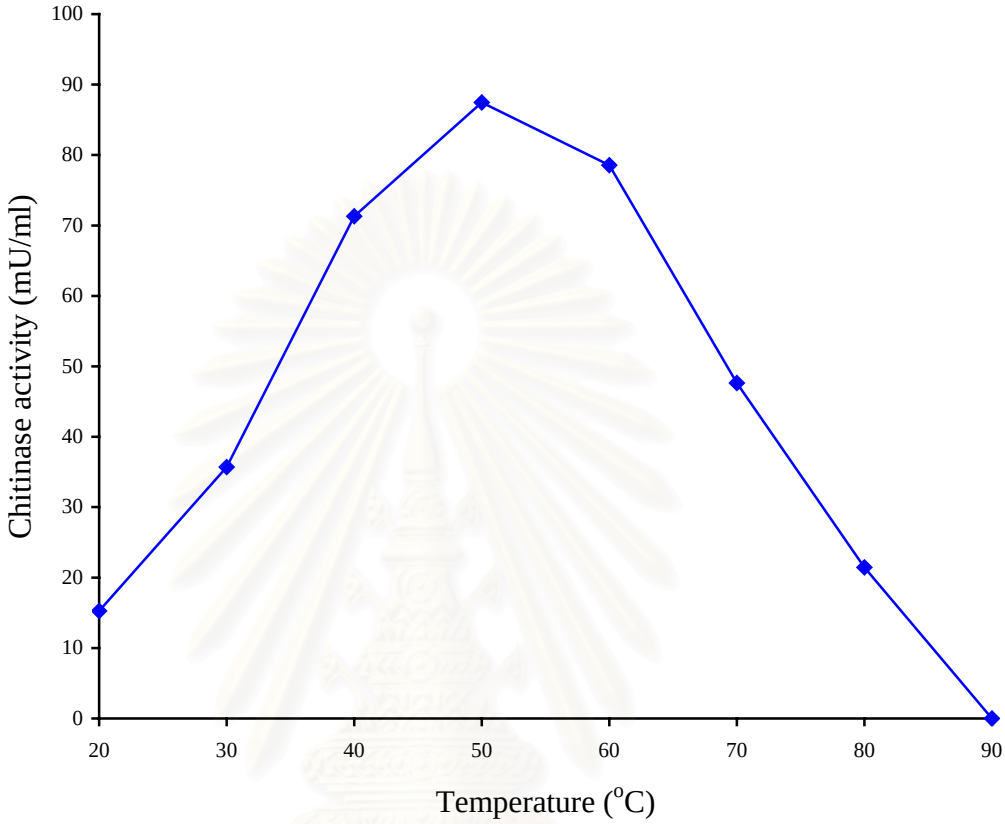

Figure 3.22 Optimum temperature of Chi66

Effect of temperature on recombinant chitinase activity was determined. Enzyme activity was assayed at 20-90°C in 0.15 M Tris-HCl pH 7.0 and colloidal chitin was used as substrate.

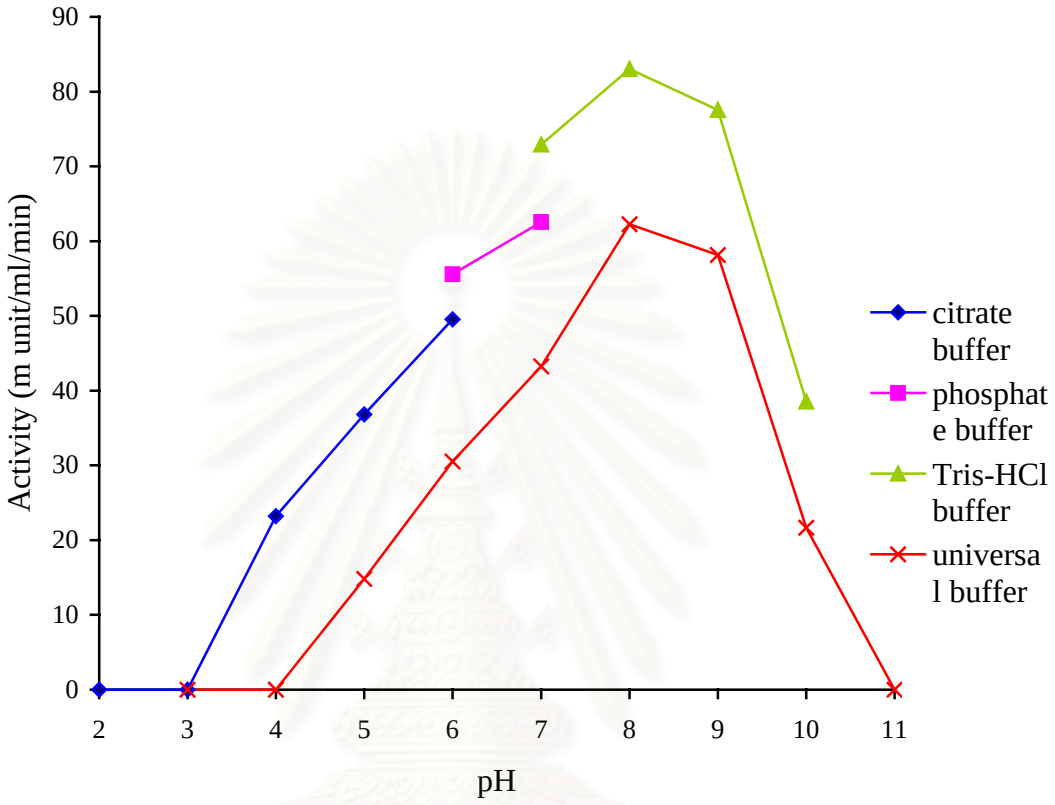

Figure 3.23 Optimun pH of Chi66.

Effect of various pHs on recombinant chitinase activity were assayed at pH 2-11 at 50°C. Colloidal chitin was used as substrate.

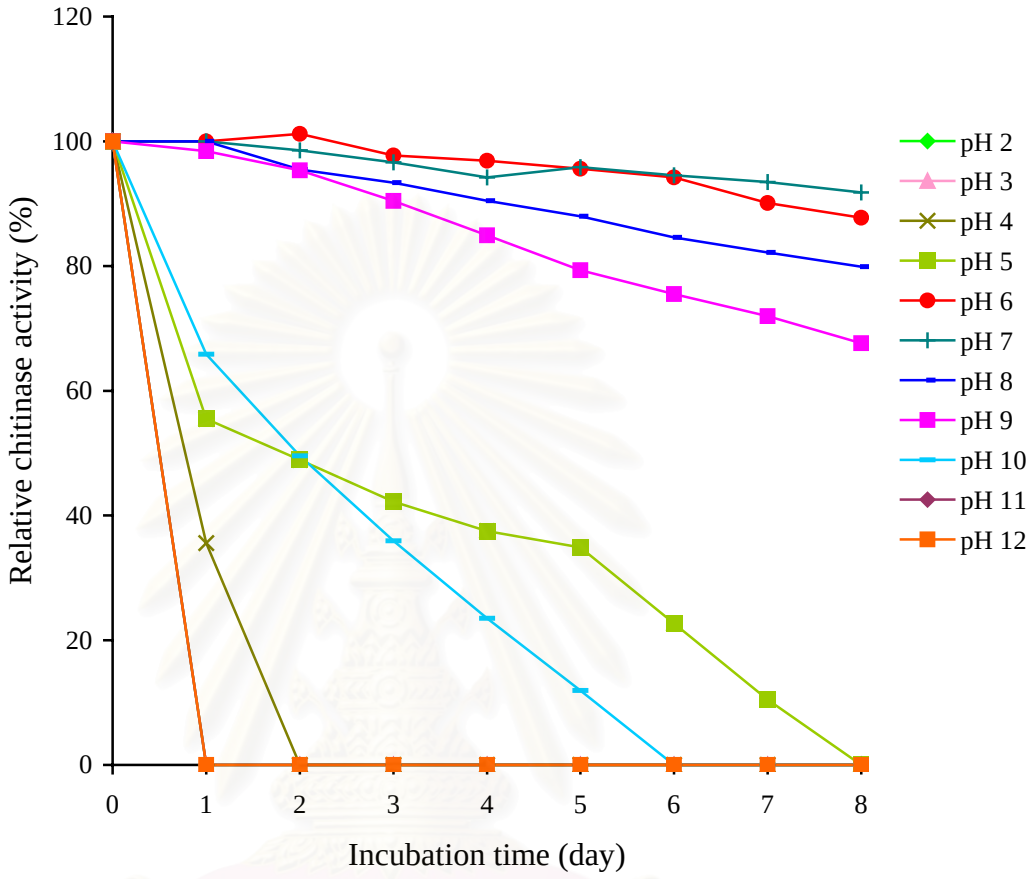

Figure 3.24 Effects of pH on the stability of crude CHI66 produced by *E. coli* Top-10 harboring pSNchi66

Crude CHI66 was incubated at 4°C on various pH for 2 months days. The residual chitinase activity was assayed in 0.15 M Tris-HCl, pH 7.0 at 50°C using colloidal chitin as substrate.

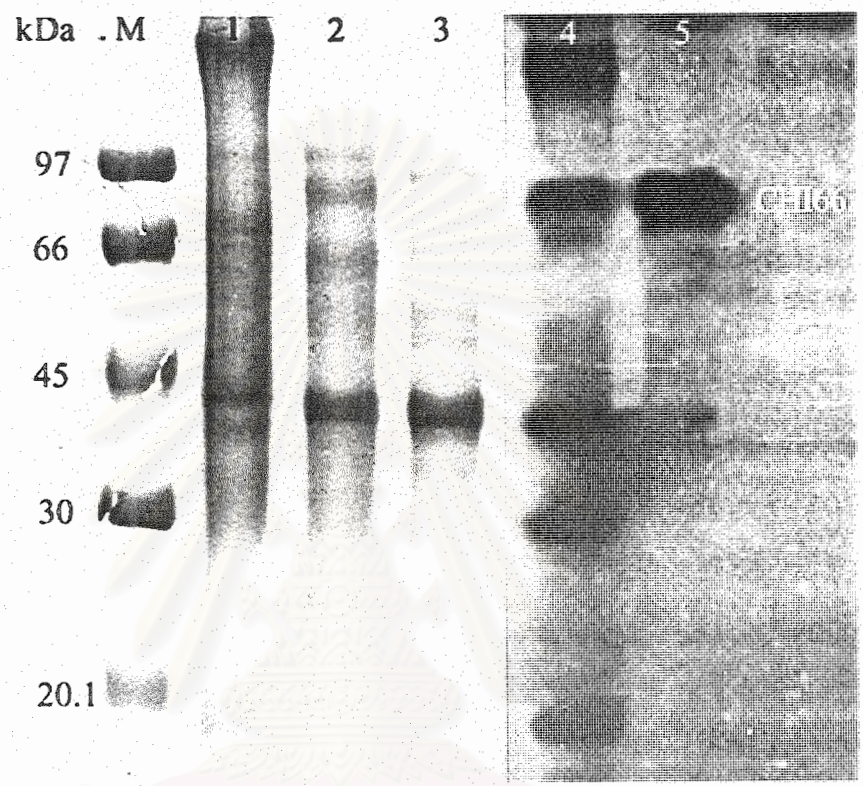

Figure 3.25 CHI66 produced by *E. coli* harboring pSNchi66

E. coli Top-10 carrying pSNchi66 was grown in colloidal chitin minimal medium containing 100 μg/ml ampicillin at 37°C for 4 days. *Bacillus* sp. PP8 was grown in colloidal chitin minimal medium at 37°C for 4 days.

Lane 1-3: protein stain and Lane 4-6: activity staining

Lane M: molecular weight marker

Lane 1 and 4: culture medium of *Bacillus* sp. PP8

Lane 2 and 5: culture medium of *E. coli* harboring pSNchi66

Lane 3 and 6: culture medium of *E. coli* harboring pBS/SK$^-$

Crude recombinant enzyme was incubated with various substrates at 50^{o}C and in reaction buffer pH 7.0 for chitinase assay. As shown in table 3.2 the degradation of amorphous chitin i.e. colloidal chitin was about 80% relative to the value of regenerated chitin. Crude enzyme also hydrolyzed powdered chitin and soluble chitosan (80%DD) but the relative degradation was about 30% and 4%, respectively. Essentially, this enzyme did not hydrolyze flaked chitin, which was differed from the crude enzyme of *Bacillus* sp.PP8.

Table 3.2 Degradation of some carbohydrates by crude enzyme of *Bacillus* sp.PP8

Carbohydrates	Relative degradation (%)
Glycol chitin	15.36
Colloidal chitin	81.25
Regenerated chitin	100.00
Powdered chitin	31.67
Flaked chitin	0
Soluble chitosan (80%DD)	4.12
Soluble chitosan (100%DD)	0

Where %DD stand for % deacetylation

Analysis of hydrolysis products

Hydrolysis product of chitinase was examined using colloidal chitin as the substrate. As shown in figure 3.25, colloidal chitin was hydrolyzed to GlcNAc and $(GlcNAc)_2$ as a major product (95% of total product).

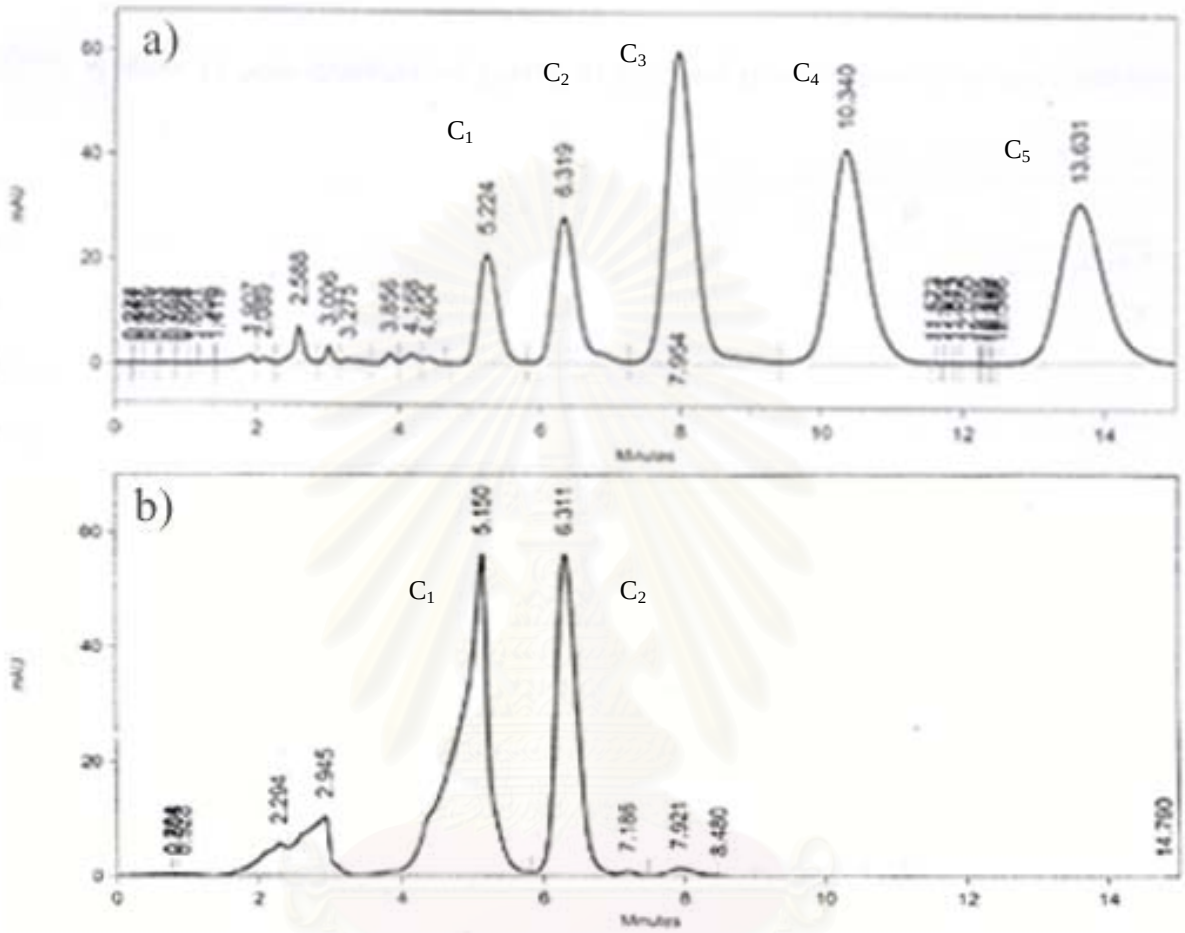

Figure 3.26 Hydrolysis product of CHI66

HPLC chromatogram of colloidal chitin hydrolysis by CHI66 at 50°C for an overnight.

a) The standard GlcNAc-$(GlcNAc)_5$ C_1:GlcNAc, C_2:$(GlcNAc)_2$, C_3:$(GlcNAc)_3$, C_4:$(GlcNAc)_4$ and C_5:$(GlcNAc)_5$

b) HPLC profiles of chitin oligosaccharide produced by crude chitinase from CHI66

Expression of *chi66* in *E. coli.* by pET19b(+)

chi66 was *in vitro* amplified using a two primer set , which were designed according to the available chitinase gene from *Bacillus* sp. PP8. The cloning site of the *Nco*I and *Bam*HI was incorporated into forward and reverse primers, respectively. The PCR was carried out as described in Materials and Methods. An amplified gene product, which primer annealing was varied from 50 to 60^{o}C, was examined on 0.7% agarose electrophoresis. As shown in Figure 3.26, there were nonspecific PCR products contaminated in the reaction. Thus, the correct PCR product with 2.0 kb expected size were recovered and purified from gel by excising the band and using QIAquick gel extraction kit. The purified PCR product was restriction digested by *Nco*I and *Bam*HI and ligated to the proper sites of the pET19b to generate an in-frame recombinant plasmid. The recombinant plasmids were transformed in to *E. coli* Top-10 and the transformants were screened onto a LB/Ampicillin (100μg/ml). 12 clones were selected for plasmid extraction and restriction digesting analyzed (Figure 3.27). The plasmids, which contained the appropriate insert, were retransformed to expression host, *E. coli* BL21(DE3). The retransformed cells were plating in LB-colloidal chitin/Ampicillin (100 μg/ml) agar containing 1 mM IPTG for phenotype analysis. BL21(DE3) produced a large clear zone after 12 hours of plating. However, BL21(DE3) pLysS did not produce a clear zone (Figure 3.28). The recombinant cells obtained from the same clone with those produced clear zones around colony were sequenced by using CEQ^{TM}8000 Genetic Analysis system to confirm the DNA sequence surround the cloning site by using T7 promoter as a sequence primer, as shown in Figure 3.29.

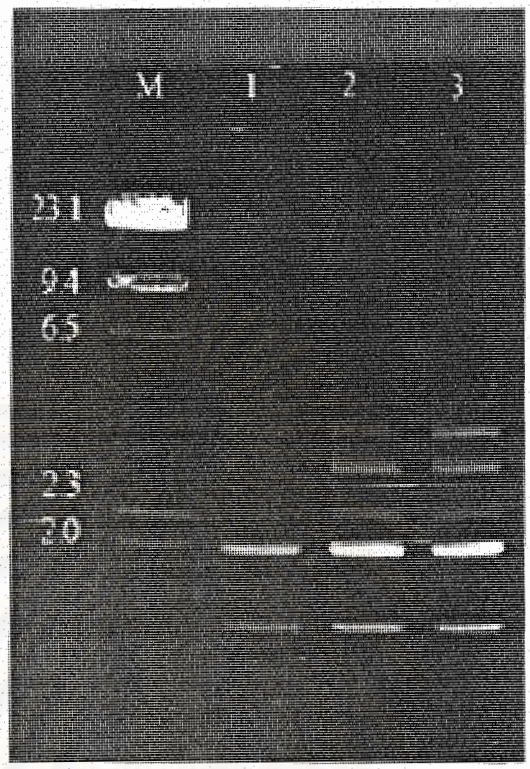

Figure 3.27 The PCR products of *chi66* for in-frame cloning to pET19b

0.7% Agarose gel electrophoresis pattern of PCR product amplified of *chi66* by using pSN*XX*-3.0 as a template. pF*Nco*I and pR*Bam*HI were used as PCR primers for in-frame cloning to pET19b. The annealing temperatures varied in the range of 50-60°C.

Lane M: λ/*Hind*III Marker,
Lane 1: PCR products when 50°C annealing temperature was used.
Lane 2: PCR products when 55°C annealing temperature was used
Lane 3: PCR products when 60°C annealing temperature was used

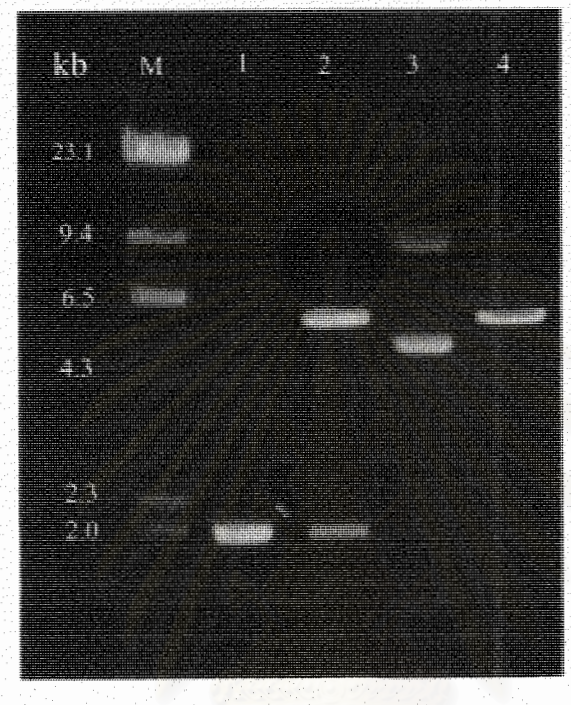

Figure 3.28 Subcloning of *chi66* into pET19b

0.7%agarose gel electrophoresis pattern of purified PCR product and pETCHI66 digested pattern.

Lane M: λ/HindIII

Lane 1: purified PCR product

Lane 2: pETchi66/*Nco*I+*Bam*HI

Lane 3: pETchi66 undigested

Lane 4: pET19b(+)/*Nco*I+*Bam*HI

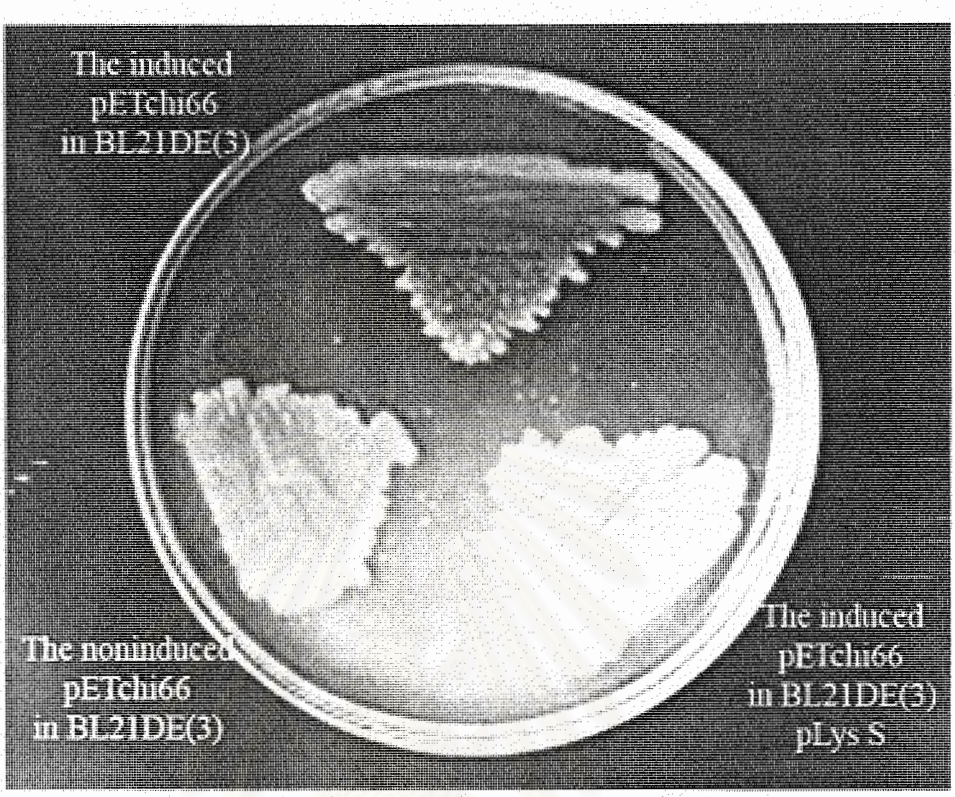

Figure 3.29 Chitinase expression of pETCHI66 in *E. coli* BL21(DE3)

pETCHI66 was transformed to *E. coli* BL21DE(3) and BL21DE(3) pLysS and grown on colloidal chitin LB/Ampicilin agar at 37°C for an overnight.

The top cells: *E. coli* BL21DE(3) harboring pETchi66 were induced by 1 mM IPTG

The left bottom cells: *E. coli* BL21DE(3) harboring pETchi66

The right bottom cells: *E. coli* BL21DE(3)pLysS harboring pETchi66 were induced by 1 mM IPTG

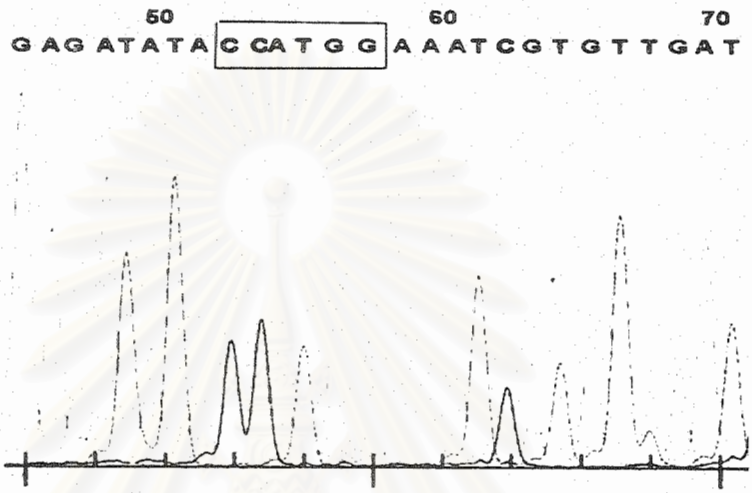

Figure 3.30 The sequence at the start site junction of chi66 in pET19b

The DNA sequence surrounding start site junction was sequenced using a primer complementary to the T7 promoter as a sequencing primer. The yellow and pink box are shown *Nco*I site and a codon for the methionine and glutamic acid, which was changed from lysine.

plate and inoculated in 200 ml LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphinicol in 1000 ml Erlenmeyer flask. The cell culture was incubated with shaking at 37°C until OD_{600} reached 0.7. A 100 ml of uninduced control was removed. To the remainder, IPTG from 0.5 M stock was added to the final concentration of 1 mM and the incubation continued for 36 hours. 1 ml of culture medium was collected at 6 hours interval for 48 hours. The chitinase activity, expressed by both BL21 (DE3) and Rosetta (DE3) were detected at 12 hour of cultivation and gradually increased after further cultivation. Although, Rosetta (DE3) produced 3 folds higher chitinase activity, when compared to *Bacillus* sp. PP8 but it was still less than the activity produced by BL21 (DE3) (6 folds higher than of *Bacillus* sp. PP8), as showed in Figure 3.30.

When *E. coli* BL21 (DE3) was used as expression host, the optimal temperature for chitinase expression by this strain was determined. The BL21 (DE3) was grown in LB/ampicillin at 30°C and 37°C, OD_{600} and chitinase activity of the medium fraction was examined at 0, 4, 8, 12, 16, 24, 36,48 and 60 hours of cultivation. The supernatant of culture medium showed a gradual increased in chitinase activity and reached its maximum after 2.5 days of cultivation (Figure 3.31). When the expression was carried out at 30°C, there was a higher chitinase activity than that at 37°C.

At 30°C, *E. coli* BL21 (DE3) was grown in 100 ml LB, 2xLB and terrific broth containing 100 μg/ml ampicillin in 1000 ml Erlenmeyer flask for a good aeration rate. Chitinase activity of the medium fraction was examined at 0, 4, 8, 12, 16, 24, and 36 hours of cultivation. Each medium was not different in chitinase activity, which was shown in Figure 3.32.

The best condition for overexpression of chitinase by pET19b was growing *E. coli* BL21 (DE3) harboring pETchi66 in LB medium at 30°C and induced the expression by 1mM IPTG for 60 hours. The medium fraction was examined at 0, 4, 8, 12, 16, 24 and 36 hours of cultivation by SDS-PAGE followed by activity staining, as showed in Figure 3.33.

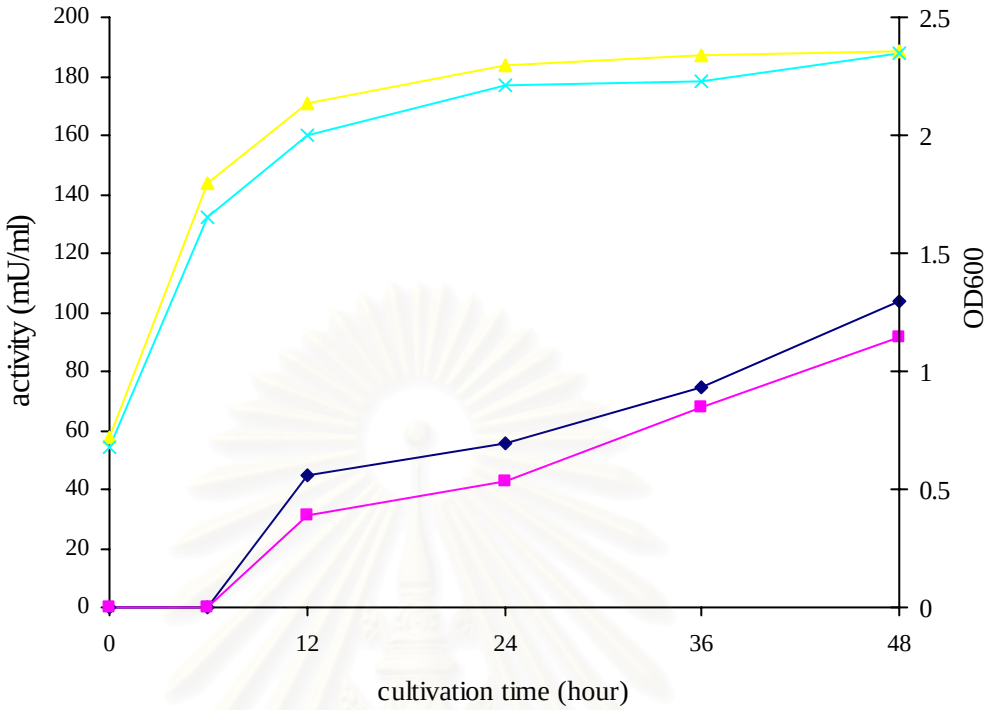

Figure 3.31 Relation between Chitinase activity and cell growth in *E. coli* BL21(DE3) and Rosetta(DE3)

E. coli BL21(DE3) and Rosetta(DE3) harboring a pETchi66 was grown in LB/100 μg/ml ampicillin (and 34 μg/ml chloramphinicol for *E. coli* Rosetta(DE3)) at 30°C. Chitinase expression was induced by 1mM IPTG. Medium fractions of the cell culture were collected at interval time. Cells growth were monitored by OD600 and chitinase activity was assayed at 50°C in 0.15 M Tris-HCl, pH 7.0 and usedcolloidal chitin as substrate.(*cell growth of *E. coli* Rosetta(DE3), ▲cell growth of *E. coli* BL21(DE3), ■chitinase activity from *E. coli* Rosetta(DE3) harboring pETchi66, ♦chitinase activity from *E. coli* BL21(DE3) harboring pETchi66)

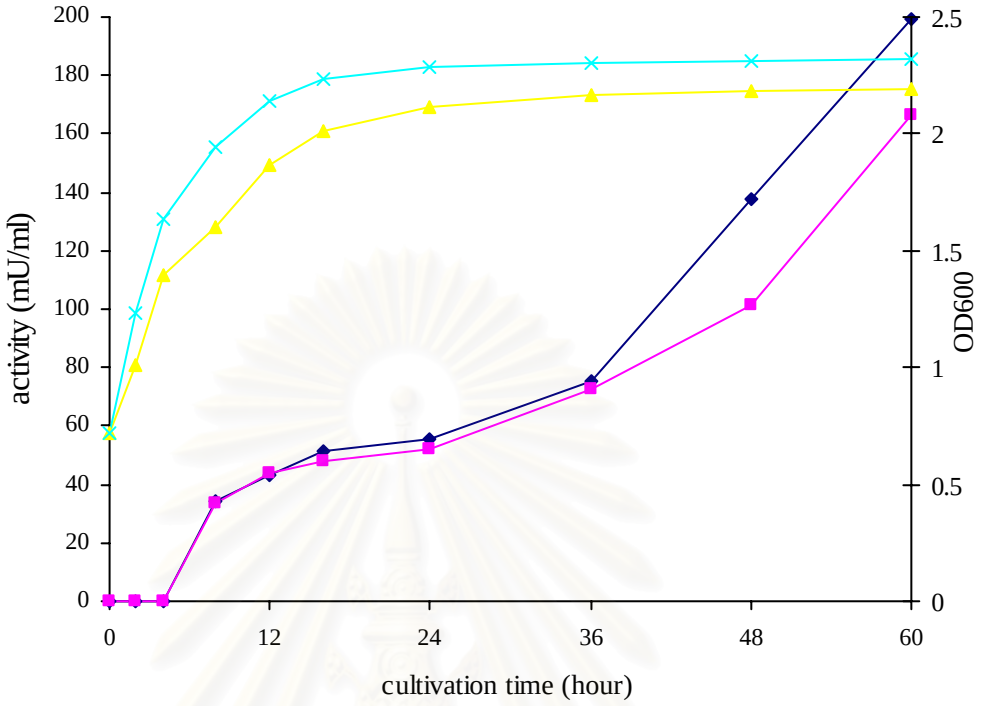

Figure 3.32 Relation between Chitinase activity and cell growth in different temperature

E. coli BL21(DE3) harboring a pETchi66 was grown in LB/ampicillin at 30 and 37°C. Chitinase expression was induced by 1mM IPTG. Medium fractions of the cell culture were collected at interval time. Cells growth were monitored by OD600 and chitinase activity was assayed at 50°C in 0.15 M Tris-HCl, pH 7.0 and used colloidal chitin as substrate.(*cell growth at 37°C, ▲cell growth at 30°C, ■chitinase activity when grew at 37°C, ♦chitinase activity when grew at 30°C)

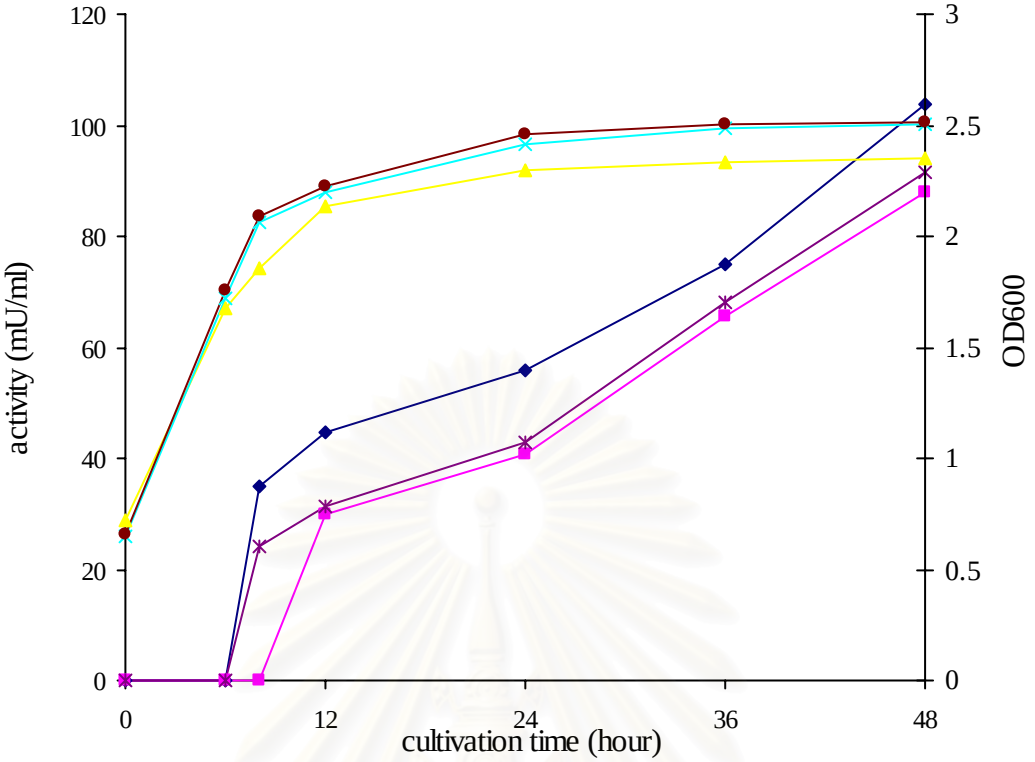

Figure 3.33 Relation between Chitinase activity and cell growth in different medium

E. coli BL21(DE3) harboring a pETchi66 was grown in LB/ampicillin at 30°C. Chitinase expression was induced by 1mM IPTG. Medium fractions of the cell culture were collected at interval time. Cells growth were monitored by OD600 and chitinase activity was assayed at 50°C in 0.15 M Tris-HCl, pH 7.0 and used colloidal chitin as substrate.(*cell growth in LB medium, ▲cell growth in 2X LB medium, ● cell growth in terrific broth, ♦chitinase activity in LB medium, ■chitinase activity in 2X LB medium , * chitinase activity in terrific broth)

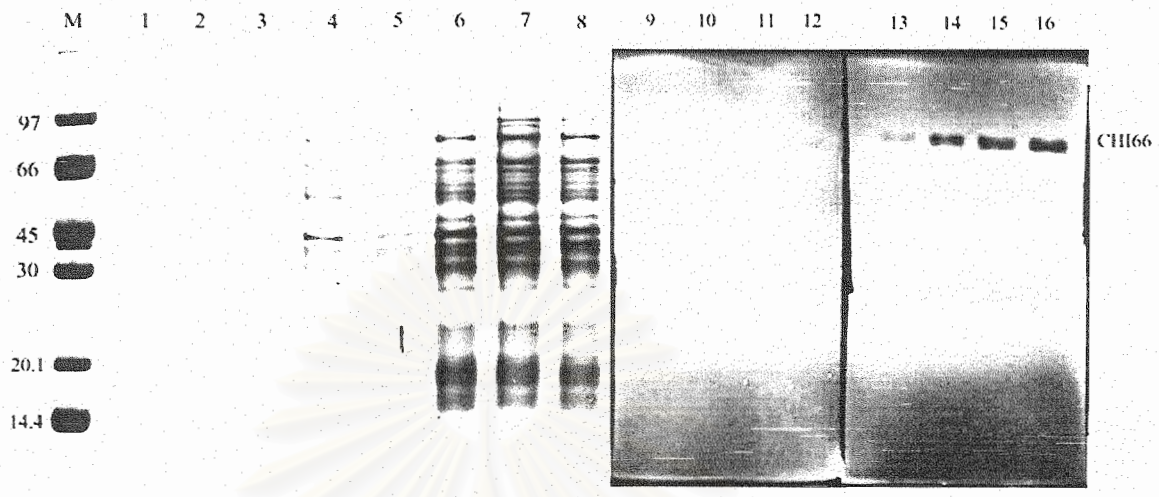

Figure 3.34 Chitinase expression using pET system in *E. coli* BL(DE3) cell harboring by pETchi66

E. coli BL21(DE3) harboring a pETchi66 was grown in LB/ampicillin at 30°C. Chitinase expression was induced by 1mM IPTG. Medium fractions of the cell culture at time interval were analyzed by 12.5%SDS-PAGE followed by activity staining

Lane 1-8:	protein staining and Lane 9-16: activity staining
Lane M:	molecular weight marker
Lane 1and 9:	*E. coli* BL21(DE3) harboring a pET19b(+)
Lane 2 and 10:	*E. coli* BL21(DE3) harboring a pETchi66 at 0 hour
Lane 3 and 11:	*E. coli* BL21(DE3) harboring a pETchi66 at 4 hours
Lane 4 and 12:	*E. coli* BL21(DE3) harboring a pETchi66 at 8 hours
Lane 5 and 13:	*E. coli* BL21(DE3) harboring a pETchi66 at 12 hours
Lane 6 and 14:	*E. coli* BL21(DE3) harboring a pETchi66 at 16 hours
Lane 7 and 15:	*E. coli* BL21(DE3) harboring a pETchi66 at 24 hours
Lane 8 and 16:	*E. coli* BL21(DE3) harboring a pETchi66 at 36 hours

*Nco*I and *Sal*I was incorporated into forward and reverse primer, respectively. The PCR was carried out as described in Materials and Methods by using the pSN*XX*-3.3 as a template. An amplification gene product was examined on 0.7% agarose electrophoresis. As shown in Figure 3.34, there was some nonspecific PCR products contaminated in the reaction. Thus, the expected PCR product with 1.8 kb in size was recovered and purified from gel by band excision and using the QIAquick gel extraction kit. The purified PCR product was restriction digested by *Nco*I and *Sal*I and ligated to the proper sites of the pTrcHis-2C to generate the in-frame recombinant plasmid. The recombinant plasmids were transformed into *E. coli* Top-10 and the transformants were screened on a LB/Ampicillin (100μg/ml) plate. 12 clones were selected for plasmid extraction and restriction digestion analysis (Figure 3.35). The plasmids, which contained the appropriate inserted were plating in LB-colloidal chitin/Ampicillin (100 μg/ml) agar containing 1 mM IPTG for phenotype analysis E. coli Top-10 harboring pTrcchi66 produced a large clear zone when incubated at 37^{o}C overnight (Figure 3.36).

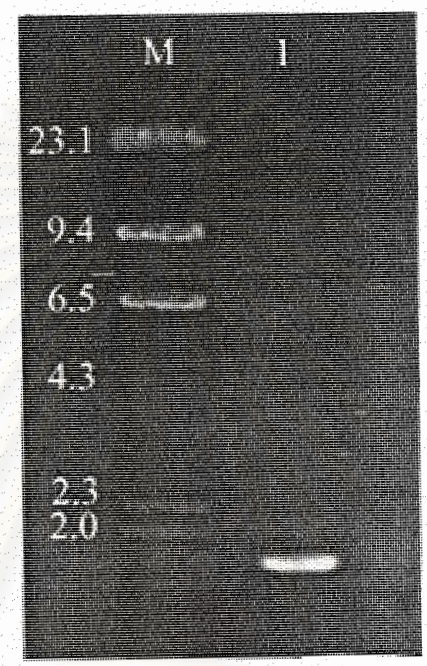

Figure 3.35 The PCR product of *chi66* for in-frame cloning to pTrcHis2-C

0.7% Agarose gel electrophoresis pattern of PCR product of *chi66* by using pSN*XX*-3.0 as a template. pF*Nco*I and pR*Sal*I were used as PCR primers for in-frame cloning to pTrcHis2-C. 81°C annealing temperature was used.

Lane M: λ/*Hind*III marker

Lane 1: PCR products

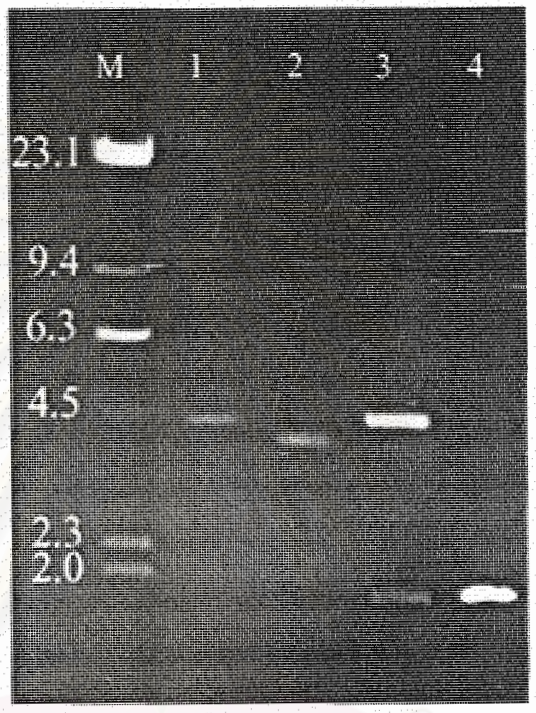

Figure 3.36 Subcloning of *chi66* into pTrcHis2-C

0.7% agarose gel electrophoresis pattern of purified PCR product and pETCHI66 digested pattern.

Lane M: λ/HindIII

Lane 1: pTrcHis2 C/*Nco*I+*Sal*I

Lane 2: pTrcchi66 undigested

Lane 3: pTrcchi66/*Nco*I+*Sal*I

Lane 4: purified PCR product

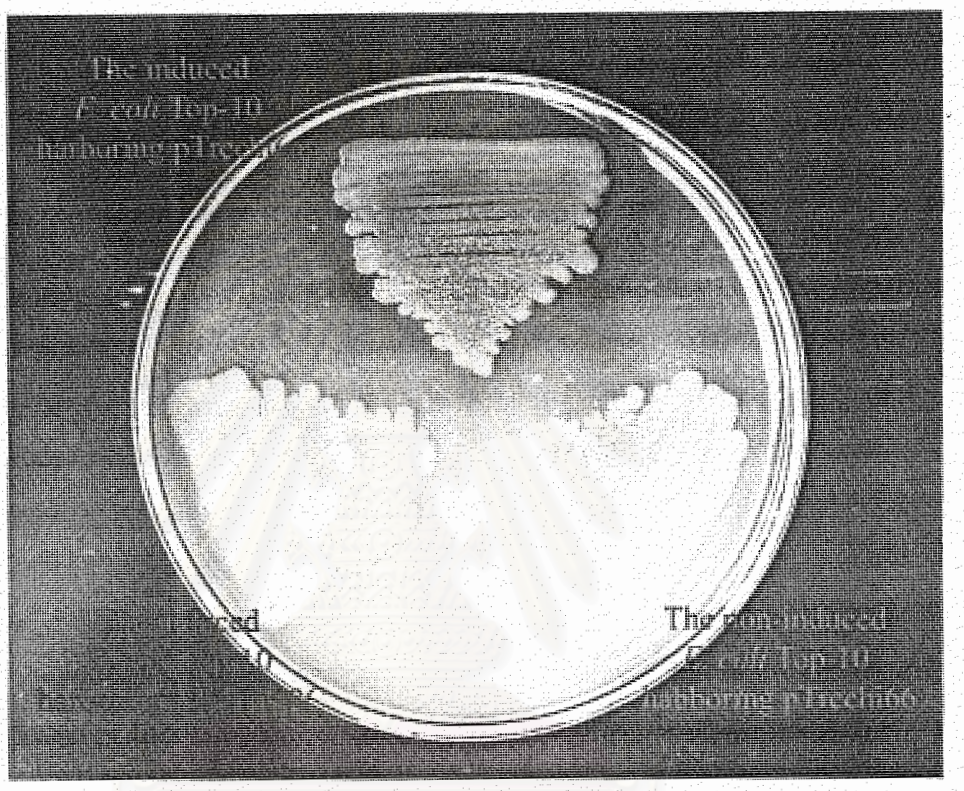

Figure 3.37 Chitinase expression using pTrcHis2-C in *E. coli* Top-10 harboring pTrcchi66

pTrcchi66 was transformed to *E. coli* Top-10 and grown on colloidal chitin LB/Ampicillin agar at 37°C for an overnight.

The top cells: *E. coli* Top-10 harboring pTrccchi66 was induced by 1 mM IPTG

The left bottom cells: *E. coli* Top-10 harboring pTrccchi66 was uninduced

The right bottom cells: *E. coli* Top-10 harboring pTrccchi66 was induced by 1 mM IPTG

0.25, 0.5, 0.75 and 1.00 mM, and continue the incubation at 30^{o}C for 60 hour, 0.5 ml of culture medium was collected for chitinase activity assayed at 0, 4, 6, 8, 12, 16, 24, 36, 48 and 60 hour. 1 mM IPTG was the best appropriate concentration for induction of chitinase expression, as showed in Figure 3.38, hence it was used in the temperature optimization.

To determined, the optimal temperature for chitinase expression by pTrcchi66, the *E. coli* Top-10 harboring the pTrcchi66 was cultured at 30^{o}C and 37^{o}C. OD_{600} were monitored for the cell growth. The supernatant of culture medium showed gradually increased chitinase activity and reached its maximum after 2.5 days of cultivation. The higher chitinase activity (182.38 mU/ml) was detected in culture medium of the *E. coli* Top-10 harboring the pTrcchi66, which was cultured at 30^{o}C (Figure 3.39).

The best condition for overexpression of chitinase by pTrcHis2-C was grew *E. coli* Top-10 harboring pTrcchi66 at 30^{o}C and induced the expression by 1mM IPTG for 60 hours. The medium fraction was examined at 0, 4, 8, 12, 16, 24 and 36 hours of cultivation by SDS-PAGE followed by activity staining, as showed in Figure 3.40.

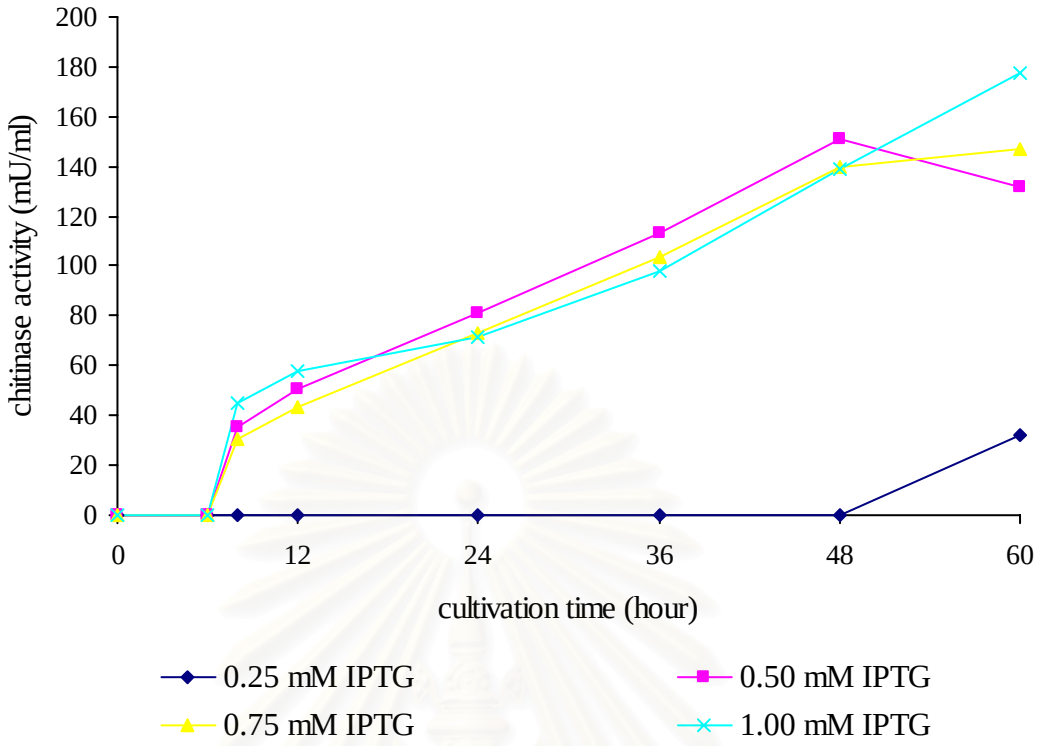

Figure 3.38 Chitinase activity was induced by various IPTG concentration.
E. coli Top-10 harboring a pTrcchi66 was grown in LB/ampicillin at 30°C. Chitinase expression was induced by 0.00-1.00 mM IPTG. Medium fractions of the cell culture were collected at interval time. Cells growth were monitored by OD600 and chitinase activity was assayed at 50°C in 0.15 M Tris-HCl, pH 7.0 and used colloidal chitin as substrate.

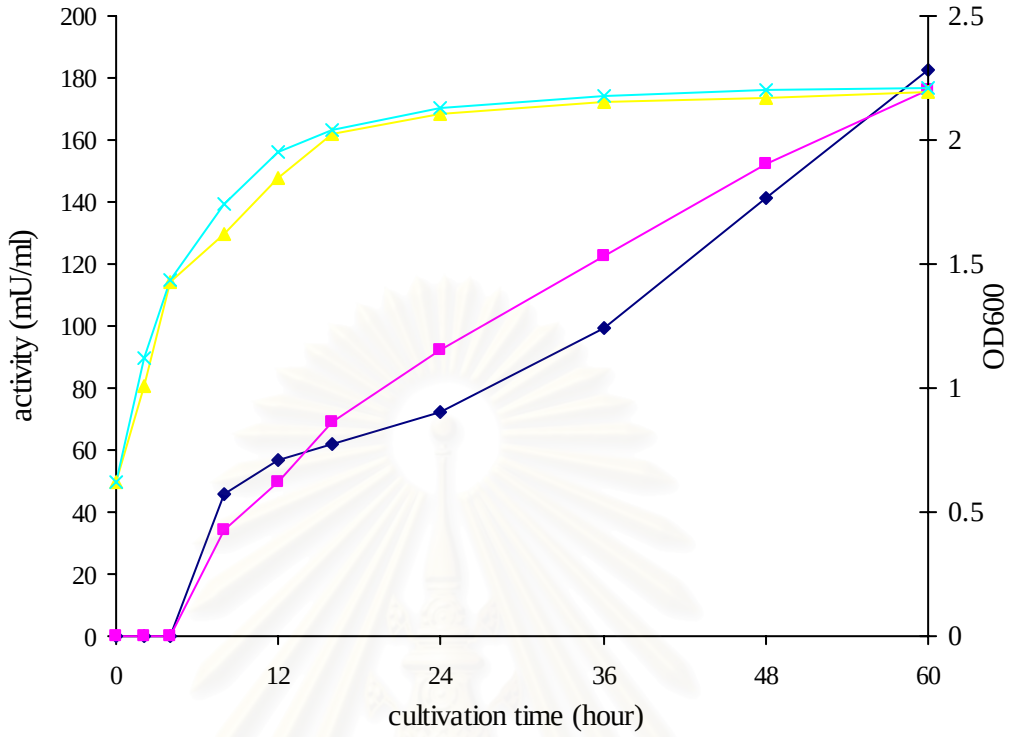

Figure 3.39 Relation between Chitinase activity and cell growth in different temperature

E. coli Top-10 harboring a pTrcchi66 was grown in LB/ampicillin at 30 and 37°C. Chitinase expression was induced by 1mM IPTG. Medium fractions of the cell culture were collected at interval time. Cells growth were monitored by OD_{600} and chitinase activity was assayed at 50°C in 0.15 M Tris-HCl, pH 7.0 and used colloidal chitin as substrate.(*cell growth at 37°C, ▲cell growth at 30°C, ■chitinase activity when grew at 37°C, ♦chitinase activity when grew at 30°C)

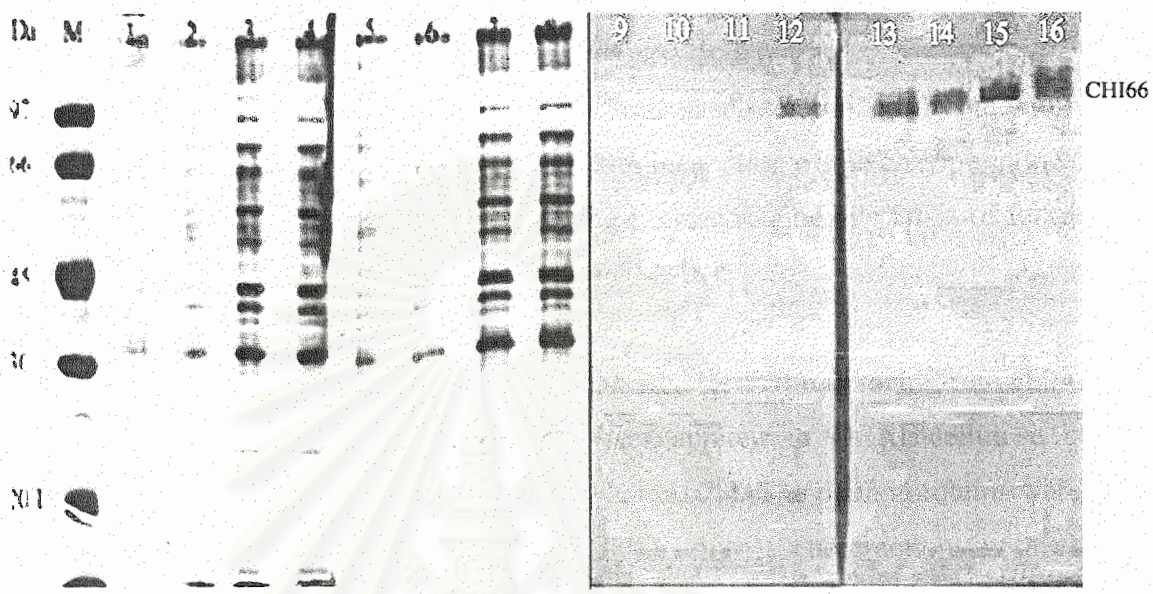

Figure 3.40 Chitinase expression by using pTrc system in *E. coli* Top-10 harboring pTrcchi66. *E. coli* Top-10 harboring a pTrcchi66 was grown in LB/ampicilin at 30°C. Chitinase expression was induced by 1mM IPTG. Medium fraction of the cell culture at interval time were analyzed by 12.5%SDS-PAGE followed by activity staining

Lane 1-8:	a protein staining and Lane 9-16: activity staining
Lane M:	molecular weight marker
Lane 1and 9:	*E. coli* Top-10 harboring a pTrcHis2-C
Lane 2 and 10:	*E. coli* Top-10 harboring a pTrcchi66 at 0 hour
Lane 3 and 11:	*E. coli* Top-10 harboring a pTrcchi66 at 4 hours
Lane 4 and 12:	*E. coli* Top-10 harboring a pTrcchi66 at 8 hours
Lane 5 and 13:	*E. coli* Top-10 harboring a pTrcchi66 at 12 hours
Lane 6 and 14:	*E. coli* Top-10 harboring a pTrcchi66 at 16 hours
Lane 7 and 15:	*E. coli* Top-10 harboring a pTrcchi66 at 24 hours
Lane 8 and 16:	*E. coli* Top-10 harboring a pTrcchi66 at 36 hours

The optimum temperature and pH of crude chitinase from pETchi66 in *E. coli* BL2(DE) under standard assay condition was identical to those from *E. coli* Top-10 harboring pSN*XX*-3.3.

In addition, temperature stability was also determined by measuring the residual activity after incubation at 4^{o}C. After incubation of the enzyme in the 50 mM Tris-HCl, pH 7.0 and 8.0 for 2 months, the enzyme retained its 80 % of full activity, as showed in Figure 3.41.

Purification of CHI66 produced by *E. coli* BL21(DE3) harboring pETchi66

CHI66, which 4.5 in pI value was applied to DEAE-cellulose column, previously equilibrated with 50 mM Tris-HCl pH 7.0. The bound proteins were eluted with an increasing concentration of NaCl (0-1.5 M). The fractions with chitinase activity were eluted at 0.75 M NaCl. The active fractions were pooled and concentrated to 40 folds. For adsorption to phenyl-Sepharose column, The concentrated DEAE-cellulose fraction was saturated with $(NH_4)_2SO_4$ to a final concentration of 1.5 M and applied to the column, which was previously equilibrated with 50 mM Tris-HCl containing 1.5 M $(NH_4)_2SO_4$. The unbound proteins were eluted by a 10-bed volume. The bound proteins were eluted with a 10-bed volume with decreasing in $(NH_4)_2SO_4$ concentration. Chitinase activity was found in unbound fraction. The total activity remained about 70% of the full activity as well as the total protein, as shown in Table 3.3 and Figure 3.42 and 3.43.

Due to an ineffective purification fold, we carried out a chitin adsorption by using 0.25 M and 0.5 M Tris-HCl pH 7.0 as a binding buffer. CHI66 effectively bound to the colloidal chitin when 0.5 M buffer was used, as shown in Table 34 and Figure 3.44.

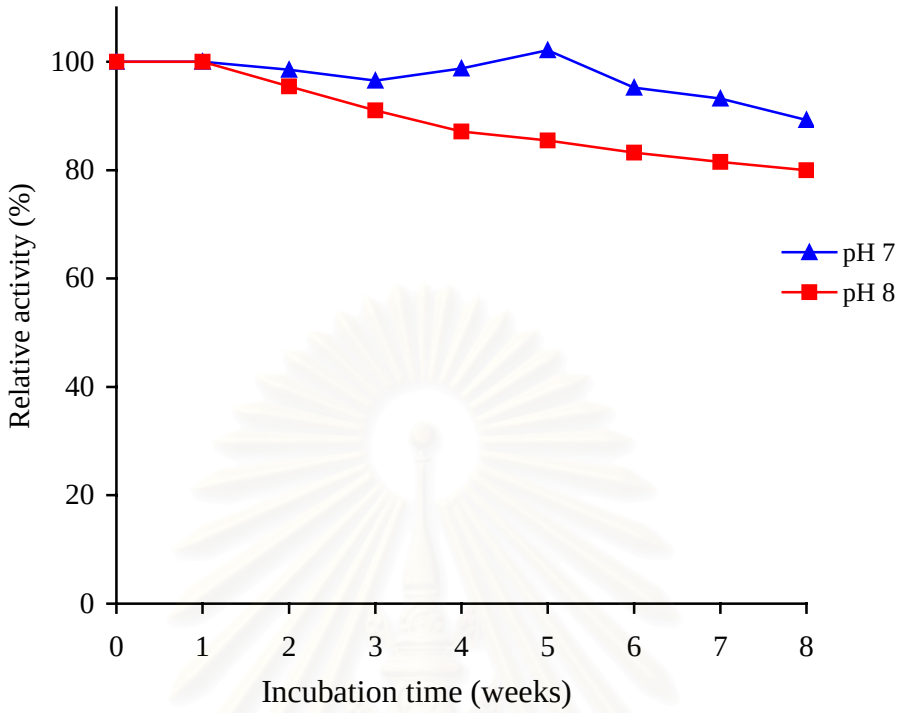

Figure 3.41 Storing of crude chitinase from pETchi66

Crude chitinase from in *E. coli* BL21(DE) harboring pETchi66 was storing at 4^{o}C for 2 months. The residual activity was examined every week. Enzyme activity was assayed at 50^{o}C in 0.15 M Tris-HCl, pH 7.0 by using colloidal chitin as substrate.

Table 3.3 Purification of CHI66 produced by *E. coli* BL21(DE3) using column chromatography

Purification steps	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U/ml)	Specific activity (U/mlmg)	Fold	Yield (%)
Culture medium	200	0.51	102.0	178.54	1.75	1	100
DEAE-cellulose	20	4.34	86.7	182.18	2.10	1.2	120
Phenyl-Sepharose	40	1.95	78.0	89.12	1.14	0.6	65.1

Table 3.4 Chitin adsorption

Purification steps	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U/ml)	Specific activity (U/mlmg)	Fold	Yield (%)
Culture medium	200	0.60	120.0	180.68	1.51	1	100
Chitin adsorption	20	0.91	18.1	163.62	9.01	6.0	90.6

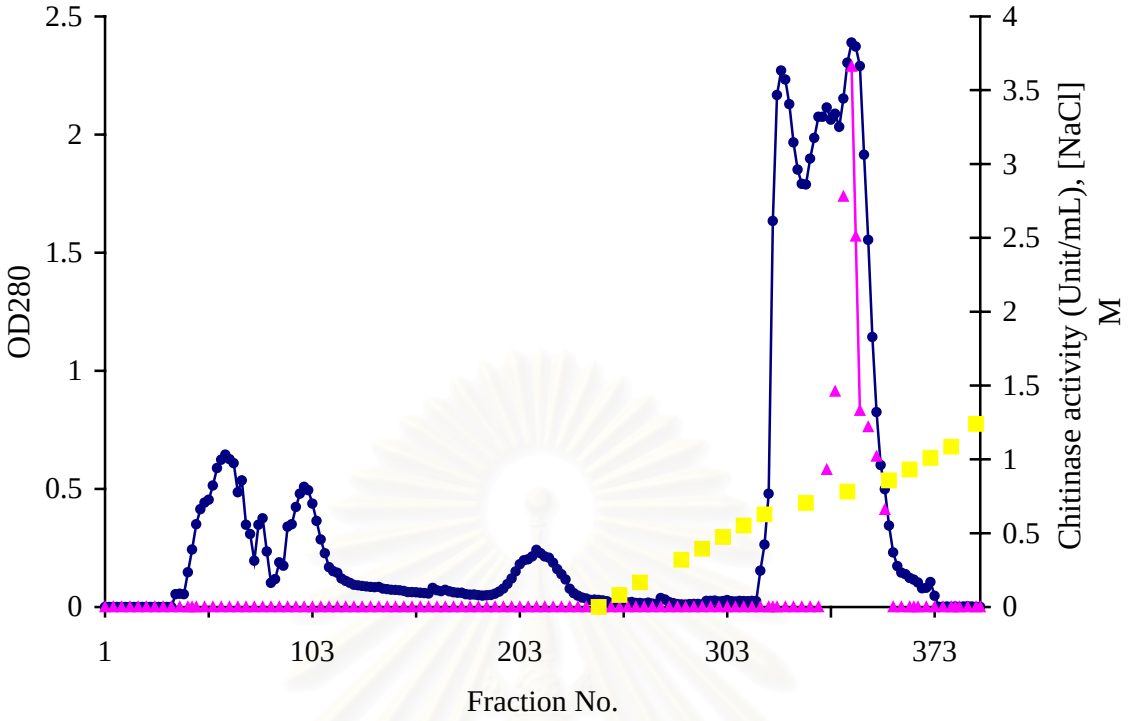

Figure 3.42 DEAE-chromatography profile of CHI66.

CHI66 from *E. coli.* BL21(DE3) harboring pETchi66 was applied to DEAE-cellulose by using 50 mM Tris-HCl, pH 7.0 as a column buffer. Flow rate was 1 ml/min with 4 ml fraction size. The bound proteins were eluted by increasing the NaCl concentration (0-1.5 M). (• : OD280, ■ : [NaCl], ▲ : chitinase activity)

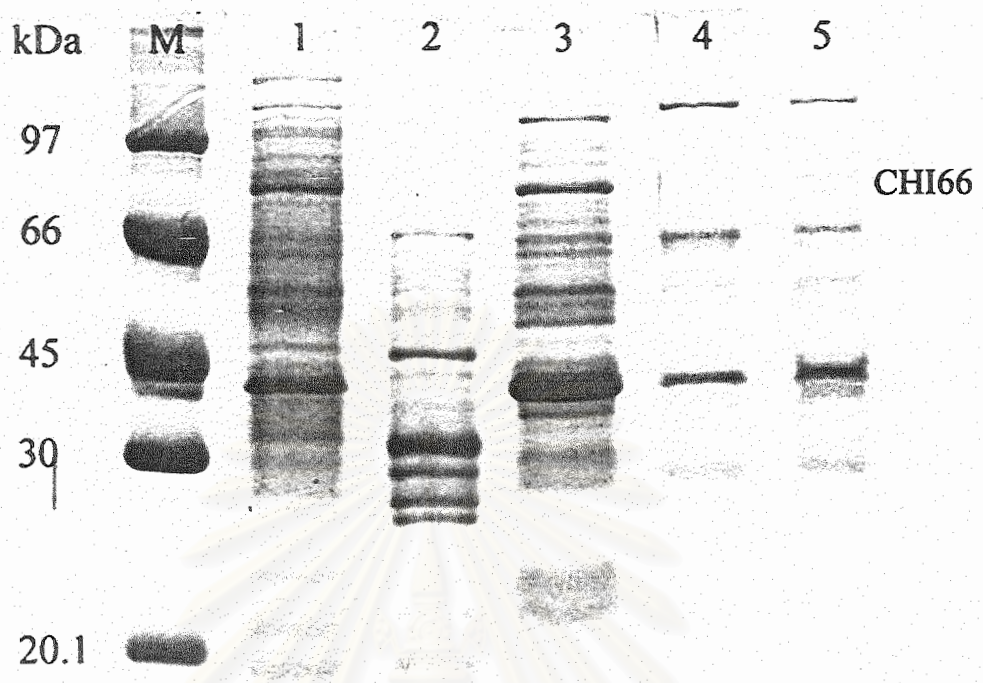

Figure 3.43 Purification of CHI66 by column chromatography

12.5% SDS-PAGE of bound and unbound fractions of crude CHI66 produced by *E. coli* BL21(DE3) harboring pETchi66on DEAE-cellulose and unbound fraction on Phenyl-Sepharose column.

Lane M: Molecular weight marker

Lane 1: Crude CHI66 produced by *E. coli* BL21(DE3) harboring pETchi66.

Lane 2: DEAE-cellulose unbound fraction of crude CHI66

Lane 3: DEAE-cellulose bound fraction of crude CHI66

Lane 4: Supernatant of DEAE-cellulose bound fraction of crude CHI66, which was saturated with $(NH_4)_2SO_4$.

Lane 5: Phenyl-Sepharose fraction

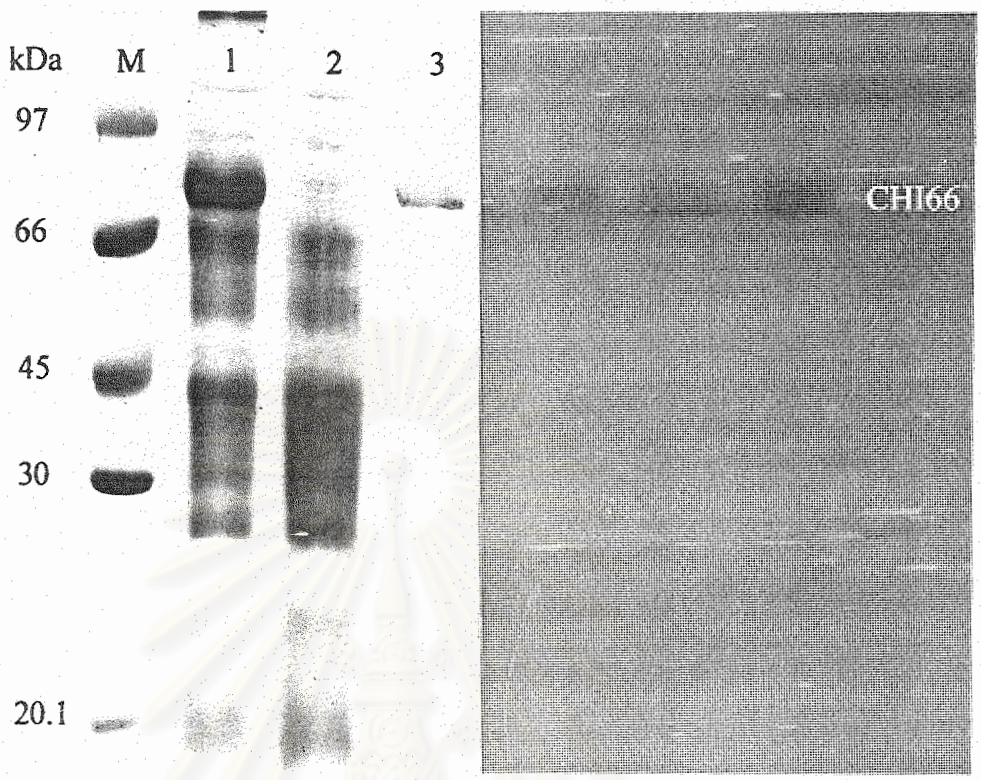

Figure 3.44 Chitin adsorption of CHI66

A 10 fold concentrated CHI66 from *E. coli* BL21(DE3) harboring pETchi66 was bound to colloidal chitin in 0.5 M Tris-HCl, pH 7.0 at 4°C for 8 hours.

Where Lane 1-3: protein staining Lane 3-6: activity staining

Lane M: Molecular weight marker

Lane 1, 4: Crude CHI66 from *E. coli* BL21(DE3) harboring pETchi66

Lane 2, 5: Unbound protein

Lane 3, 6: Partial purified CHI66, which bound to colloidal chitin

CHAPTER IV

DISSCUSSION

Identification of the bacterial strain PP8

Characteristics of strain PP8 are lies between to *circulans* and *coagulans* species. Bacteria PP8 is a gram positive, which form ellipsoidal subterminal spore. PP8 is a rod shape bacillus. The interior of its colony has a circular motion, which is an unique feature of the *circulans* species. However, strain PP8 grew at 55^{o}C. It hydrolyzed glycogen and produced acetone when grew in VP medium, which are the importance characteristics of the *coagulans* species.

16S rRNA gene of strain PP8 was *in vitro* amplified. The nucleotide sequence of 16S rRNA gene was compared to others, which deposited in GenBank. Sequence with most similarity with 16S rRNA gene of strain PP8 was 16S rRNA gene from *B. lantus,* with 97% identity, next was *B. licheniformis* with 95% identity. *Bacillus* was divided into 5 groups base on 16S rRNA gene sequence as show in Figure 4.1. *B. circulans, B. coagulans, B. lantus* and *B. licheniformis* are membered of group 1, which is the largest group. The members of this group have almost the same morphology and biochemical properties.

However, *B. lantus* has not been reported to produced chitinase or chitosanase. Whereas, six distinct chitinases were detected in the culture medium of *B. circulans* WL-12 (Watanabe, 1990). Both of *B. coagulans* (Yoon, 2002) and *B. circulans* (Saito, 1999) produced a single chitosanase in culture medium similar to *Bacillus* sp. PP8

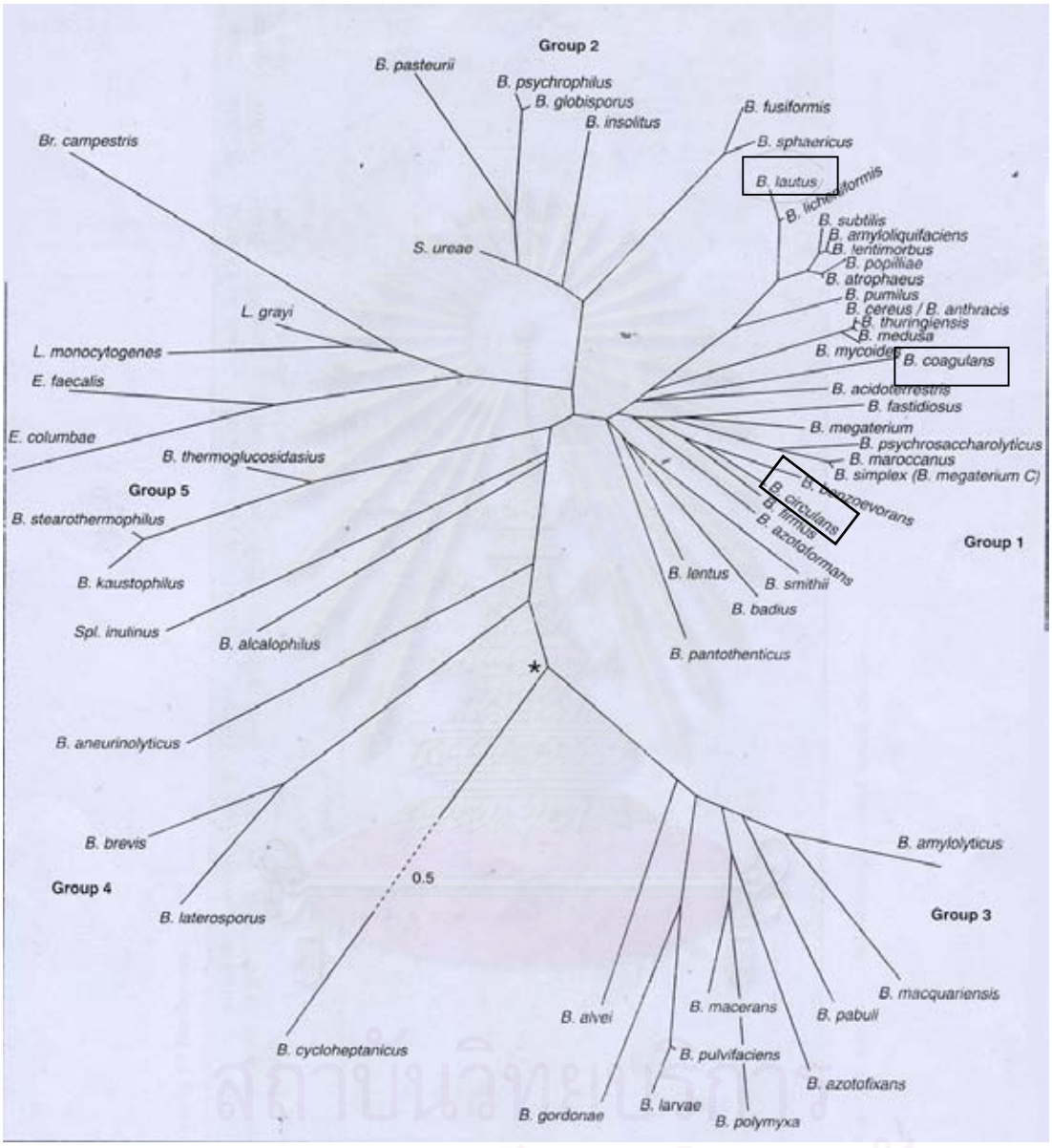

Figure 4.1 Phylogenic tree of 16S rRNA sequence from 51 *Bacillus* spp. Phylogenic tree of 16S rRNA sequence from 51 *Bacillus* spp Based on 16S rRNA sequence analysis. Genus abbreviations; *B., Bacillus; Br., Brochothrix; E., Enterobacter., L., Listeria; S., Sporosarcina; Spl., Sporolactobacillus.*

Chitinase and chitosanase production by *Bacillus* sp. PP8

When grew in colloidal chitin minimal medium *Bacillus* sp. PP8 produced at least two activities, chitinase and chitosanase, to degrade chitin. This degradation of chitin occur in nature by two pathways. The first pathway, crystalline chitin hydrolyzing enzyme, which was produced in the early stage of cultivation, hydrolyzed chitin to amorphous chitin or chitin oligomer. Then the amorphous chitin hydrolyzing enzyme, which was produced later in of cultivation, hydrolyzed amorphous chitin or chitin oligomer to chitin monomer and used its as carbon source. The second pathway, chitin deacetylase converted chitin oligomer to chitosan, which has a swollen form and could more accessible for hydrolyze by chitosanase.

When the crude enzyme from PP8 was analyzed by SDS-PAGE followed by activity staining, we observed five bands of 145, 66, 55, 40 and less than 15 kDa in size containing chitinase activity. These chitinases were not a degradation product of a larger enzyme but they are a different chitinases. Each chitinase were produced in different time indicated that they may be produce for different purposes. A 45 kDa chitinase, which was produced in the first day of cultivation was produced for hydrolyzed crystalline chitin. The 55 kDa chitinase and 66 kDa chitinase, which were produced in the second day of cultivation. They were produced for the hydrolysis of amorphous chitin or chitin oligomer. This synergism on chitin degradation was similar to the chitinase system on chitin degradation from *S. marcescens* 2170. It produced chitinase A, B and C. ChitinaseA was a most active enzyme toward insoluble chitin, but chitinaseC was the most active toward soluble chitin (Suzuki, 2002).

When various types of chitinous source were added in the culture medium (0.02% colloidal chitin, 0.2% powdered chitin, 2% flaked chitin, 0.02% colloidal chitosan, 2% flake chitosan, 0.2% GlcNAc and 0.2%GlcN), the enzymes produced by *Bacillus* sp.PP8 significantly differed for each chitinous source. The highest chitinase activity was detected in the medium containing colloidal chitin. The other forms of chitin and GlcNAc can induce chitinase production, However, chitinase was not induced by chitosan. On the other hand, the highest chitosanase activity was detected in the medium containing colloidal chitosan. The other chitosans and chitins also induced significant levels of chitosanase, but it was much lower than observed in the medium containing colloidal chitosan. From this result suggested that chitinase and chitosanase are regulated from separate promotor.

Characterictics of chitinase from *Bacillus* sp. PP8 and CHI66 from *E. coli* Top-10 harboring pSNchi66

The peak ratio between GlcNAc and $(GlcNAc)_2$ of colloidal chitin hydrolytic products by crude chitinase from *Bacillus* sp. PP8 was 2:1, whereas it was 1:1 when chitinase from *E. coli* Top-10 harboring pSNchi66 was used suggested that *Bacillus* sp. PP8 produced other chitinase, which preferred to hydrolyze chitin oligomer to monomer.

Chitinase from *Bacillus* sp. PP8 showed the two pH optimum in acid and basic range. While, CHI66 produced by *E. coli* Top-10 harboring pSNchi66 showed a single pH optimum at pH 8.0 suggested *Bacillus* sp. PP8 produced more than one chitinase to ensued that it hydrolyzed chitin in every milieu.

The optimum temperature of crude chitosanase was 40-70^{o}C. The optimum pH of crude chitosanase was 7.0. The optimum pH/temperature and thermostability property of this chitosanase were similar to the characteristics of chitosanase *from B. coagulans* TCH-2 (Yoon, 2002). The crude enzyme has the highest activity on 100%DD soluble chitosan more than that do on 80%DD soluble chitosan indicated that chitosanase prefer to hydrolyze the linkage between GlcN-GlcN than GlcNAc-GlcN or GlcN-GlcNAc.

Deletion of pST847 for chitinase gene location and nucleotide sequencing.

To locate the chitinase gene, the pST847 was subjected to restriction mapping. The deleted fragment was subcloned. A series of deletion mutants were constructed. There are four construct pSN*XP*-6.3, pSN*XX*-3.0, pSN*BB*-4.5 and pSN*BP*-4.5. All deletion derivatives were introduced into *E. coli* Top-10, and transformants were selected by overnight incubation at 37^{o}C on LB/ampicillin. The positive clones were plating on LB/colloidal chitin agar, only pSN*XX*-3.0 produced clear zone around colony. Therefore, pSN*XX*-3.0, which was a chitinase producing clone was designated as pSNchi66. pSNchi66 produced a larger clear zone than pST847. It may result from a loss of its repressor binding site or other negative reguratory elements (Tsujibo, 1999).

Expression of *chi66* in *E. coli.* by using two expression system pET19b and pTrcHis2 C.

A primer set was designed from an available sequence of *chi66*. The forward primer, which incorporated the *Nco*I site in to the pFChi66-*Nco*I primer resulted in mutation of the second amino acid residue (lysine to glutamic acid). Although a positive amino acid became a negatively charged one, it did not have any or a little effect in protein folding because it is in a signal peptide, which was cleaved by signal peptidase before the nascent peptide chain was fold to functional chitinase.

Optimized expression of chitinase gene

Comparative study of the expression by pET19b and pTrcHis2 C expression system were examined. They was not a significantly difference in expression level. Chitinase from both expression system was a higher activity than chitinase activity of enzyme produced by *E. coli* harboring pNSchi66. A chitinase was secreted better at 30^{o}C. Both system requied 1.0 mM IPTG to full expression level. When the pET system was used, *E. coli* BL21(DE3) and Rosetta were act as expression host, but *E. coli* BL21(DE3) pLysS did not express chitinase, may result of more stringent control of *E. coli* BL21(DE3) pLysS than the other cell lines.

Purification of CHI66 expressed by *E. coli* BL21(DE3) harboring pETchi66

CHI66 was applied to DEAE-cellulose column. The fractions with chitinase activity were eluted at 0.75 M NaCl. The active fractions were pooled and concentrated to 40 folds. For adsorption by Phenyl-Sepharose column, The concentrated DEAE-cellulose fraction was saturated with $(NH_4)_2SO_4$ and applied to the column. The unbound proteins were by a 10-bed volume. The bound proteins were eluted with a 10-bed volume with decreasing in $(NH_4)_2SO_4$ concentration. The total activity remained about 70% of the full activity as well as the total protein. The activity of the DEAE-cellulose fraction was higher than those from culture broth may result of the removal of some molecule pressed its activity. When applied the DEAE-fraction on a hydrophobic column, almost proteins were unbound to bind the matrix, resulted in low purification by Phenyl-Sepharoese column.

Due to an ineffective purification fold, we carried out a chitin adsorption by using 0.25 M and 0.5 M Tris-HCl pH 7.0 as a binding buffer. A 0.5 M buffer was a

more effective binding buffer than a 0.25 M suggested that CHI66 binds to substrate via hydrophobic interaction.

CHAPTER V

CONCLUSION

Chitinolytic producing enzyme bacterium, *Bacillus* sp. PP8 was isolated from soil collected from PP Island Krabi province, Thailand. When grew in medium containing chitin *Bacillus* sp. PP8 produced both chitinase and chitosanase but when it grew in medium containing chitosan only chitosanase activity was detected. The optimum pH/temperature of the chitinase and chitosanase was 8.0/50^{o}C and pH 7.0/40^{o}C, respectively. GlcNAc and $(GlcNAc)_2$ are major products of colloidal chitin hydrolyzed by crude chitinase from *Bacillus* sp. PP8. When the culture medium was analyzed by SDS-PAGE followed activity staining, there are five bands containing chitinolytic activity, a 145, 66, 55, 45 and less than 15 kDa in size but only single band of 47.5 kDa showed chitosanase activity.

A chitinase encoding gene was cloned by short gun cloning using pBluescript/SK^{-} and transformed to *E. coli.* The nucleotide sequence revealed a 1797 open reading frame that encoding 599 amino acids with signal peptide corresponding to 66.2 kDa, was called CHI66. The highest similar of CHI66 from *Bacillus* sp.PP8 was the CHI65 from *B. lichenformis* with 97%identity.

The CHI66 gene expression was preformed using pET19b and pTrcHis-2C in *E. coli* BL21 (DE3) and Top-10, respectively. The highest chitinase activity was found in the culture medium of *E. coli* BL21 (DE3) harboring pETChi66, which incubated with shaking at 30^{o}C, 300 rpm for 60 hr after induction with 1 mM IPTG. Chitinase activity of CHI66 was confirmed by activity staining. The optimum pH, temperature and product of colloidal chitin hydrolysis of the recombinant chitinase were corresponding to the crude chitinase from *Bacillus* sp. PP8. CHI66 was stable at 4^{o}C in 0.15 M Tris-HCl pH 7-8 for 2 months.

CHI66 was partial purified by single step purification, chitin adsorption using 0.5 M Tris-HCl, pH 7.0 as a binding buffer and the 90% of activity was recovery by this process.

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APPENDIX A

Characcteristics of the bacterial strain PP8

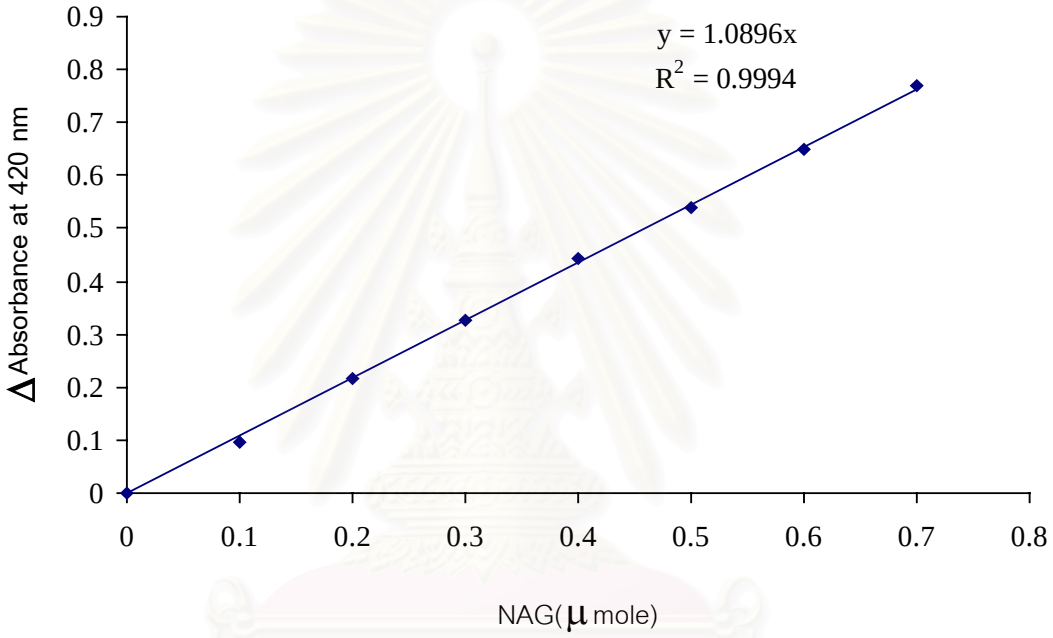
Characteristics	Reaction	Characteristics	Reaction
Gram reaction	+VE	- Esculine	+
Fermentative production		- Arbutin	+
of acid from:		- Salicine	+
- Glycerol	+	- Cellobiose	+
- Erythritol	-	- Maltose	+
- D-arabinose	+	- Lactose	+
- L-arabinose	+	- Melibiose	+
- Ribose	+	- Sucrose	+
- D-xylose	+	- Trehalose	+
- L-xylose	-	- Inuline	+
- Adonitol	-	- Melezitose	+
- β-methyl-D-xylose	+	- D-raffinose	+
- Galactose	+	- Starch	+
- D-glucose	+	- Glycogen	+
- D-fructose	+	- Xylitol	-
- D-mannose	+	- β-gentiobose	+
- L-sorbose	-	- D-turanose	+
- Rhamnose	+	- D-lyxose	-
- Dulcitol	-	- D-tagatose	-
- Inositol	+	- D-fucose	-
- Mannitol	+	- L-fucose	+
- Sorbitol	-	- D-arabinose	-
- α-methyl-d-mannoside	+	- L-arabinose	+
- α-methyl-d-glucoside	+	- Gluconate	+
- *N*-acetyl-glucosamine	+	- 2-keto-gluconate	-
- Amygdaline	+	- 5-keto-gluconate	+

Remark: +ve = Gram positive bacteria + = Positive reactio - = Negative reaction

APPENDIX B

Standard curve *N*-acetyl glucosamine for chitinolytic enzyme assay by colorimetric method.

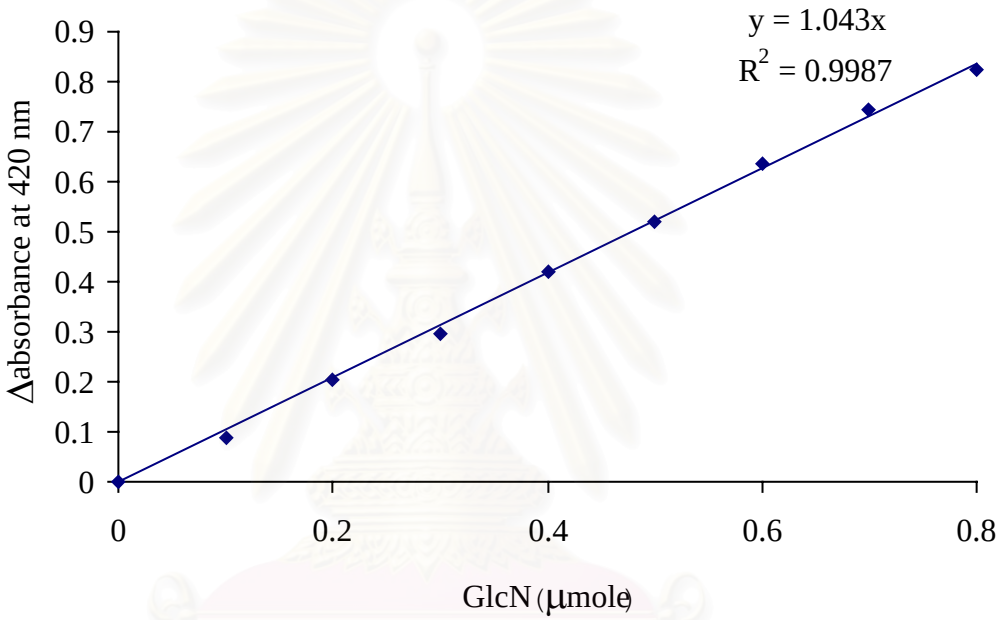
Standard curve for GlcNAc was made by mornitoring the absorbance at 420 nm of standard concentration GlaNAc according to the Schale's method.



APPENDIX C

Standard curve glucosamine for chitosanase assay by colorimetric method.

Standard curve for GlcN was made by mornitoring the absorbance at 420 nm of standard concentration GlcN according to the Schale's method.



APPENDIX D

Preparation of colloidal chitin

A 400 mL of concentrated hydrochloric acid is poured into 10 g chitin at 0 ^{o}C with vigorous stirring. After homofeneneous dispersion of chitin has been reached, the mixture is heated gently up to 37^{o}C with moderate shaking. The viscosity of the mixture increases rapidly and then, within a few minutes, begins to decrease. As the viscoscity decreases, the appearance of the mixture becomes clearer. At the stage when a small amount of chitin is still undissolved, the mixture is filtered through gauze and the filtrate is poured into 4 litres of deionized water below 4^{o}C. The solution becomes turbid because of the reprecipitation of chitin, and then the suspention is kept overnight at 4^{o}C. The supernatant is then decanted out and the remaining mixture is centrifuged at 4^{o}C. The residue is washed with deionized water until the washing become nutral. The acid-free residue is added into 100 mL of deionized water and resuspended with vigorous shaking to prepare so-call colloidal chitin solution. Chitin content of the solution is dertermined by drying a sample and reported in g/mL or %(W/V).

APPENDIX E

Preparation of regenerated chitin

A alkali chitin is prepared by suspending 2.0 g of chitin powder in 50 g of 40%(w/w)aqueous sodium hydroxide. The mixture is allowed to stand at ambient temperature for 2 hr under reduced pressure. Then 150 g crushed ice is added and stirre at 0^{o}C, and a clear alkali chitin solution is obtained. The solution is diluted by adding equal volumn of distilled water. Thr pH is adjusted to nutral and the solution becomes turbid because of the reprecipitation of chitin. The regernerated chitin is washed with deionized water to reduced the ionic strenght.

APPENDIX F

Preparation of colloidal chitosan

Colloidal chitosan is perpared from 70%DA chitosan or 90%DA chitosan. A 5 g chitosan is completely dissolved in 400 mL of 0.1 M hydrochloric acid with vigorous stirring. The chitosan in acid solution is digested by either chitinase or chitosanase depending on its %DA, when 70%DA chitosan is used as substrate, chitinase is an appropiated enzyme to hydrolyse this chitosan. A 0.5 unti of chitinase from *Bacillus licheniformis* SK1 is poured into 400 mL of acid chitosan solution and then incubated at 50^{o}C for an overnight. A digested chitosan is adjusted pH to 7.4 by 0.5 M potassium hydroxide and made volume to 500 mL, the final concentration of this colloidal chitosan is 1% (w/v)solution. When 90%DA chitosan is used for perpared acid chitosan solution, chitosanase is an appropiated enzyme to digest 90%DA chitosan. In this case 5 untis of chitosanase from *Bacillus circulans* PP8 is poured into 400 mL of acid chitosan solution and incubated at 50^{o}C for an hour. Then the acid chitosan solution is adjusted pH to 7.4 by 0.5-1.0 M potassium hydroxide, at this time the solution becomes turbid because of the reprecipitation of chitosan. Finnally the colloidal chitosan is made volume to 500 mL and the finnal concentration of colloidal chitosan becomed 1%(w/v) solution.

APPENDIX G

Preparation of denaturing polyacrylamide gel electrophoresis & chitinase or chitosanase activity staining gel electrophoresis.

Solution A (Acrylamide Stock Solution)

a. 29.2 g acrylamide
b. 0.8 g *N, N'*-dimethylene-bis-acrylamide

Add ultrapure water to make 100 mL and stir until completely dissolved.

Solution B (4X Separating Gel Buffer)

a. 75 mL of 2 M Tris-HC, pH 8.8 (final concentration is 1.5 M)
b. 4 mL of 10%SDS (final concentration is 0.4%)
c. 21 mL of ultrapure water (final volume is 100 mL)

Solution C (4X Stacking Gel Buffer)

a. 50 mL of Tris-HCl, pH6.8 (final concentration is 0.5 M)
b. 4 mL of 10%SDS (final concentration is 0.4%)
c. 46 mL of ultrapure water (final volume is 100 mL)

10% Ammonium persulfate, 1 mL

a. 0.1 g of ammonium persulfate
b. 1 mL of ultrapure water

Stable for months in a capped tube in the refrigerator.

10X Electrophoresis Buffer, 1 liter

a. 30 g of Tris (final concentration is 0.25 M)
b. 144 g of glycine (final concentration is 1.92 M)
c. 10 g of SDS (final concentration is 1%)
d. Ultrapure water to make 1 liter

5X Sample Buffer, 10 mL

a. 0.6 mL of a M Tris-HCl, pH 6.8 (final concentration is 60 mM)
b. 5 mL of 50%(v/v)glycerol (final concentration is 25 %)
c. 2 mL of 10%SDS (final concentration is 2%)
d. 0.5 mL of 2-mercaptoethanol (final concentration is 14.4 mM)
e. 1 mL of 1%(w/v) bromophenol blue (final concentration is 0.1%)
f. 0.9 mL of ultrapure water

2.5% substrate (chitin or chitosan)

a. 0.1 g ethylene glycol chitn or ethylene glycol chitosan
b. 4 mL ultrapure water

Chemical	10%Seperating Gel (μL)	2.5%Stacking Gel (μL)
Solution A	1875	670
Solution B	1250	-
Solution C	-	1000
2.5%Substrate	100	-
10%ammonium persulfate	60	40
TEMED	20	10
Ultrapure water	1695	2300
Total	5000	4000

5X Sample Buffer, 10 mL

a. 0.6 mL of a M Tris-HCl, pH 6.8 (final concentration is 60 mM)
b. 5 mL of 50%(v/v)glycerol (final concentration is 25 %)
c. 2 mL of 10%SDS (final concentration is 2%)
d. 0.5 mL of 2-mercaptoethanol (final concentration is 14.4 mM)
e. 1 mL of 1%(w/v) bromophenol blue (final concentration is 0.1%)
f. 0.9 mL of ultrapure water

2.5% substrate (chitin or chitosan)

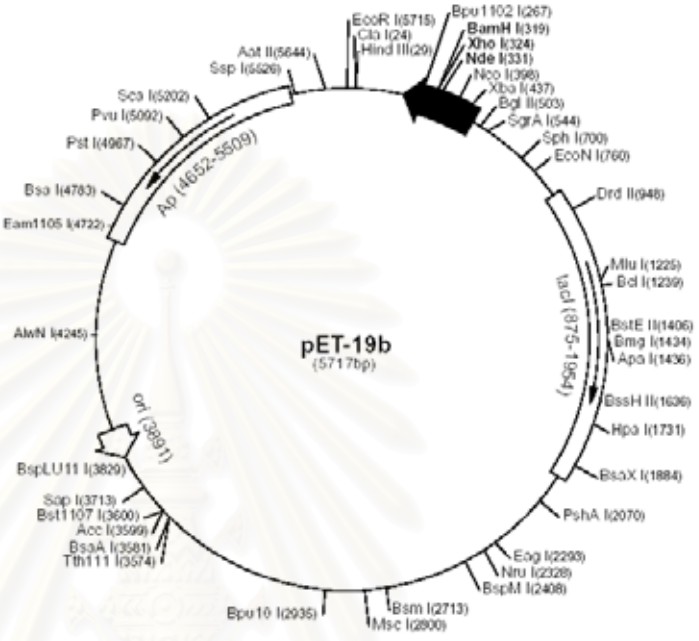
a. 0.1 g ethylene glycol chitn or ethylene glycol chitosan
b. 4 mL ultrapure water

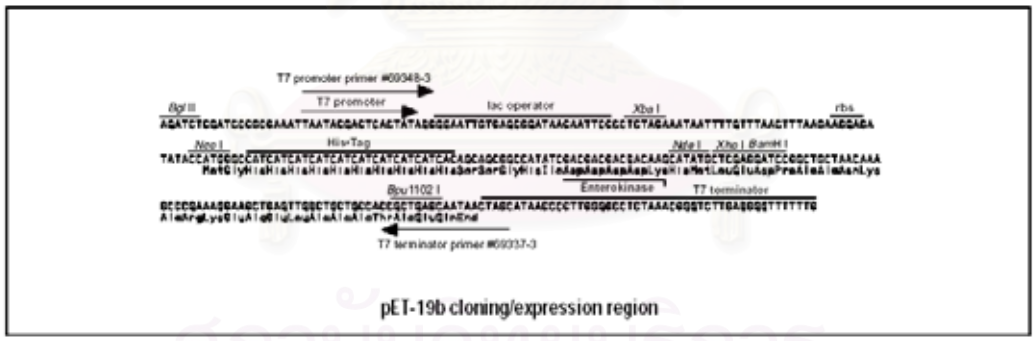
Chemical	10%Seperating Gel (μL)	2.5%Stacking Gel (μL)
Solution A	1875	670
Solution B	1250	-
Solution C	-	1000
2.5%Substrate	100	-
10%ammonium persulfate	60	40
TEMED	20	10
Ultrapure water	1695	2300
Total	5000	4000

APPENDIX I

Restriction map of pET19b

pET-19b sequence landmarks	
T7 promoter	472-488
T7 transcription start	471
His•Tag coding sequence	366-395
Multiple cloning sites (*Nde*I -*Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	875-1954
pBR322 origin	3891
bla coding sequence	4652-5509



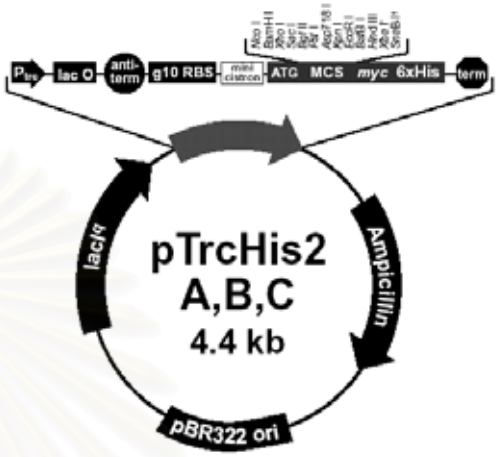


pET-19b cloning/expression region

APPENDIX J

Restriction map of pTrcHis2-C

Comments for pTrcHis2 A
4406 nucleotides
trc promotor region: bases 190-382
-35 region: bases 193-198
-10 region: bases 216-221
lac operator (*lac*O): bases 228-248
*rrn*B antitermination signal: bases 264-333
gene 10 region: bases 346-354
Ribosome binding site: bases 369-373
pTrcHis forward priming site: bases 370-390
Minicistron ORF: bases 383-409
Reinitiation RBS: bases 398-403
Expression ATG: bases 413-415
Multiple cloning site: bases 411-464



myc epitope: bases 471-503
Polyhistidine tag: bases 516-533
*myc*His reverse priming site: bases 508-527
*rrn*B T_1 and T_2 transcriptional terminators: bases 639-796
Ampicillin resistance ORF: bases 1076-1936
pBR322 origin: bases 2081-2754
Lac Repressor (*lac*Iq) ORF: bases 3285-4367

pTrcHis2 C MCS

pTrcHis forward priming site; RBS; Mini cistron; RBS; *Nco* I

```
361  AAAATTAAAG AGGTATATAT TA ATG TAT CGA TTA AAT AAG GAG GAA TAA ACC
                              Met Tyr Arg Leu Asn Lys Glu Glu ***
```

*Bam*H I; *Xho* I; *Sac* I; *Bgl* II; *Pst* I; *Asp*718 I; *Kpn* I; *Eco*R I; *Bst*B I; *Hin*d III; *Sna*B I

```
413  ATG GATCCGAGCT CGAGATCTGC AGCTGGTACC ATATGGGAAT TCGAAGCTTA CGTA
     Met
```

myc epitope tag; *Sal* I

```
461  GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT AGC GCC GTC GAC CAT
     Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His
```

ProBond™ binding domain

```
510  CAT CAT CAT CAT CAT TGA GTTTA
     His His His His His *** 
```

BIOGRAPHY

Miss. Santhana Nakapong finished her B.SC. in Biochemistry from Kasetsart University in 2001. She graduated her M.Sc. in Biochemistry at Chulalongkorn University in 2004.