การออกแบบดีเอ็นเอไพรเมอร์เพื่อโคลนยีนไคทิเนสจาก *Bacillus licheniformis* PR-1

นายประกานต์ ฤดีกุลธำรง

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DESIGN OF DNA PRIMERS FOR CHITINASE GENE CLONING FROM *Bacillus licheniformis* PR-1

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ไคทิเนส (EC 3.2.1.14) เป็นเอนไซม์ที่เร่งปฏิกริยาการย่อยสลายไคทิน *Bacillus licheniformis* PR-1 ที่แยกได้จากดินในประเทศไทย ผลิตเอนไซม์ไคทิเนสได้ และผลิตเอนไซม์สูงสุดในวันที่ 8 และ 5 เมื่อเลี้ยงในอาหารที่มีคอลลอยด์ดัลไคทินที่มี yeast extract 0.05 % และ 0.25 % ตามลำดับ ค่าความเป็นกรด-ด่างและอุณหภูมิที่เหมาะสมที่ทำให้ไคทิเนสมีแอคติวิตีสูงสุด คือ 5.0 และ 70 ˚C ตามลำดับ เอนไซม์ไคติเนสย่อยสลายคอลลอยด์ดัลไคทินได้ดีที่สุด รองลงมาคือ powder chitin, 80 % DD chitosan, flake chitin และ regenerated chitin ตามลำดับ ผลิตภัณฑ์ที่ได้จากการย่อยสลายคอลลอยด์ดัลไคทินได้เป็น chitobiose และ *N*-acetylglucosamine โดยมี chitobiose เป็นผลิตภัณฑ์หลัก เมื่อทำการแยกโปรตีนโดย SDS-PAGE และย้อมแอคติวิตี พบโปรตีนที่มีไคทิเนสแอคติวิตี 3 แถบที่มีขนาดต่างๆ คือ 70, 65 และ 58 กิโลดาลตัล ตามลำดับ ดีเอ็นเอไพรเมอร์ BP-I, II, V, VI, VII, VIII, IX, BP-F และ BP-R ที่ออกแบบมีความจำเพาะต่อยีนไคติเนส family 18 ของ *Bacillus spp.* และสามารถใช้เพิ่มจำนวนยีนไคทิเนสได้ครบทั้งยีน เมื่อนำโครโมโซมัลดีเอ็นเอมาตัดแบบไม่สมบูรณ์แล้ว ทำ shotgun cloning พบสองโคโลนีจาก 8,000 โคโลนี ที่สามารถเกิดวงใสบนอาหารเลี้ยงเชื้อแข็งที่มีคอลลอยด์ดัลไคทิน โดยทรานสฟอร์แมนท์มีพลาสมิดที่มีชิ้นดีเอ็นเอขนาด 5 และ 3 kb แทรกอยู่ มีเพียงทรานสฟอร์แมนท์ที่มีพลาสมิดที่ชิ้นดีเอ็นเอขนาด 5 kb แทรกอยู่ตั้งชื่อว่า pPRChi65 เท่านั้นที่มีการผลิตเอนไซม์ออกมาหลังจากรีทรานส์ฟอร์มเข้า *E.coli* JM109, DH5α และ XL-1 blue และผลิตเอนไซม์ได้ดีที่สุดใน JM109 โปรโมเตอร์ของ pPRChi65 สามารถถูกยับยั้งได้เมื่อเลี้ยง *E.coli* JM109 ในอาหาร LB ที่มีกลูโคสอยู่ เมื่อทำการศึกษาสมบัติบางประการของเอนไซม์ CHI65 พบว่าสภาวะที่เหมาะสมที่มีแอคติวิตีมากที่สุดอยู่ที่ค่าความเป็นกรด-ด่าง 5.0 และอุณหภูมิ 60˚C ตามลำดับ เมื่อหา open reading frame พบว่ามีสองยีน คือ *Chi*65 ที่มีขนาด 1,779 คู่เบส เมื่อนำมาแปลรหัสเป็นกรดอะมิโนจะได้ กรดอะมิโน 592 ตัว ซึ่งมีขนาดประมาณ 65,100 Da มี isoelectric point เท่ากับ 5.84 และในอีกยีนหนึ่งเป็น chitodextrinase ซึ่งได้ไม่ครบทั้งยีน กรดอะมิโนของ *Chi65* ที่ได้มีความคล้ายคลึง 89 % และ 79% กับยีนไคติเนสจาก *B. liceniformis* M1-1 และ *B. subtilis* ตามลำดับ ส่วนการศึกษาการย่อยไคทินของ *Chi65* พบว่าเอนไซม์ไคติเนสย่อยสลายคอลลอยด์ดัลไคทินได้ดีที่สุด รองลงมาคือ powder chitin, flake chitin, 80 % DD chitosan และ regenerated chitin ตามลำดับ ผลจาก SDS-PAGE และย้อมสีแอคติวิตีพบแถบโปรตีนที่มีไคทิเนสแอคติวิตี 3 แถบ มีขนาด 70, 65 และ 58 kDa เหมือนกับ *B. licheniformis* PR-1 และยังพบอีกว่าผลิตภัณฑ์ที่ได้จากการย่อยสลายได้คอลลอยด์ดัลไคทินเป็น chitobiose และ *N*-acetylglucosamine เช่นเดียวกัน

หลักสูตร……เทคโนโลยีทางชีวภาพ…….. **ลายมือชื่อนิสิต** ……...……...

สาขาวิชา …...เทคโนโลยีทางชีวภาพ…..… **ลายมือชื่ออาจารย์ที่ปรึกษา**…….

ปีการศึกษา……2544……..… .. **ลายมือชื่ออาจารย์ที่ปรึกษาร่วม** ……-……..

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KEY WORD: CHITINASE / *Bacillus licheniformis* PR-1 / PRIMER / PCR / CLONING

PRAKARN RULDEEKULTHAMRONG: DESIGN OF DNA PRIMER FOR CHITINASE GENE CLONING FROM *Bacillus licheniformis* PR-1

THESIS ADVISOR: RATH PICHYANGKURA, Ph. D., 111pp.

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Chitinase (EC3.2.1.14) is an enzyme that catalyzes the degradation of chitin. *Bacillus licheniformis* PR-1, isolated from soil in Thailand, is capable of producing chitinase. Chitinase from *B. licheniformis* PR-1 produced maximum chitinase activity in 8 and 5 day when cultured on colloidal chitin minimum medium with 0.05% and 0.25 % yeast extract, respectively. The optimum pH and temperature of crude chitinase was pH 5.0 in citrate buffer and 70 ^{o}C, respectively. Crude chitinase hydrolyzed colloidal chitin the best followed by powder chitin, 80 % DD chitosan, flake chitin and regenerated chitin respectively. Products from this enzyme, analyzed by HPLC, were a mixture of chitobiose $(GlcNAc)_2$ and N-acetylglucosamine (GlcNAc); chitobiose was the major products. SDS-PAGE and activity staining of crude enzymes showed three bands with chitinase activity. The estimated molecular weights of the major chitinase species were 70, 65 and 58 kDa. Designed primers (BP-I, II, V, VI, VII, VIII, IX, BP-F and BP-R) that were specific for familly 18 chitinases among *Bacillus sp*., were able to amplified full length chitinase genes. Shotgun cloning of the *Pst*I partially cut genomic DNA of *B. licheniformis* PR-1 was performed. Two transformants from 8,000 colonies showed clear zones with inserted fragment of 5 and 3 kb, respectively. Only one plasmid, pPRChi65, had chitinase activity and produced highest chitinase activity in *E.coli* JM109. The promotor of *Chi*65 could be suppressed when grown in LB medium with glucose. Crude chitinase from pPRChi65 was characterized. The optimum pH and temperature were pH 5.0 in citrate buffer and 60 ^{o}C, respectively.

Two open reading frame were found in the 5 kb inserted. One is 1,779 bp long encoding for a protein 593 amino acids, which correspond to 65,100 Da with isoelectric point of 5.84 and the other is not a complete sequence for chitodextrinase. The amino acid comparison indicated *Chi*65 is 89 % similar to chitinase from *B. licheniformis* TP-1 followed by 79 % chitinase from *B. subtilis*. Crude chitinase hydrolyzed colloidal chitin the best followed by 80 % DD, powder chitin, flake chitin and regenerated chitin.

SDS-PAGE and activity staining of crude enzyme of recombinant clone showed three bands with were chitinase activity. The molecular weights were approximately 70, 65 and 58 kDa, which is the same as crude chitinase from *B. licheniformis* PR-1.

Determination of hydrolytic products by HPLC, found a mixture of chitobiose and GlcNAc from pPRChi65.

Program……. Biotechnology…	Student's signature……..……
Field of study..Biotechnology...	Advisor's signature……..……
Academic year… 2001……..	Co-advisor's signature……. -…….

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ABBREVIATIONS

A	Absorbance
bp	Base pair(s)
CCMM	Colloidal chitin minimum medium
cm	Centimetre(s)
°C	Degree celcius
dATP	Deoxyadenosine 5’ triphosphate
dCTP	Deoxycytidine 5’ triphosphate
dGTP	Deoxyguanidine 5’ triphosphate
dNTP	Deoxyribonucleotide triphosphate
dTTP	Deoxythymidine 5’ triphosphate
DNA	Deoxyribonucleic acid
et al.	Et. Alii (latin), and others
hr	Hour(s)
Fig.	Figure
kb	Kilobase(s)
kDa	Kilodalton(s)
L	Litre
M	Molar(s)
μCi	Microcurie
μg	Microgram
μL	Microlitre
mg	Milligram
mL	Millilitre
mU	Milliunit
min	Minute
ng	Nanogram
ORF	Open Reading Frame
ORI	Origin of replication
PCR	Polymerase Chain Reaction
rpm	Revolution per minute
GlcNAc	N-acetyl-D-glucosamine
$(GlcNAc)_2$	N,N’-diacetyl-D-glucosamine (chitobiose)

CHAPTER I

INTRODUCTION

Chitin

Chitin, a natural biopolymer and the most abundant compound in the world after cellulose, is a major structural component of fungal cell walls and the exoskeletons of invertebrates including insects, arachnids, crustaceans and extracellular polymer of some bacteria (Muzzarelli, 1977). It is also found in common foods such as grain, yeast, and mushrooms. In nature, chitin has been estimated annual production between 10^{10} and 10^{11} tones. The highest amount of chitin with respect to total dry weight is found in crustaceans. Thus crustacean shells were used as main source of chitin by most chemical industries.

The structure of chitin

The chemical structure of chitin, similar to that of cellulose, is a straight chain homopolymer of β-1,4-linked-2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine, GlcNAc). This structure resembles cellulose with the C-2 hydroxyl group replaced by an acetamido residue. Chitin can be processed into many derivative, such as chitosan, chitin oligosaccharide and chitosan oligosaccharide. The most readily available being chitosan, which is formed when chitin is heated in 40-50 % (w/w) NaOH (Subasinghe, 1995). Chitosan is deacetylated at various degrees. Chitosan is composed primarily of glucosamine, 2-amino-2-deoxy-D-Glucose (Deshpande, 1986).

The structure of chitin determined by polarized light and electron microscope indicated that chains of chitin usually orientate in a high degree of order (Kramer and Koga, 1986). X-ray diffraction was the first to show the crystalline nature of chitin (Tracey, 1957). The chitin or polysaccharide chains are assembled into microfibrils forming into a crystalline structure via intramolecular hydrogen bond. The comparisons of the X-ray data for chitin from different sources have revealed three polymeric forms; α, β and γ-form (Table 1).

Alpha-form, which are arranged in an antiparallel, the polymer chains are tightly bonded. Different to parallel arrangement, β-form, and γ-forms was mix strands between antiparallel and parallel, has less crystal1inity than alpha chitin, is easily dispersed in water, and is more easily degradable by lysozyme and chitinase than colloidal chitin as shown in Figure 2 (Blackwell, 1988).

The α-form, found most in nature, is more stable than the others. Most chitin including those from fungi, insect and crustaceans are classified as α-form. The second form, β-chitin, has been found in four sources: spine of the polychaete *Aphrodite*, pen of squid *Loligo*, the tubes of *Pogonophora*, and the spines of certain marine diatoms. The last form, γ-chitin, has been reported from the stomach lining of *Loligo* and probably in coelenterates (Tracey, 1957)

The application of chitin and its derivatives

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

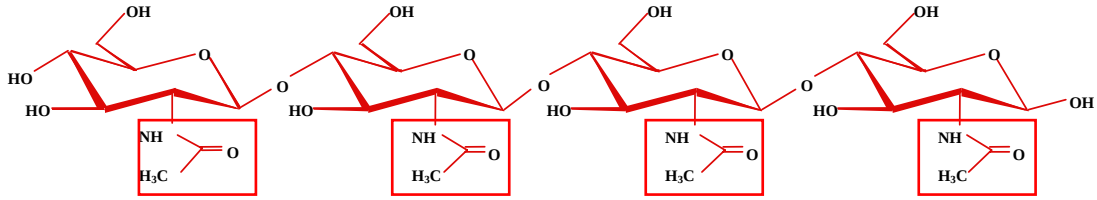
Chitin and chitosan have been extensively examined and tested by researchers world-wide in a wide range of medical applications (H.K. and Meyers, 1995), food and nutrition uses, cosmetics, beauty aids and other new discoveries as shown in Table 2. Today, more than two million people mainly in the U.S. and Japan, take chitin and chitosan as dietary supplements. Researchers are regularly discovering new applications for chitin and the range of products expands yearly.

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

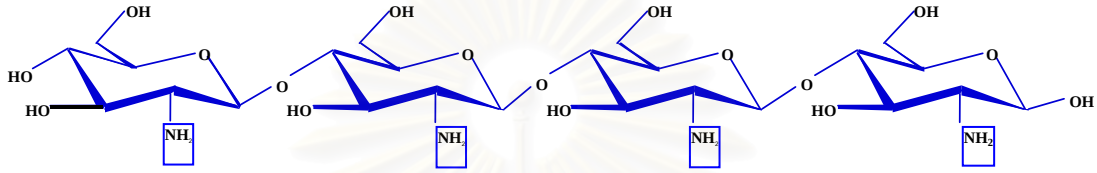
The production of N-acetylglucosamine (GlcNAc) can be also achived by enzymatic reaction (Aiba, 1994). The enzyme hydrolysis of chitin gives GlcNAc is performed by a chitinolytic system, the action of which is known to be synergistic and consecutive (Deshpande, 1986).

Table 1 Sources of chitin in different organisms (Muzzarelli, 1977)

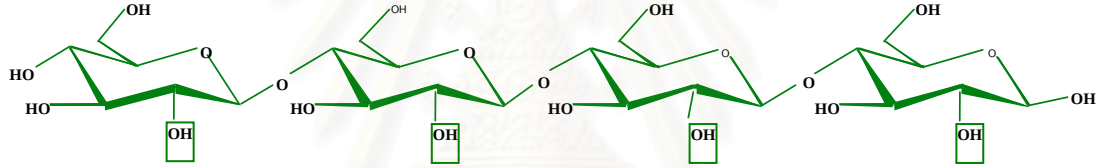
Sources of chitin	(%) chitin	Type of chitin
Crustacea (shrimp)		α-chitin
▪ Crangon	69.1	
▪ Alaskan	28.0	
Insect		α-chitin
▪ May beetle	16.0	
▪ Pieris (sulfur butterfly)	64.0	
▪ Colcoptera (beetle)	27-35	
▪ Diptera (true fly)	54.8	
▪ Bombyx (silk worm)	44.2	
▪ Calleria (wax worm)	33.7	
Mollusca		β-chitin
▪ Squid ,Octopus	41	
▪ Oyster shell	3.6	
▪ Clamshell	6.1	
Fungi		α-chitin
▪ *Aspergillus niger*	42.0	
▪ *Aspergillus phoenicis*	23.7	
▪ *Pennicilium notatum*	18.5	
▪ *Histoplasma capsulatum*	25.8	
▪ *Histroplasma farciminosum*	40.0	
▪ *Mucor rouxi*	44.5	
▪ *Mortierella vinacea*	22.0	



CHITIN

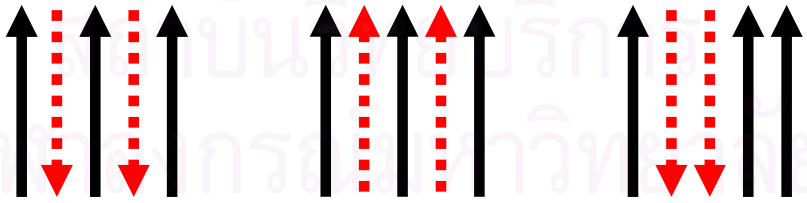


CHITOSAN



CELLULOSE

Figure 1 **The structure of chitin, chitosan and cellulose**



(A) α- Chitin **(B)** β- Chitin (**C)** γ- Chitin

Figure 2 **Model of α- Chitin, β- Chitin and γ- Chitin**

The poly N-acetylglucosamine chains $[\text{N-acetylglucosamine}]_n$ were represented by arrows.

Table 2 Current applications of Chitin and its derivatives

Medical; ▪ Bandages, Sponges ▪ Artificial Blood Vessels ▪ Blood Cholesterol Control ▪ Tumor Inhibition ▪ Membranes ▪ Dental/Plaque Inhibition ▪ Skin Burns/Artificial Skin ▪ Eye Humor Fluid ▪ Contact Lens ▪ Controlled Release of Drugs ▪ Bone Disease Treatment **Pulp and Paper;** ▪ Surface Treatment ▪ Photographic Paper ▪ Carbonless Copy Paper **Membranes;** ▪ Reverse Osmosis ▪ Permeability Control ▪ Solvent Separation **Water Treatment;** ▪ Removal of Metal Ions, dyes, Pesticides and phenols ▪ Flocculant/Coagulant: ▪ Proteins, amino Acids ▪ Filtration	**Food indutries;** ▪ Removal of Dyes, Solids, Acids ▪ Preservatives ▪ Color Stabilization ▪ Animal Feed Additive ▪ Emulsifying agent ▪ Clarification and deacidification of fruits and beverages ▪ Dietary fibre ▪ Beverages and organic acid **Agriculture;** ▪ Seed Coating ▪ Leaf Coating ▪ Hydroponics/Fertilizer ▪ Controlled Agrochemical Release **Cosmetics and Toiletries;** ▪ Make-up Powder ▪ Nail Polish ▪ Moisturizers ▪ Fixtures ▪ Bath Lotion ▪ Face, Hand and Body Creams ▪ Toothpaste ▪ Foam Enhancing **Biotechnology;** ▪ Enzyme Immobilization ▪ Protein Separation ▪ Chromatography ▪ Cell Recovery ▪ Cell Immobilization

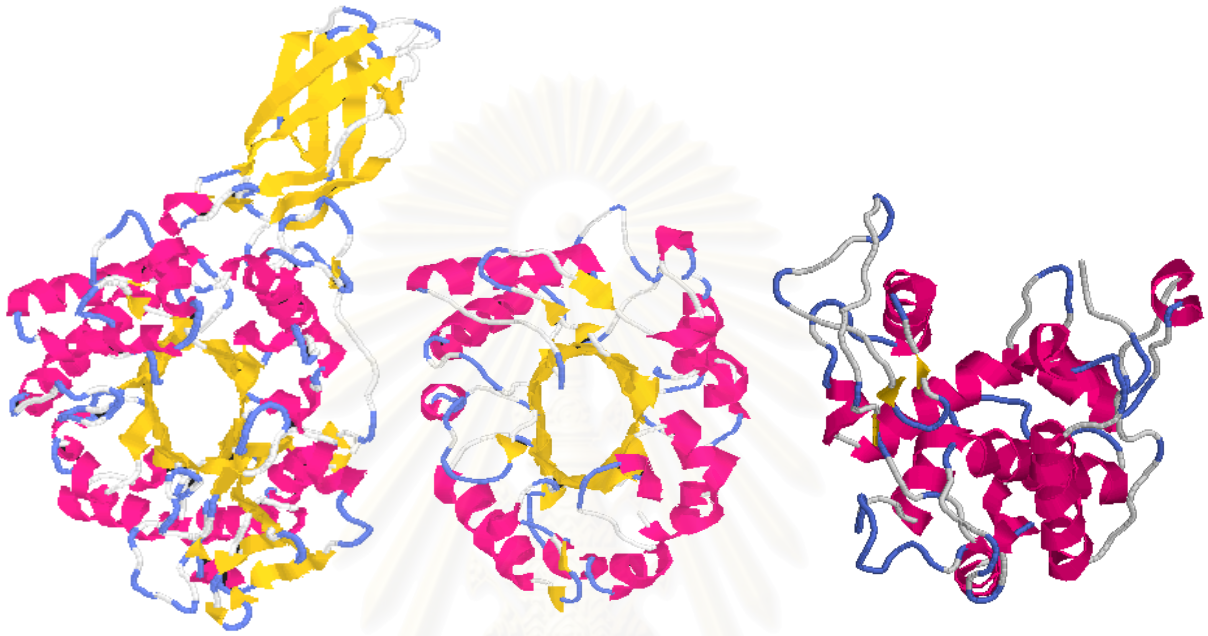
Chitinase

Chitinase (EC 3.2.1.14), is a glycosyl hydrolase that hydrolyzed N-acetyl-D-glucosamine (1,4)-β-linkages in chitin. Chitinase are found in a wide variety of organisms that possess chitin as well as organisms that does not possess chitin such as bacteria, plants, and vertebrates. The roles of chitinases in these organisms are diversed (Jolles and Muzzarelli, 1999).

Invertebrates require chitinases for partial degradation of old exoskeletons. Fungi produce chitinases to modify chitin, which is used as an important cell wall component, on the other hand, higher plants use chitinases for auto defensive machanisms against insects and fungal pathogens (Boller, 1985). Bacteria produce chitinases to digest chitin and utilize it as carbon source for their growth.

At the beginning chitinases were classified into exo- and endo-chitinases. These activities may be defined as follows: exo-chitinase catalyses processive release of diacetylchitobiose units from the non-reducing ends of chitin chains; endo-chitinase randomly catalyses hydrolysis of N-acetylglucosaminide (1,4)-□-linkages of chitin within the chains (Robbins, *et al.* 1988).

Based on their amino acid sequences, 3D structures and machanisms, chitinases are grouped into two district glycosyl hydrolase families (families 18 and 19; Henrissat and Bairoch, 1993). Family 18 includes chitinases from bacteria, fungi, viruses, and animals and chitinases from class III and V in plants. The crystal structures of these chitinases, e.g., chitinase A from *Serratia marcescens* (Perrakis *et al*. 1994), hevamine from *Hevea brasiliensis* (Termisscha van Scheltirga *et al.*, 1994), are shown in Figure 3A and 3B. Family 19 chitinases are mainly found in plants and some bacteria. The structure of family 19, barley chitinase is shown in Figure 3 C.



(A) **(B)** **(C)**

Figure 3 The 3D structure cartoon drawing of a chitinase
display from RasWin Molecular graphics (Rasmol) ver. 2.4.
β–sheet is colored in yellow, α–helix in pink and turns in blue.
(A) = Chitinase A from *Serratia marcescens* (family 18)
(B) = Chitinase from hevamine (family 18)
(C) = Chitinase from barley (family 19)

Mechanisms of chitinases action

Chitinases act by hydrolytically cleaving the β-glycosidic linkages between GlcNAc residues of chitin. In general, this hydrolysis can occur in one of two ways, either with retention or with inversion of anomeric configuration of the product. Extensive studies of the mechanism of lysozyme (family 19) demostrated that glycoside hydrolysis required two acid residues (Glu 36 and Asp 52), both are protonated. The consensus view of the mechanism (Scheme I, Figure 4) involves protonation of the β-(1,4)- glycosidic oxygen atom, leading to an oxocarbenium ion intermediate, which is stabilized by the secondary carboxylate group (either through covalent or electrostatic interactions). Nucleophilic attack by water yields the hydrolysis product, which retains the initial anomeric configuration. This is commonly referred to as the double displacement mechanism of hydrolysis.

The crystal structure of barley chitinase, a family 19 plant endochitinase shows structural similarities with hen lysozyme, suggesting an analogous double displacement mechanism (Hart *et al*., 1995). However, subsequent analysis of the anomeric products for family 19 chitinases show that an inversion of the anomeric configuration accompanies these reactions (Fukamizo *et al*., 1995 and Iseli *et al*., 1996). This observation rules out the double displacement mechanism of hen lyzoyme. A possible mechanism explaining inversion is a concerted single displacement reaction (McCarter and Withers, 1994 and Davies and Henrissat, 1995) in which a bound water molecule acts as the nucleophile.

This binding mode, together with the catalytic residues, is shown in scheme II, Figure 5. Two carboxylates were hypothesized to be responsible for catalysis, Glu67 as catalytic acid and Glu89 as a pararelled base. Hydrolysis would occur between sugars in sites D and E, a convention developed for hen lysozyme (Blake *et al*., 1967; Kelly *et al*., 1979). The importance of these two residues to catalysis has since been confirmed by site-directed mutagenesis (Andersen *et al*., 1997). The mechanism was hypothesized to be an inverting one because the space between the “second carboxylate”, Glu89, and the susceptible glycosidic bond demanded that attacking water be interposed (Hart *et al*., 1995). This inverting mechanism was confirmed using nuclear magnetic resonance (NMR) to follow the anomeric hand of the product sugars which were α (Hollis *et al*., 1997).

This result is consistent with similar work showing that chitinase from *Dioscorea opposit* (yam) proceeds with inversion of product (Fukamizo *et al*., 1995). It is reasonable to assume that family 19 chitinases all work in this way. As indicated in Figure 5, scheme II, 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 involves Glu89 acting as a base to polarize the attacking water molecule, whereas Glu67 acts as an acid to protonated O4 of the leaving sugar.

Family 18 chitinases have not been studies as extensively as those from family 19. They were reported to yield hydrolysis products which retain the anomeric configuration at C1′ (Brameld *et al*., 1998; Brameld and Goddard, 1998 and Yannis *et al*., 2001) and two proposed catalytic mechanisms. The earlier proposed catalytic mechanism that invoked substrate assistance (Brameld *et al*., 1998). That is, the N-acetyl group at position 2 for the scissile sugar may itself facilitate the reaction via formation of a transient oxazolinium intermediate (Terwisscha *et al*., 1995 and Drouillard *et al*., 1997). Chitinase A from *Serratia marcescens* have been investigated. The first step of the acid-catalyzed hydrolysis mechanism of this enzyme involves proton transfer from Glu315 (Perrakis *et al*., 1994).

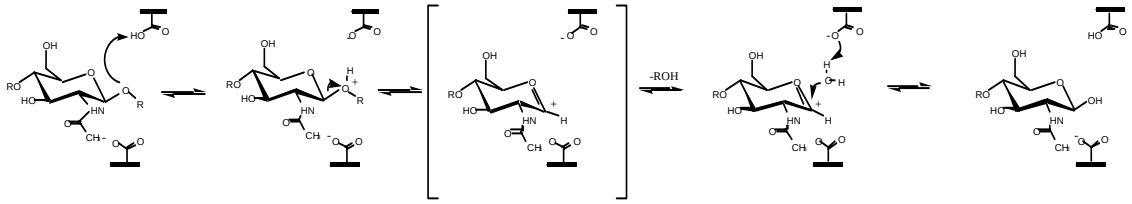
Evidence in support of this includes the observation that Glu315 is completely conserved in family 18 chitinases. In addition, site directed mutagenesis of the corresponding Glu residue in other family 18 chitinase to a Gln was reported to essentially eliminate chitinase activity (Watanabe *et al*., 1993). The likelihood of proton transfer in these reactions primarily depends on the distance between the proton donor and acceptor. The assumption that Glu315 is the donor, the proton acceptor is the β-(1,4)-glycosidic oxygen between sugar residues -1 and +1, which spanning the active site where cleavage occurs.

The conformation of the sugar in the active site is important for catalysis. It has evident that extended N-acetyl geometry of the -1-chair conformation places the glycosidic oxygen too far from Glu315 for efficient proton transfer. In contrast, the -1-boat geometry of the sugar residue places the proton near the glycosidic oxygen and occasionally much closer when a direct hydrogen bond is made. From this evidence,

one may concluded that the binding of a chitin substrate in the -1-boat geometry is not compatible with protonation. Glu315, Asp313 and Asp311 were predicted as important amino acid residues in the active site of chitinase A of *Serratia marcescens*.

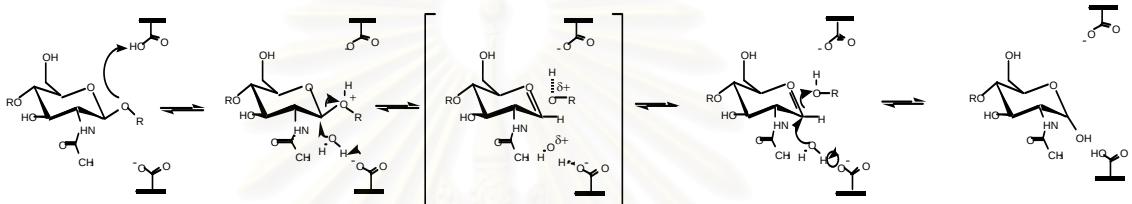
The last proposed catalytic mechanism in chitinase A from *Serratia marcescens* suggests that residues Asp313 and Try390 along with Glu315 play a central role in the catalysis (Yannis *et al*., 2001). Yannis *et al*. proposed that after the protonation of the substrate glycosidic bond, Asp313 that interacts with Asp311 flips to its alternative position where it interacts with Glu315 thus forcing the substrate acetamido group of -1 sugar to rotate around the C2-N2 bond. As a result of these structural changes, the water molecule that the hydrogen-bonded to try390 and the NH of the acetamido group is displaced to a position that allows the completion of hydrolysis (Scheme III). In this mechanism, we can not observe an oxazoline ring intermediate, the acetomido group of -1 sugar comes close to O5 atom in a way that could allow a modified “substrate assisted” reaction.

Scheme I



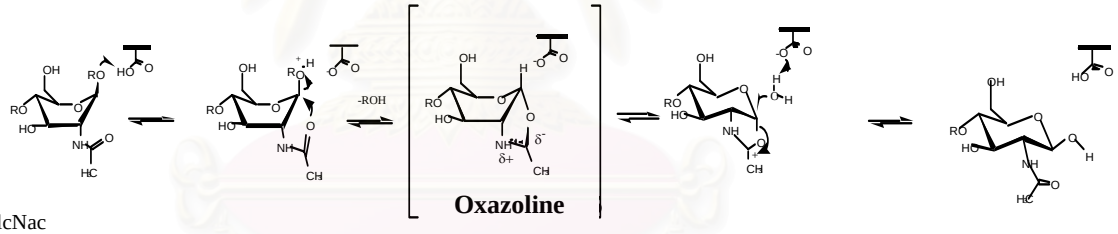
R=GlcNac **Oxocarbenium**

Scheme II



R=GlcNac **Oxocarbenium**

Scheme III



R=GlcNac **Oxazoline**

Scheme IV

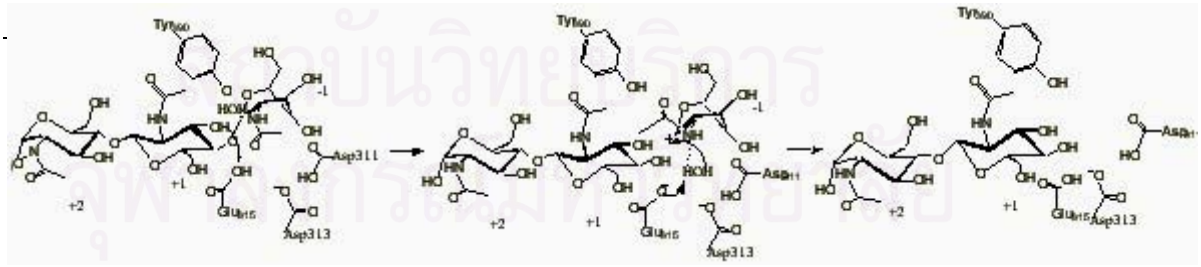


Figure 4 Chitinase mechanisms of lysozyme (scheme I), family 19 (scheme II) and family 18 (scheme III, IV)

Applications of chitinase

Among bacteria, high chitinase activity has been observed in species of *Streptomyces, Serratia, Vibrio* and *Bacillus* (Cody., 1989, Monreal and Reese, 1969, Romaguera *et al*., 1992, Wortman *et al*. 1986). Practical applications of chitinase include its uses in the preparation of protoplasts from fungi (Yabuki *et al*., 1984), as a protective agent against plant-pathogenic fungi (Sundheim *et al*., 1988) and in the production of chitiooligosaccharides as biologically active substances (Usui *et al*., 1987).

Chitinases are reported to dissolve cell walls of various fungi, a property that has been used for the generation of fungal protoplasts (Ramaguera *et al.,* 1993). Chitinases from *Streptomyoes* was found to be active in the generation of protoplasts from *Asperigillus oryzae* and *Fusarium solani* (Skujins *et al*., 1965). Chitinase-producting organisms are used in agriculture as an effective biocontrol agent against a number of phytopathogenic fungi (Elad *et al*., 1982), *Aeromonas caviae* controlled infection by *Rhizoctonia solani, Fusarium oxysporum* in cotton and *Sclerotium rolfsii* in beans by producing chitinase. Chitinase produced by *Serratia marcescens* was effective against pathogenic fungi *S. rolfsii* and larvae of *Galleria mellonela* (Lysenko, 1976). A culture filtrate of *Aphanocladium album* strongly inhibited growth of *Necteria haematcocca* in the pea (Kunz *et al*., 1992).

These organisms are effectively used in the bioconversion process to treat shellfish waste and also to obtain value-added products from such wastes. Commercially, chitinases from different sources are available from Sigma Chemical Co. (USA). The approximate cost is U.S. 105 $ for 10 U (400-1200 units/g solid). Thus, chitinolytic enzymes have been purified and cloned from many microorganisms, and their enzymatic properties have been investigated.

Characterization and molecular cloning of chitinase

In recent years, various chitinases were isolated, purified and characterized from microorganisms such as *Bacillus circulans* WL-12 (Wanatabe *et al*., 1990), *Serratia marcescens* (Nawani and Kapadnis, 2001) and an *Aeromonas sp*.10S-24 (Ueda *et al.*, 1992). Brurberg *et al*. (1994) purified chitinase from an *E.coli* culture carrying the *chiA* gene of *S. marcescens* using single step hydrophobic interaction chromatography. In Thailand Chanpen *et al.* (1999) had recently purified and

characterized chitinase from *Bacillus licheniformis* No.4.1. The research on purification and characterized of chitinases were summarized in Table 3.

A wide range of molecular weight from 30 to 120 kDa is observed in bacteria and fungi. Some of these small chitinases may possibly be processed from a larger enzyme by limited proteolysis (Radwan *et al*., 1994). Most of the chitinases were active at a wide pH range from 4.0-8.0 and 40-55 °C in temperature.

Several genes encoding chitinases in many bacterium were cloned and sequenced such as chitinase A1 and C from *Bacillus circulans* WL-12 (Wanatabe *et al*., 1990), Chitinase C of *Serratia marcescens* (Suzuki *et al*., 1999), an *Aeromonas caviae* (Sitrit *et al*., 1995) and *Alteromonas sp*.strain O-7 (Tsujibo *et al*., 1992). In Thailand, chitinase from *Bacillus licheniformis* TP-1 was also successfully cloned (Tantimavanich, 1997). A summary of all researchs on cloning of microbial chitinase genes gave in Table 4.

Bacillus licheniformis

Bacillus licheniformis is a ubiquitous, saprophytic, soil bacterium which is thought to contribute to nutrient cycling due to its ability to produce a wide variety of enzymes. *B. licheniformis* has been used for industrial production of proteases, amylases and antibiotics (Claus and Berkeley, 1986).

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

B. licheniformis is not a frank human pathogen, but has on several occasions been isolated from human infections. Diseases attributed to *B. licheniformis* included bacteremia, opthalmitis following trauma, and their reports of food poisoning based on circumstantial evidence (Tabbasa and Tarabay, 1979). However, the literature suggested that there must be immunosuppression of the host, or there must be trauma

(especially to the eye) followed by inoculation in high numbers (Farrar, 1963), before infection can occur (Claus and Berkeley, 1986). *B. licheniformis* does not produce significant quantities of extracellular enzymes or other factors that would predispose it to cause infection. Unlike several other species in the genus, *B. licheniformis* does not produce toxins.

The use of *B. licheniformis* for industrial production of enzymes should not cause environmental hazards. First, the number of microorganisms released from the fermentation facility is low. In addition, *B. licheniformis* is ubiquitous in the environment, and the releases expected from fermentation facilities operating under the conditions of this exemption will not significantly increase population of this microorganism in the environment. Therefore, although *B. licheniformis* may be associated with livestock abortions, the use of this microorganism in fermentation facilities will not substantially increase the frequency of this occurrence, even if a scenario for high exposure to *B. licheniformis* released from the fermentation facility to livestock could be envisioned.

In conclusion, the used of *B. licheniformis* in fermentation facilities for production of enzymes or specialty chemicals presents low risk. Although not completely innocuous, *B. licheniformis* presents low adverse effects to human health or the environment.

In this study, the chitinase from bacteria was observed because chitinase activity has been found in a wide variety of bacteria, easy cultivation, large quantity of enzyme production and most of them secrete enzyme out of the cell which simplify the purification. We study *Bacillus licheniformis* PR-1 because this bacteria is an active producer of chitinase in our lab. In addition, the study of Chanpen *et al.* (1999) and Tantimavanich (1997) showed that it can lead to application for biocontrol. For cloning, there are few reports about chitinase from *Bacillus licheniformis* availible. Recently, polymerase chain reaction (PCR) technique has been applied for sequencing and cloning. Therefore, using PCR technique for cloning and identifying chitinase gene is the main interest for my study.

Table 3 Comparion of the characteristics of purified Chitinase from several Microorganisms.

Source Organism	Molecular weight	Optimum pH	Optimum temp (°C)	pI	Reference
Aeromonas hydrophilia H-2330	62,000	5.0-8.0	40	4.0	Hiraga *et al*., 1997
Aeromonas sp. 10S-24					Ueda *et al*., 1992
Chitinase I	115,000	4.0	50	7.9	
Chitinase II	112,000	4.0	50	8.1	
Alteromonas sp. strain O-7 Chitinase A	70,000	8.0	50	3.9	Tsujibo *et al*., 1992
Bacillus circulans No.4.1	45,000	8.0	40	5.1	Chanpen., 1999
Bacillus circulans WL-12					Wanatabe *et al*., 1990
Chitinase A1	74,000	5.0	N.D.	4.7	
Chitinase A2	69,000	N.D.	N.D.	4.5	
Chitinase B1	38,000	N.D.	N.D.	6.6	
Chitinase B2	38,000	N.D.	N.D.	5.9	
Chitinase C	39,000	N.D.	N.D.	8.5	
Chitinase D	52,000	N.D.	N.D.	3.9	

Table 3 (Continued)

Source Organism	Molecular weight	Optimum pH	Optimum temp (°C)	pI	Reference
Bacillus licheniformis B-6839					Lesya *et al*., 1996
Chi66	66,000	All	70		
Chi62	62,000	4.5-5.5 and	90	All	
Chi53	53,000	9.0-9.5	70	4.5-4.7	
Chi49	49,000		70		
Bacillus licheniformis X-7u					
Chitinase I	89,000	6.0	70	N.D.	Takayanagi., 1991
Chitinase II	76,000	6.0-10.0	70-80	N.D.	
Chitinase II	66,000	5.0-10.0	70-80	N.D.	
Chitinase IV	59,000	5.0-10.0	70-80	N.D.	
Clostridium paraputrificum					Morimoto *et al*., 1997
Chitinase B	87,000	6.0	45	N.D.	
Streptomyces erythraeus	30,000	5.0	N.D.	3.7	Hara *et al*., 1989

Table 3 (Continued)

Source Organism	Molecular weight	Optimum pH	Optimum temp (°C)	pI	Reference
Streptomyces thermoviolaceus OPC-250					Tsujibo *et al*., 2000
Chi30	30,000	4.0	60	3.8	
Streptomyces RC1071	70,000	8.0	40	N.D.	Gomes *et al*., 2001
Streptomyces sp. J13-3	31,000	6.0	45	3.9	Okasaki *et al*., 1995
Chitinase B1	38,000	N.D.	N.D.	5.9	
Serratia marcescens BJ2000					Brurberg *et al*., 1982
Chitinase A	61,000	4.0-7.0	N.D.	6.4	
Serratia marcescens NK1	57,000	6.2	47	N.D.	Nawani and Kapadnis, 2001
Serratia marcescens QMB1466	58,000	4.0-7.0	N.D.	6.4	Roberta *et al*., 1982
Chitinase A					
Xanthomonas sp. strain AK	64,000	4.0	35	N.D.	Yamaoka *et al*., 1999
Chitinase A	48,000	6.0	0	N.D.	
Chitinase B					

Table 4 Molecular cloning of chitinase genes

Source Organism	Host	Vector	DNA insert Sizes (kb)	ORF (kb)	Reference
Aphanocladium album	*E.col*	pBL1	8.0	1.9	Blaiseau *et al*., 1992
Aeromonas hydrophilia	*E.coli*	pUC18	5.0	2.5	Tsujibo *et al*., 1992
Aeromonas caviae	*E.col*	pBluesccript II SK	4.5	2.6	Sirit *et al*., 1995
Alteromonas sp. strain O-7	*E.coli*	pUC18	5.0	2.5	Tsujibo *et al*., 1992
Bacillus circulans WL-12					
Chitinase A1	*E.col*	pKK223-3	4.0	2.1	Wanatabe *et al*., 1990
Chitinase C	*E.coli*	pUC19	2.8	1.5	Wanatabe *et al*., 1995
Bacillus licheniformis TP-1	*E.coli*	pGEM7Zf(+)	5.5	1.8	Tantimavanich., 1997
Bacillus circulans C-2	*E.coli*	pUC19	3.0	N.D	Zeng H, Wang Y, Zhang Y
Clostridium paraputrificum					
Chitinase B	*E.coli*	pMW119	5.1	2.5	Morimoto *et al*., 1997
Enterobacter agglomerans	*E.coli*	pGEM-T	2.2	1.7	Chernin *et al*., 1997

Table 4 (Continued)

Source Organism	Host	Vector	DNA insert Sizes (kb)	ORF (kb)	Reference
Streptomyces thermoviolaceus OPC-520					
Chi30	*E.coli*	pUWL219	3.4	1.0	Tsujibo *et al*., 2000
Serratia marcescens BJL200					
Chitinase B	*E.coli*	pBluescript II SK	4.5	2.6	Sirit *et al*., 1995
Serratia marcescens 2170					
Chitnase C	*E.coli*	pUC119	4.5	1.4	Suzuki *et al*., 1999
Serratia liquefaciens					
Chitinase B	*E.coli*	pSJ12	4.6	1.5	Woytowich *et al*., 2000

CHAPTER II

MATERIALS AND METHODS

Equipments

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

Autopipette: Pipetman, Gilson, France

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

Centrifuge, microcentrifuge high speed: Model MC-15A, TOMY SEIKO CO.LTD., TOKYO, Japan

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

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

Gene Pulser™: Bio-Rad, U.S.A.

HPLC Model water™ 600 system

Incubator: Model 1H-100, Gallenkamp, England

Incubator shaker controlled environment: Psyco-threm, New Brunswick Scientific Co., U.S.A.

Incubator shaker: Model G76, New Brunswick Scientific Co., Inc. Edison, U.S.A.

Incubator, water bath: Model M20S, Lauda, Germany

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

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

Microcentrifuge tube 1.5 mL, Bioactive, Thailand

pH meter: Model PHM95, Radiometer Copenhegen, Denmark

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

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

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

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

Spectrophotometer UV-240, Shimadzu, Japan, and Du series 1050, Beckman, U.S.A.

Thin-wall microcentrifuge tubes 0.2 mL, Axygen Hayward, U.S.A.

UV transluminator: Model 2011 Macrovue, SanGabriel California, U.S.A.

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

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

Water bath, shaking: Heto lab Equipment, Denmark

Chemicals

Acetonitrile: (Methyl cyanide) Sigma, U.S.A.

Acrylamide: Merck, U.S.A.

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

Aqua sorb: Fluka, Switzerland

Ammonium persulphate: Sigma, U.S.A.

Ammonium sulphate: Sigma, U.S.A.

Ampicillin: Sigma, U.S.A.

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

β-mercaptoethanol: Fluka, Switerland

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

Bovine serum albumin (BSA): Sigma, U.S.A.

Bromphenol blue: Merck, Germany

Chloroform: BDH, England

Coomassie brilliant blue R-250: Sigma, U.S.A.

Dialysis tubing: Sigma, U.S.A.

di-Potassium hydrogen phosphate anhydrous: Carlo Erba Reagenti, Italy

di-Sodium ethylenediaminetetra acetate: M&B, England

DNA marker: Lamda(λ)DNA digest with *Hin*d III: GIBCOBRL, U.S.A.

Ethidium bromide: Sigma, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

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

Flake chitin: Sigma, U.S.A.

Fluorescent Brightener 28: Sigma, U.S.A.

Glacial acetic acid: Carlo Erba Reagenti, Italy

Glycine: Sigma, U.S.A.

Glycol chitin: Seikaguku Corporation, Japan

Glucose: BDH, England

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

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

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

Megnesium sulphate 7 hydrate: BDH, England

Megnesium chloride: BDH, England

Methanol: Merck, Germany

N, N'-dimethyl-formamide: Fluka, Switzerland

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

NNN'N'-tetramethyl-1,2-diaminoethane (TEMED): Carlo Erbo Reagenti, Italy

Phenol: BDH, England

Phosphoric acid: Mallinckrodt, U.S.A.

Potassium ferricyanide: BDH, England

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

QIAquick Gel Extraction Kit: QIAGEN, Germany

QIAquick Plasmid Extraction Kit: QIAGEN, Germany

Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy

Sodium citrate: Carlo Erba Reagenti, Italy

Sodium chloride: Carlo Erba Reagenti, Italy

Sodium dodecyl sulfate (SDS): Boehringer Mannheim Gmbtt, Germany

Sodium hydroxide: Merck, Germany

Sucrose: Sigma, U.S.A.

Tris (hydroxymethyl) aminomethane: Carlo Erba Reagenti, Italy

TritonX-100: Merck, Germany

Tryptone: DIFCO, U.S.A.

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

Yeast extract: DIFCO, U.S.A.

Enzymes and Restriction enzymes

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

Lysozyme: Sigma, U.S.A.

Proteinase K: Sigma, U.S.A.

Restriction enzymes: GIBCOBRL, U.S.A. and New England Biolabs, Inc., U.S.A.

RNase: Sigma, U.S.A.

Tag polymerase: Pacific science, France

T4 DNA ligase: New England Biolabs, Inc., U.S.A.

Bacterial strains

Bacillus licheniformis PR-1, isolated from Southeast Asian soil, was screened for chitinase and chitinase gene amplification.

E.coli DH5α with genotype *F′,* ∅80δ lacZΔM15, Δ(lacZYA-argV169), endA1, recA1, hsdR17 ($r_{K-}m_{K+}$), deoR, thi-1, supE44, λ^-gyrA96, relA1 was purchased from GIBCOBRL, U.S.A.

E.coli XL-1-Blue with genotype recA1, relA1, endA1, gyrA96, thi-1, hsdR17, supE44, lac[*F′,* proAB, lac/^{9}ZΔM15Tn10(Tet^r)] was purchased from GIBCOBRL, U.S.A.

E.coli JM109 with genotype *F′* [traD36, proAB, laclqZM15], λ^-, endA1, gyrA96, hsdR17($r_{K-}m_{K+}$), mcrB$^+$, recA1, relA1, Δ(lac-proAB), thi, supE44 was purchased from GIBCOBRL, U.S.A.

Bacillus licheniformis SK-1, *Bacillus circulans* PP8, *Paenibacillus Compinasensis* (PT), *Bacillus ciculans* A11 (RB) and *Peanibacillus* (BT) were isolated from Southeast Asian soil and used for whole chitinase gene amplification.

Media Preperation

Luria-Bertani (LB) medium

LB consists of 1.0% tryptone, 0.5% yeast extract and 1.0% NaCl. pH was adjusted to pH 7.2-7.4 with NaOH or HCl. For solid medium, 2 % agar was added.

Terrific medium

Terrific medium consists of 1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.23% (w/v) KH_2PO_4 and 1.25% (w/v) K_2HPO_4.

Colloidal chitin minimum medium (CCMM)

Colloidal chitin minimum medium was used as enzyme production medium containing 0.02% (w/v) dry weight of colloidal chitin, 0.05% (w/v) yeast extract, 0.1% (w/v) $(NH_2)SO_4$, 0.03% (w/v) $MgSO_4.7H_2O$, 0.6% (w/v) KH_2PO_4 and 1% (w/v)

K_2HPO_4 adjusted pH to 7.2-7.4. For solid medium, 2% (w/v) agar was added. (Preperation of colloidal chitin see Appendix A.)

All medium above were steriled by autoclaveing at 121 °C for 15 min.

Plasmid vectors

Plasmid pBluescript SK^- (Stratagene) was used as a cloning vector for cloning and subcloning of chitinase gene into *E. coli*.

Plasmid pGEM®-T Easy (Promega) was used for cloning PCR fragments into *E. coli*.

Cultivation of Bacteria

A single colony of *Bacillus licheniformis* PR-1 was grown in 1.5-2 mL LB medium at 37 °C with 250 rpm rotation shaking about 12-16 hours as an inoculumn.

Identification of bacterium producing chitinase

Bacteria *B.licheniformis* PR-1 was cultured on colloidal chitin minimum medium plate and then identified by biochemical methods at TISTR. The identity was confirmed by 16S rRNA sequence comparison. The amplification method described below.

Polymerase Chain Reaction (PCR) amplification

Primer for amplifying 16S rRNA gene.

A partial DNA sequence for the 16S rRNA gene was amplified by using pA (5'-AGAGTTTGATCCTGGCTCAG-3') for forward primer and pH' (5'-AAGGAGGTGATCCAGCCGCA-3') for reverse primer (Ulrike *et al.*, 1989).

Primers for amplification of partial and full lenght chitinase gene

The first set of primers used for partial chitinase gene amplification were degenerate primers designed from N-terminal amino acid sequences of familly 18 chitinases from *B.circulans* chi41, *B.licheniformis* TP, *B.subtilis, B.circulans* chi1, *B.circulans* A1, *B.thuringiensis* and *B.cereus* chiB. All constructed primers are shown in Table 5.

All PCR products were analysed by agarose gel electrophoresis and sequencing as describe in chapter III.

The conditions for 16S rRNA and chitinase gene amplification are described in Table 6.

Table 5 Nucleotide sequences and *Tm* (°C) of all primers used in chitinase gene amplification.

Forward Primer	**Sequence (5'⟶ 3')**	***Tm* (°C) [2°(A+T)+4°(G+C)]**	**Remark**
FPSK$^-$	GGTGGCGGCCCGCTCTAGAAC	68	For pBlueScript II SK$^-$ sequencing
BP-I	AAYTAYGCDTTYGCDGAYATHTGYTGGRANGG	84	For partial gene amplification
BP-II	TTYGAYGGNGTNGAYYTNGAYTGGGARTA	76	For partial gene amplification and sequencing
BP-VIII	AAYATCATGACNTAYGAYTTYAAYGGNGGNTGG	86	For partial gene amplification
BP-F	GTTTTCCCTTGTTGTCTTC	54	For whole gene amplification
BP-IX	CATGGGAAAAGGGCG	48	For gene sequencing
Reverse Primer	**Sequence (5'⟶ 3')**	***Tm* (°C) [2°(A+T)+4°(G+C)]**	**Remark**
BP-VII	NCCNTYCCARCADATRTCHGCRAAHGCRTARTT	86	For partial gene amplification
BP-V	RTAYTCCCARTCNARRTCNACNCCRTCRAA	76	For partial gene amplification
BP-VI	CCANCCNCCRTTRAARTCRTANGTCATGATRTT	86	For partial gene amplification
BP-R	CTCTTTATCGTTTTCTATCC	54	For whole gene amplification

Abbreviations ; **Y** = C,T **R** = A,G **D** = A,G,T **H** = A,C,T **N** = A,C,G,T

Table 6 PCR condition in each step

Corporated primers	Predenaturation	Denaturation	Annealing	Extension	Final extension	Number of cycle
16S rRNA pA+pH'	94 °C, 5 min	94 °C, 1 min	55 °C, 2 min	72 °C, 3 min	72 °C, 5 min	25-30
Partial chitinase gene BP-I+BP-V BP-II+BP-VI BP-I+BP-VI	94 °C, 4 min	94 °C, 1 min	60 °C, 2 min	72 °C, 3 min	72 °C, 5 min	25-30
Whole chitinase gene BP-F+BP-R	94 °C, 4 min	94 °C, 1 min	50 °C, 2 min	72 °C, 2 min 30 sec	72 °C, 5 min	25-30

After final extension, the PCR products were kept at 4 °C.

Partial characterization of crude chitinase from *B.licheniformis* PR-1

Enzyme production

The inoculumn was diluted 1:100 into 100 mL of colloidal chitin medium in a 1,000 mL Erlenmeyer flask and cultivated for 5 days at 37°C with 250 rpm rotary shaking.

Cells were removed by centrifugation at 8,000 rpm for 15 min at 4°C. Crude chitinase enzyme was kept at 4°C for characterization.

Determination of chitinase activity

For this study, bacterial strains producing chitinase were screened by formation of clear zone around the bacterial colony on colloidal chitin minimum medium plate.

Crude chitinase activity was determined by reducing sugar producing activity assay (Colorimetric method), modified from Schale's method (Imoto, 1971).

Chitinases activity was assayed by measuring the reducing sugar (Imoto and Yagishita, 1971) produced from 1.5 mL reaction containing 0.1 g/mL of substrate and 100-500 μL of enzyme in final concentration 0.1 M buffer of optimum pH. The mixture was incubated at optimum temperature for 1 hour, then heated to 100 °C for 15 min to stop the reaction.

An appropriate amount of reaction mixture was adjust to the final volume of 1.5 mL with 0.1 M buffer then mixed with 2 mL of color reagent, made by dissolving 0.5 g of potassium ferricyanide in 1 litre of 1.5 M Na_2CO_3. The mixture was heated in boiling water for 15 min. After cooling at room temperature, small particles were removed from the mixture by centrifugation at 5,000 g for 10 min.

Measuring absorbance (A_1) at 420 nm by a spectrophotometer versus distilled water. A blank value (A_0) was obtained when denatured enzyme (heating in boiling water for 20-30 min) was used instead of the enzyme in the reaction. The difference between A_0 and A_1 was used to determine the amount of GlcNAc from standard curve (see Appendix I).

One unit (U) of enzyme activity was defined as the amount of an enzyme able to liberate 1 μmol product (N-acetylglucosamine) per min. Specific activity was defined as units per mg protein of an enzyme sample.

Determination of protein concentration

Protein concentration was determined by dye binding method according to Bradford (1976), using bovine serum albumin as standard (see standard curve in Appendix H).

One hundred microlitres of sample was mixed with 5 mL of Bradford working solution (5x) and left for 5 min before recording the absorbance at 595 nm.

One litre of Bradford working solution (5x) consists of 100 mg Coomassie Brilliant Blue G-250, 50 mL of 95% (v/v) ethanol, 10 mL of 85% (v/v) phosphoric acid (H_3PO_4). The volume was adjusted to 1 litre by distilled water.

Optimum pH

The optimum pH of crude chitinase was measured after incubation in 0.1 M buffer at pH range 3.0-10. The buffers used were 1 M sodium citrate buffer (pH from 3.0-6.0), 1 M sodium phosphate buffer (pH from 5.0-8.0) and 1 M Tris-HCl buffer (pH from 7.0-10).

The 1.5 mL reaction mixture contains 60 μl of 2.5 mg/mL (2.5% dry weight) colloidal chitin (total 10 mg), 150 μL of 1 M buffer (final concentration 0.1 M), 100 μL crude enzyme and distilled water was added to adjust volume to 1.5 mL. The reaction was incubated at 37°C for 45 min.

The enzyme activity was assayed by measuring the increasing reducing property of colloidal chitin suspension during chitinolysis.

Optimum temperature

The optimum temperature of crude chitinase was determined in 0.1 M citrate buffer pH 5.0 which is optimum pH of this enzyme.

The reaction mixture containing 10 mg/mL colloidal chitin, 0.1 M buffer, 100 μL crude enzyme and distilled water was added to adjust volume to 1.5 mL. The reaction mixture was incubated for 45 min at temperature range of 30-80 °C. The enzyme activity was assayed by measuring the increasing reducing property of colloidal chitin suspension during chitinolysis.

Substrate specificity

The activity of chitinase was assayed on chitin-related compounds, including colloidal chitin, powder chitin, 80% deacetylated chitiosan, flake chitin and regenerated chitin by measuring reducing sugar produced from a mixture composed of 10 mg/mL of substrate in 0.1M citrate buffer pH 5.0 (optimum pH). The mixture was incubated at 70°C (optimum temperature).

Estimation of molecular weight of chitinase by SDS-PAGE

The molecular weight of chitinase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by activity staining of chitinase. SDS-PAGE was performed by the method of Trudel and Asselin (Trudel *et al*., 1989) using a 10% acrylamide gel containing 0.001% (w/v) glycol chitin.

Enzyme was denatured by heating at 100°C for 10 min in sample loading dye containing 15 % (w/v) sucrose and 2.5 % (w/v) SDS in 125 mM Tris–HCl pH 6.7, 15 % (v/v) β-mercaptoethanol and 0.01 % bromophenol blue (w/v) as tracking dye. The gel was ran as for the Davis system. Low molecular weight protein marker range 14.4-97 kDa was used. After electrophoresis, proteins were stained with 0.25 % (w/v) Coomassie Brilliant Blue R-250 at room temperature for 2 hours then destaining with a mixture of 10 % (v/v) acetic acid and 10 % (v/v) methanol for 2-3 hours or until gel background was clear.

The protein band containing chitinase activity after SDS-PAGE was detected by incubating gel overnight at 37 °C in 100 mM Tris-HCl buffer (pH 7.0) containing 1 % (v/v) Triton X-100. The gel was stained with 0.01 % (w/v) Fluorescent Brightener 28 in 500 mM Tris-HCl pH 8.9 for 10 min and then destained with distilled water. Activity of chitinase was visualized as the dark zone under UV light.

Detection of hydrolytic products produced by chitinase from *B. licheniformis* PR-1

For this experiment, in the total of 2.5 mL reaction mixture consisted of 40 mg/mL colloidal chitin, 800 μL of crude chitinase, 250 μL of 1 M citrate pH 5.0 and 250 μL distilled water, incubated at 70 °C overnight, then added another 800 μL

crude chitinase after 12 hours of digestion. The digested products at the end of 24 hours were boiled for 15 min and centrifuged at 8,000 rpm for 10 min.

Then 300 μL of the supernatant was withdrawn and mixed with 700 μL of acetonitrile, and filtered through a 0.45 micron filter. The products were analysed by HPLC. The conditions used were; Shodex Asahipak NH2P-50 column, mobile phase 300 mL water: 700 mL acetonitrile, sample injected volume 20 μL, flow rate 1.0 mL/min at 25 °C.

General methods in cloning of chitinase gene (Maniatis *et al*., 1982)

Extraction of chromosomal DNA of bacteria strain PR-1

A single colony of *B.lichenifromis* PR-1 grown on colloidal chitin minimum medium was cultured in 10 mL LB broth at 37 °C with 250 rpm shaking for overnight. The cultured was transferred in 1.5 mL microcentrifuge tube, centrifuged at 10,000 rpm for 5 min, discarded supernatant and resuspended cells with 100 μL SET buffer [25 mM (w/v) Tris pH 8.0, 10 mM (w/v) EDTA and 50 mM (w/v) sucrose]. The cell suspension was added with 400 μL SET buffer with 5 mg (w/v) of lysozyme, incubated at 37 °C for 1 hr. Then 5 μL of 10% (w/v) SDS was added followed by 3 μL of 20 mg/mL (w/v) protinase K, gently mixing by invert tube, and incubated at 50 °C for overnight.

The cell debris was separated from the supernatant by centrifugation at 12,000 rpm for 10 min. After that, 50 μL of 3M sodium acetate was added and gently mixed following by equal volume of phenol-chloroform-isoamyl alcohol (25:24:1,v/v/v) and gently mixed. The mixture was separated into 2 phases by centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a fresh tube. This process was repeated several times till no white precipitate was observed between the 2 phases.

The upper aqueous phase was transferred to a new tube and added 2.5 volume of room temperature absoluted ethanol. After mixing by inversion, genomic DNA was collected by centrifugation at 12,000 rpm for 10 min. The genomic DNA was washed with 70% ethanol, air-dried and then dissolved in 100 μL of TE buffer (10 mM (w/v) Tris-HCl and 1 mM (w/v) EDTA, pH 8.0), incubated 37 °C overnight. Store the DNA at 4°C.

The purity of DNA was calculated from absorbance at 260 nm divided by absorbance at 280 nm (A_{260}/A_{280}). The range between 1.8-2 was good for further used.

Plasmid DNA preparation by alkaline extraction

Plasmid DNA vectors, pBluescript KS^- was extracted by alkaline lysis method (Maniatis *et al*., 1982).

A single colony of *E.coli* containing pBluescript SK^- was inoculated into 1.5 mL of LB broth, containing ampicillin 150 μg/mL and incubated at 37 °C with shaking 250 rpm overnight. The culture was transferred into 1.5 mL microcentrifuge tube and centrifuged at 8,000 rpm for 1 min. The supernatant was discarded, then 100 μl of solution I [25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 50 mM glucose and 0.5% (w/v) lysozyme] was added, mixed by vigorous vortexing and placed on ice for 15 min. 200 μL of freshly prepared solution II [0.2 N NaOH and 1% (w/v) SDS] was added for cell lysis and DNA denaturation and mixed gently by invert tube, incubating on ice for 10 min.

The mixture was added with 150 μL of solution III (3 M sodium acetate, pH 4.8) for renaturation, mixed gently and placed on ice for 30 min. The tube was centrifuged at 12,000 rpm for 10 min to separate cell debris. The supernatant was transferred into a new 1.5 mL microcentrifuge tube, added an equal volume of a solution containing phenol:chloroform:isoamyl alcohol (25:24:1,v/v/v), mixed and centrifuged at 12,000 rpm for 10 min. The upper aqueous phase was transferred to a new tube.

The plasmid DNA was precipitated by adding 2.5 volumes of absolute ethanol then mixed well and kept at –80 °C for at least 10 min. The mixture was centrifuged at 12,000 rpm for 10 min. The plasmid DNA was washed with 70 % (v/v) ethanol, evaporated trace ethanol at 50 °C and then dissolved in 50 μL of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0) containing 20 μg/mL RNase A and incubated at 37 °C for overnight. Store the plasmid DNA at -20°C.

The purity of plasmid DNA was calculated from absorbance at 260 nm divide by absorbance at 280 nm (A_{260}/A_{280}). The range between 1.8-2 was good for further used.

Agrarose gel electrophoresis

Agarose gel electrophoresis was used to analyze plasmid and DNA fragments, obtained from restriction enzyme digestion. The size was estimated from standard curve of DNA markers, fragments of Lamda DNA digested with *Hin*dIII range 23-0.6 kb and 100 bp ladder range 1.5-0.1 kb. The standard curve was plotted between logarithmic fragment sizes and their relative mobilities.

The concentration of agarose gel used depends on the size of the DNA fragment to be separated. Generally 0.7-1.5% gel in 1xTAE buffer (0.04 M Tris-acetate and 0.001 M EDTA) was used.

DNA solution was mixed with 10-20% (v/v) of 6x loading buffer [0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF and 15 % (w/v) Ficoll 400 in distilled water). The mixture was loaded into the well of the gel that was submerged in electrophoretic chamber filled with 1xTAE. Electrophoresis was carried out at constant 50-100 volts. The duration of running depended on the size of DNA.

Generally, the gel was run until the tracking dye reached near the bottom of the gel. After electrophoresis, the gel was stained with ethidium bromide solution (5-10 μg/mL in distilled water) for 3-5 min and then destained with distilled water 2-3 times. The DNA was visualized on an UV transilluminator and photographed.

Partial digestion of chromosomal DNA

The chromosomal DNA of *B.licheniformis* PR-1 was partially digested with *Pst*I for 30 min under conditions recommended by the manufacturer and separated on 1% agarose gel in TAE buffer (0.004M Tris-acetate and 0.001M EDTA) at 100 volts. Lamda DNA cut with *Hin*dIII (λ/*Hin*dIII) was used as size standard. The gel segment corresponding to the size between 2 and 6 kb was cut out, DNA fragments in the gel were recovered by using QIA quick Gel Extraction Kit (Qiagen, Germany).

Recombinant DNA construction

Both 2-6 kb chromosomal DNA fragments and dephosphorylated *Pst*I-digested *pBluescript* SK^- were mixed together in a ratio of DNA: vector from 2:1. In total 20 μl, 2 μl of 10x ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP) was added to the mixture, adjusted volume to 20 μl with

steriled distilled water. One microlitre of T4 DNA ligase (10 units) was added into the reaction mixture. The reaction was incubated for 18-20 hours at 18-20 °C. Ligation products were kept at –20 °C.

Competent cells preparation

The competent *E. coli* XL-1 blue was prepared according to the method of Sambrook *et al.* (1989). A single colony of *E. coli* XL-1blue was cultured as the starter in 12 mL of LB broth [1 % (w/v) tryptone, 0.5% (w/v) yeast extract, and 1 % (w/v) NaCl] and incubated at 37 °C with shaking 250 rpm for overnight. One percent of starter was inoculated into 1,000 mL of LB broth [1 % (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5 % (w/v) NaCl] and the culture was incubated at 37 °C with shaking 250 rpm for 2-3 hours until the optical density at 600 or 650 nm (OD_{600} or OD_{600}) of the cells reached 0.5-0.6 or 0.4-0.5 respectively.

The cells were chilled on ice for 15-30 min and harvested by centrifugation at 8,000 rpm for 15 min at 4 °C. The supernatant was removed as much as possible. The cell pellet was washed with 1 volume of cold steriled water (800 mL), resuspended by gently mixing and centrifuged at 8,000 rpm for 15 min at 4 °C. The supernatant was discarded. The pellet was washed and centrifugation further with 400 mL of cold steriled water, followed by 20 mL of ice cold steriled 10 % (v/v) glycerol, and finally resuspended in a final volume of 1.6-2.0 mL ice cold steriled 10 % (v/v) glycerol. This cell suspension was divided into 40 μl aliquots and stored at -80 °C until used.

These competent cells were good for at least 6 months under these conditions.

Electrotransformation

The competent cells were thawed on ice. Fourty microlitres of the cell suspension was mixed with 1.5 μL of the ligation mixture, mixed well and placed on ice for 1 min. The mixture of cell and DNA was electroporated in a cold 0.2 cm cuvette with apparatus setting as follows; 25 μF, 200 Ω of the Pulse controller unit and 2.50 kV.

After one pulse was applied, the cells were immediately resuspended with 1 mL of LB medium [1.0 % (w/v) tryptone, 0.5 % (w/v)yeast extract and 1.0 % (w/v)

NaCl] and transferred to a sterile test tube. The cell suspension was incubated at 37 ° C with shaking at 250 rpm for 1 hour. All of the cells in the suspension was spread on the 0.02% (dry weight) colloidal chitin agar plates, which contained 100 mg/mL ampicillin, 30 μL of 25 mg/mL iso-1-thio-β-D-galactopyranoside (IPTG) and 30 μL of 25 mg/mL chloro-3-indolyl-β-D-galactopyranoside (X-gal), incubated in 37 °C incubator for 1 week.

The positive transformants with insertions were screened for plasmid harboring chitinase gene by phenotype screening described below.

Detection of chitinase gene (phenotype screening)

Transformants that might harboring chitinase gene were detected by the formation of clearing zone around the colonies on screening medium which consisted of 0.25% yeast extract, 0.02% colloidal chitin, 100 μg/mL (w/v) ampicillin, 30 μL of 25 mg/mL (w/v) IPTG and 30 μL of 25 mg/mL X-gal.

Transformants, which produced clear zone, were cultured for plasmid extraction. The plasmid was then cut with *Pst*I to determine the size of inserted fragment.

Analysis of chitinase gene

Induction and repression of chitinase synthesis

Clone I was streaked on 0.25 % yeast extract, 0.02% colloidal chitin minimum medium containing 100 μg/mL ampicillin either in the presence or absence of IPTG and X-gal, incubated at 37°C for 3 days. Chitinase activity was observed by the formation of clear zone around the colonies on CCMM plate.

Mapping of recombination plasmid containing chitinase Gene

The map of the plasmid containing chitinase gene was constructed from single and double digestion of the plasmid with restriction enzyme *Bam*HI, *Eco*RI, *Hin*dIII, *Not*I, *Pst*I and *Sal*I using the manufacturers's recommended condition (New England Biolabs and Gibco BRL). DNA products were separated on 1.2 % agarose gel at 100 volts in TAE buffer. Standard marker λ/*Hin*dIII, 100 bp ladder and pBluescript SK^- linear form were used.

Construction of pPRChi65/PS

After mapping, 7 kb fragments of pPRChi65 was cut with *Sal*I to remove 2 kb DNA fragment. This was accomplished by digesting with *Sal*I to liberate the 2 kb fragment then, religated and retransformed to *E.coli* DH5α host cell, using 0.25 % yeast extract, 0.02% colloidal chitin minimum medium containing 100 μg/mL ampicillin in the absence of IPTG and X-gal, incubated at 37°C for 3 days and observed chitinase activity by the formation of clearing zone around the colonies on plate.

***Chi*65 gene regulation in *E.* coli.**

Plasmid pPRChi65 was extracted and retransformed into 3 *E.* coli strains to confirm chitinase activity and grown on colloidal chitin minimum medium plates containing 0.25 and 0.5 % yeast extract, LB, glucose and LB+glucose to study the expression of *Chi*65 in these cells.

All plates were incubated at 37°C for 7 days and chitinase activity was observed by the formation of clear zone around the colonies on the plate.

Orientation of C*hi*65 gene detected by PCR

Primers FPSK^{-} corporated with BP-I, BPVII, BP-VI and BP-VIII were used to define the orientation of *chi*65 gene in pPRChi65.

DNA Sequencing

Primers BP-II, BP-IX and M13 universal primers were used for sequencing by automate sequencer at Bioservice unit (BSU), Thailand.

Chitinase gene amplification

Primer BP-F and BP-R were used to amplify the full lenght chitinase gene from *Bacillus licheniformis* PR-1, *Bacillus licheniformis* SK-1, *Bacillus circulans* PP8, *Paenibacillus Compinasensis* (PT), *Bacillus ciculans* A11 (RB) and *Peanibacillus* (BT).

Analysis of *Chi*65 gene

Homology search of *Chi*65 gene using Basic Local Alignment Search tool (BLAST)

The sequence of *Chi*65 was analyzed, using BLAST program from GENBANK (Altschul *et al*., 1997). To search for homologes of *Chi*65 gene and protein in other bacteria.

DNA sequence manipulation

GENETYX-WIN version 3.1 was used to analyse *Chi*65 gene for open reading frame (ORF), translated amino acid sequence and calculated molecular weight including isoelectric point of the translate protein.

Partial characterization of crude chitinase from *E.coli* containing pPRChi65

Chitinase production of *Chi*65

Transformant harboring pPRChi65 was cultivated in minimum medium containing 0.02% colloidal chitin, 0.25% yeast extract and 100 μg/mL ampicillin at 37°C with 250 rpm rotary shaking for 8 days. Crude chitinase in cultured medium was collected everyday and assayed for chitinase activity by determination of reducing sugar.

Optimum pH

The effect of pH on chitinase activity was measured at different pH values by assay method described above using colloidal chitin as a substrate.

Optimum temperature

The effect of temperature on enzyme activity was measured at different temperatures described above using colloidal chitin as a substrate.

Substrate specificity

The activity of chitinase was assayed on chitin-related compounds and the reducing sugar was measured as described above, at optimum pH (0.1M citrate buffer pH 5.0) and optimum temperature (60°C).

Detection of hydrolytic products produced by chitinase from pPRChi65 and pPRChi60/PS by HPLC

All conditions were done under the same condition of crude chitinase from *B.licheniformis* PR-1 as described above.

CHAPTER III

RESULTS

Identification of a bacterium producing chitinase.

Bacteria strain PR-1, screened from Southeast-Asian soil, generated clear zones around colonies on 0.02% colloidal chitin minimum medium plate at 37 °C indicating chitinase production (Figure 5), has been sent to identify by biochemical characterization at TISTR. The identification was confirmed by 16S rRNA comparison method as described in chapter II.

Morphological and biochemical results from TISTR were listed in Table 7. This bacteria was a gram positive bacilli which formed subterminal ellipsoidal endospores. This bacteria grew both aerobically and anaerobically on LB agar. The colonies on nutrient agar were reddish-brown in colour and had a rough surface with hair-like outgrowths. It was classification in *Bacillus* group BII (Priest, 1988), from these results, bacteria strain PR-1 was identified as *Bacillus licheniformis*.

The amplified 16S rRNA product analyzed on agarose gel electrophoresis showed a single band of PCR product, size 1.5 kb compared with 100 bp marker (Figure 7), when religated to pGEM®-T Easy vector and sequenced using pA, pD and pH' (Ulrike *et al*, 1989) as primer. The results showed that the amplified fragment was 1522 bp in length (Figure 8). By using BLAST program, we found that bacteria strain PR-1 has 99% homology with 16S rRNA of *Bacillus licheniformis* M1-1 (AB039328) 1549 bp length.

From these resulted we conclude that the bacteria strain PR-1 was *B. licheniformis*.

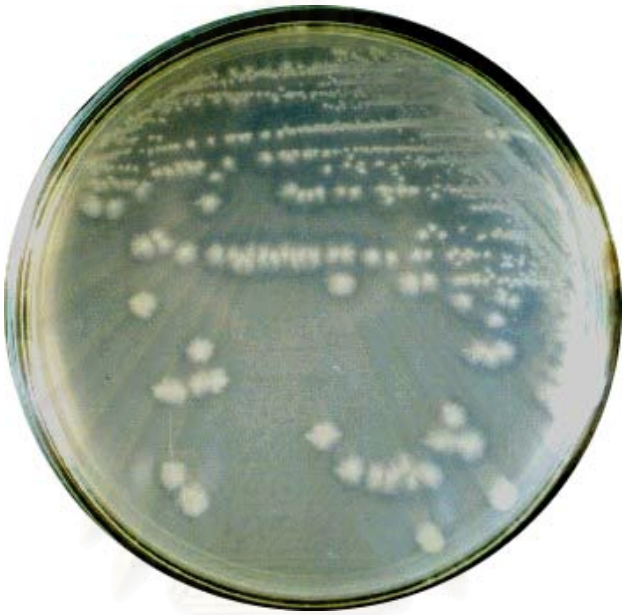


Figure 5 Colony morphology of bacteria strain PR-1 on colloidal chitin minimum medium plate

Bacillus licheniformis PR-1 was grown on minimum medium supplemented with 0.02% colloidal chitin. The culture was grown for 3 days at 37 ^{o}C.

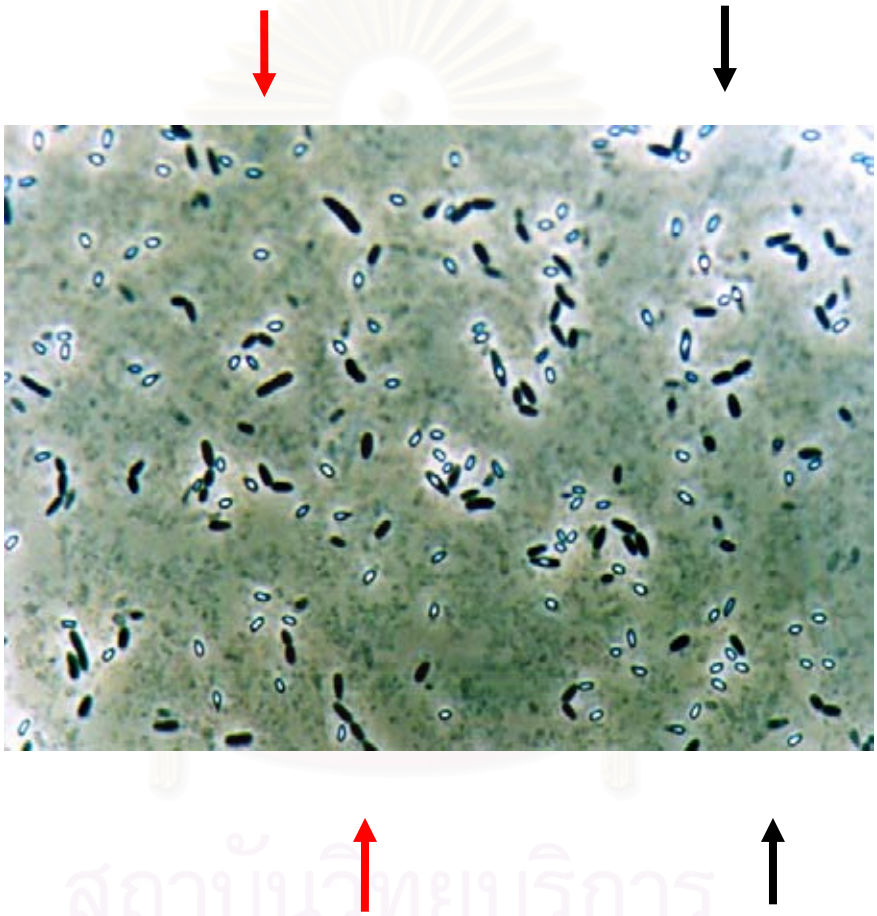


Figure 6 **Cell morphology of the bacteria strain PR-1** (x100 objective, phase-contrast microscope).
The black arrow indicated spore of these bacteria.
The red arrow indicated bacterial shape.

Table 7 Biochemical characteristics of *Bacillus licheniformis* PR-1

Characteristics	Reaction
Gram reaction	+ve
Fermentative production of acid from :	
- glycerol	+
- erythritol	-
- D-arabinose	-
- L-arabinose	+
- ribose	+
- D-xylose	+
- L-xylose	-
- adonitol	-
- β-methyl-D-xyloside	-
- galactose	-
- D-glucose	+
- D-fructose	+
- D-mannose	+
- L-sorbose	-
- rhamnose	-
- dulcitose	-
- inositol	-
- mannitol	+
- sorbitol	-
- α-methyl-D-mannoside	-
- α-methyl-D-glucoside	+
- N-acetyl-glucosamine	+
- amygdaline	+
- arbutine	+
- esculin	+

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

Table 7 (continued)

Characteristics	Reaction
Fermentative production of acid from : (continued)	
- salicine	+
- cellobiose	+
- maltose	+
- lactose	-
- melibiose	-
- sucrose	+
- trehalose	+
- inuline	-
- melezitose	-
- D-raffinose	-
- Starch	+
- Glycogene	-
- Xylitol	-
- β-gentiobiose	-
- D-turanose	+
- D-lyxose	-
- D-tagatose	+
- D-fucose	-
- L-fucose	-
- D-arbitol	-
- L-arabitol	-
- Gluconate	+
- 2-keto-gluconate	-
- 5-keto-gluconate	-

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

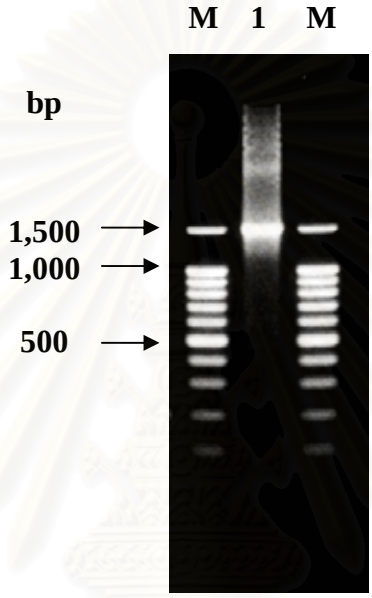


Figure 7 16S rRNA amplification product from *Bacillus licheniformis* PR-1

Lane M = 100 bp marker

Lane 1 = 16S rRNA PCR products using genomic DNA of *Bacillus licheniformis* PR-1 as a template

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                                        AGAGTTTGAT  CCTGGCTCAG-----
GCGAACGCTG  GCGGCGTGCC  TAATACATGC  AAGTCGAGCG  GACCGACGGG     50
AGCTTGCTCC  CTTAGGTCAG  CGGCGGACGG  GTGAGTAACA  CGTGGGTAAC    100
CTGCCTGTAA  GACTGGGATA  ACTCCGGGAA  ACCGGGGCTA  ATACCGGATG    150
CTTGATTGAA  CCGCATGGTT  CAATCATAAA  AGGTGGCTTT  TAGCTACCAC    200
TTGCAGATGG  ACCCGCGGCG  CATTAGCTAG  TTGGTGAGGT  AACGGCTCAC    250
CAAGGCGACG  ATGCGTAGCC  GACCTGAGAG  GGTGATCGGC  CACACTGGGA    300
CTGAGACACG  GCCCAGACTC  CTACGGGAGG  CAGCAGTAGG  GAATCTTCCG    350
CAATGGACGA  AAGTCTGACG  GAGCAACGCC  GCGTGAGTGA  TGAAGGTTTT    400
CGGATCGTAA  AACTCTGTTG  TTAGGGAAGA  ACAAGTACCG  TTCGAATAGG    450
GCGGTACCTT  GACGGTACCT  AACCAGAAAG  CCACGGCTAA  CTACGTGCCA    500
GCAGCCGCGG  TAATACGTAG  TGTGGCAAGC  GTTGTCCGGA  ATTATTGGGC    550
GTAAAGCGCG  CGCAGGCGGT  TTCTTAAGTC  TGATGTGAAA  GCCCCCGGCT    600
CAACCGGGGA  GGGTCATTGG  AAACTGGGGA  ACTTGAGTGC  AGAAGAGGAG    650
AGTGGAATTC  CACGTGTAGC  GGTGAAATGC  GTAGAGATGT  GGAGGAACAC    700
CAGTGGCGAA  GGCGACTCTC  TGGTCTGTAA  CTGACGCTGA  GGCGCGAAAG    750
CGTGGGGAGC  GAACAGGATT  AGATACCCTG  GTAGTCCACG  CCGTAAACGA    800
TGAGTGCTAA  GTGTTAGAGG  GTTTCCGCCC  TTTAGTGCTG  CAGCAAACGC    850
ATTAAGCACT  CCGCCTGGGG  AGTACGGTCG  CAAGACTGAA  ACTCAAAGGA    900
ATTGACGGGG  GCCCGCACAA  GCGGTGGAGC  ATGTGGTTTA  ATTCGAAGCA    950
ACGCGAAGAA  CCTTACCAGG  TCTTGACATC  CTCTGACAAC  CCTAGAGATA   1000
GGGCTTCCCC  TTCGGGGGCA  GAGTGACAGG  TGGTGCATGG  TTGTCGTCAG   1050
CTCGTGTCGT  GAGATGTTGG  GTTAAGTCCC  GCAACGAGCG  CAACCCTTGA   1100
TCTTAGTTGC  CAGCATTCAG  TTGGGCACTC  TAAGGTGACT  GCCGGTGACA   1150
AACCGGAGGA  AGGTGGGGAT  GACGTCAAAT  CATCATGCCC  CTTATGACCT   1200
GGGCTACACA  CGTGCTACAA  TGGGCAGAAC  AAAGGGCAGC  GAAGCCGCGA   1250
GGCTAAGCCA  ATCCCACAAA  TCTGTTCTCA  GTTCGGATCG  CAGTCTGCAA   1300
CTCGACTGCG  TGAAGCTGGA  ATCGCTAGTA  ATCGCGGATC  AGCATGCCGC   1350
GGTGAATACG  TTCCCGGGCC  TTGTACACAC  CGCCCGTCAC  ACCACGAGAG   1400
TTTGTAACAC  CCGAAGTCGG  TGAGGTAACC  TTTTGGAGCC  AGCCGCCGAA   1450
GGTGGGACAG  ATGATTGGGG  TGAAGTCGTA  ACAAGGTAGC  CGTATCGGAA   1500
GGTGCGGCTG  GATCACCTCC  TT                               1522
```

```
pA   5'   AGAGTTTGATCCTGGCTCAG     3'
pD   5'   CAGCAGCCGCGGTAATAC       3'
pF   5'   CATGGCTGTCGTCAGCTGGT     3'
pH   5'   TGCGGCTGGATCACCTCCTT     3'
```

Figure 8 **Nucleotide sequence of 16S rRNA of *Bacillus licheniformis* PR-1**

The 16S rRNA amplified was sequenced. The result 1,522 bp is shown above. The primer used for sequencing is shown in colour.

Figure 9 Nucleotide sequence alignment of 16S rRNA of *Bacillus licheniformis* PR-1 compare with *Bacillus licheniformis* M1-1

Pink colour indicates nucleotides that are different or missing from *B. licheniformis* PR-1 when compared to *B. licheniformis* M1-1.

Primers for amplification of partial chitinase gene.

The first set of primers used for partial chitinase gene amplification were degenerated primers designed from the conserved N-terminal amino acid sequence of familly 18 chitinase from *B.circulans* chi41, *B.licheniformis* TP, *B.subtilis*, *B.circulans* chi1, *B.circulans* A1, *B.thuringiensis* and *B.cereus* chiB (Figure 10).

We found at least 6 conserved regions in the chitinase gene, which were suitable for designing primers. We chose 3 regions to design the first set of primers (BP-I, BP-II, BP-V and BP-VI). To convert the amino acid sequence into genetic codon, codon usaged of amino acid from *Bacillus spp.* was used (Figure 11).

The product of PCR, when primer BP-I+BP-V and genomic DNA of *B. licheniformis* PR-1 was used in the reaction, was 400 bp in length. When primers BP-II +BP-VI was used a DNA fragment around 280 bp in length was found and after using primers BP-I+BP-VI a DNA fragment around 700 bp in length was found(Figure 13).

The approximately 700 bp PCR product was sequenced and analysed by BLAST program. We found from DNA sequencing that the exact length of the DNA fragment was 635 bp and has high similarity with chitinase gene from *Bacillus spp.* (Figure 14).

CLUSTAL W (1.81) multiple sequence alignment

```
B.circulans chi41       ---MNIVLVNKSKKFFVFSFIFVMLLSLS-FVNGEVAKADSGK-NYKIIGYYPSWGAYGR     55
B.licheniformis TP      ---MNIVLVNKSKKFFVFSFIFVMLLSLS-FVNGEVAKADSGK-NYKIIGYYPSWGAYGR     55
B.subtilis              ---MKKVFSN--KKFLVFSFIFAMILSLS-FFNGESAKASSDK-SYKIIGYYPSWGAYGR     53
B.circulans chi1        MLYKAKHMDFKKAGKVLLGLVLLLSVIIP-SFTIQSRTAEAAD-AYKIVGYYPAWAAYGR     58
B.circulans A1          MINLNKHTAFKKTAKFFLGLSLLLSVIVP-SFALQPATAEAAD-SYKIVGYYPSWAAYGR     58
B.thuringiensis         -----MAMRSQKFTLLLLSLLLFLPLFLTNFITPNLALADSPKQSQKIVGYFPSWGVYGR     55
B.cereus chiB           -------MRSQKFTLLLLSLLLFLPLFLTNFINPNLALADSPKQSQKIVGYFPSWGVYGR     53
                                ..:.: : : : :.  .  :   *.: .   **:**:*:*..***

                                          BP-I

B.circulans chi41       NFQVWDMDVSKVSHINYAFADICWEGRHGNP--DPTGPNPQTWSCQD--ENG-VIDAPNG     110
B.licheniformis TP      NFQVWDMDVSKVSHINYAFADICWEGRHGNP--DPTGPNPQTWSCQD--ENG-VIDAPNG     110
B.subtilis              DFQVWDMDASKVSHINYAFADICWEGRHGNP--DPTGPNPQTWSCQD--ENG-VIDVPNG     108
B.circulans chi1        NYNVTDIDPTKVTHINYAFADICWNGIHGNP--DPSGPNPVTWSCQN--EKSQTINVPNG     114
B.circulans A1          NYNVADIDPTKVTHINYAFADICWNGIHGNP--DPSGPNPVTWTCQN--EKSQTINVPNG     114
B.thuringiensis         NYQVADIDASKLTHLNYAFADICWNGKHGNPSTHPDNPNKQTWNCKESGVPLQNKEVPNG     115
B.cereus chiB           NYQVADIDASKLTHLNYAFADICWNGKHGNPSTHPDNPNKQTWNCKESGVPLQNKEVPNG     113
                        :::* *:* :*::*:*********:* ****  .* .**  **.*::       :.***
B.circulans chi41       TIVMGDPWIDAQKSNP--GDVWDEP----IRGNFKQLLKLKKSHPHLKTFISVGGWTWSN     164
B.licheniformis TP      TIVMGDPWIDAQKSNP--GDVWDEP----IRGNFKQLLKLKKSHPHLKTFISVGGWTWSN     164
B.subtilis              SIVMGDPWIDVQKSNA--GDTWDEP----IRGNFKQLLKLKKNHPHLKTFISVGGWSWSN     162
B.circulans chi1        TIVLGDPWIDTGKQFA--GDTWDQP----YAGNINQLNKLKQVNPNLKTIISIGGWTWSN     168
B.circulans A1          TIVLGDPWIDTGKTFA--GDTWDQP----IAGNINQLNKLKQTNPNLKTIISVGGWTWSN     168
B.thuringiensis         TLVLGEPWADVTKSYPVSGTTWEDCDKYARCGNFGELKRLKAKYPHLKTIISVGGWTWSN     175
B.cereus chiB           TLVLGEPWADVTKSYPGSGTTWEDCDKYARCGNFGELKRLKAKYPHLKTIISVGGWTWSN     173
                        ::*:*:** *. *  .  * .*::      **: :* :**   *:***:**:***:***

                                    BP-II, BP-V

B.circulans chi41       RFSDVAADPVARGNFAASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLL     224
B.licheniformis TP      RFSDVAADPVARGNFAASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLL     224
B.subtilis              RFSDVAADPAARENFAASAVNFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLL     222
B.circulans chi1        RFSDVAATSATREVFANSAVDFLRKYNFDGVDLNWEYPVSGGLDGNSKRPEDKQNYTLLL     228
B.circulans A1          RFSDVAATAATREVFANSAVDFLRKYNFDGVDLDWEYPVSGGLDGNSKRPEDKQNYTLLL     228
B.thuringiensis         RFSDMAADEKTRKVFAESTVAFLRAYGFDGVDLDWEYPGVETIPGGSYRPEDKQNFTLLL     235
B.cereus chiB           RFSDMAADEKTRKVFAESTVAFLRAYGFDGVDLDWEYPGVETIPGGSYRPEDKQNFTLLL     233
                        ****:**   :*  ** *:* *** *.******:****    : *.* *****:*:****

                                                          BP-VI

B.circulans chi41       QECVKNLMLQKQRTAREYLLTIASGASPEYVSNTELDKIAQTVDWINIMTYDFNGGWQSI     284
B.licheniformis TP      QEVRKKLDAAEAKDGKEYLLTIASGASDRYVSNTELDKIAQTVDWINIMTYDFNGGWQSI     284
B.subtilis              QDVREKLDAAEAKDGKKYLLTTVSGASPEYVSNTELDKIAETVDWINIMTYDFNGGWQSI     282
B.circulans chi1        SKIREKLDAAEAVDGKEYLLTIASGASPTYAANTELANIASIVDWINIMTYDFNGAWQKI     288
B.circulans A1          SKIREKLDAAGAVDGKKYLLTIASGASATYAANTELAKIAAIVDWINIMTYDFNGAWQKI     288
B.thuringiensis         QDVRNALNKAGAEDGKQYLLTIASGASQRYADHTELKKISQILDWINIMTYDFHGGWEAT     295
B.cereus chiB           QDVRNALNKAGAEDGKQYLLTIASGASQRYADHTELKKISQILDWINIMTYDFHGGWEAT     293
                        ..  : *       .::**** .****  *. :*** :*:  :**********:*.*:
B.circulans chi41       SAHNAPLFYDPKAKEAGVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSGCESG     344
B.licheniformis TP      SAHNAALFYDPKAKEAGVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYEGAGAVVNPA     344
B.subtilis              SAHNAPLFYDPKAKEAGVPNAETFNIESTVKRYKEAGVKADKLVLGTPFYGRGWSNCEPA     342
B.circulans chi1        SAHNAPLNADPAAASAGVPDSNTFNVAAGAQGHLNAGVPAAKLVLGVPFYGRGWDGCAQA     348
B.circulans A1          SAHNAPLNYDPAASAAGVPDANTFNVAAGAQGHLDAGVPAAKLVLGVPFYGRGWDGCAQA     348
B.thuringiensis         SNHNAALYKDPNDPAANTN----FYVDGAINVYTNEGVPVDKLVLGVPFYGRGWKSCGKE     351
B.cereus chiB           SNHNAALYKDPNDPAANTN----FHVDGAINVYTNEGVPVDKLVLGVPFYGRGWKSCGKE     349
                        * ***.*  **   *..    : :  : : : **   *****.***  .
B.circulans chi41       GHGEYQKC------GPAKEGTWE------NGVFDFSDLEKNYVNQNGYKRYWNDQAKVPF     392
B.licheniformis TP      ATENIRSA------DRRKKGRGK------MEYSTFQILKRTYVNQNGYKRYWNDQAKVPF     392
B.subtilis              DNGEYQKC------GPAKEGTWE------KGVFDFSDLEKNYINKNGYKRYWNDRAKVPF     390
B.circulans chi1        NNGQYQTCS-----GGSSIGTWE------AGSFDFYDLEANYINKNGYTRYWNDTAKVPF     397
B.circulans A1          GNGQYQTCT-----GGSSVGTWE------AGSFDFYDLEANYINKNGYTRYWNDTAKVPY     397
B.thuringiensis         NNGQYQPCKPGSDGKLASKGTWDDYSTGDTGVYDYGDLAANYVNKNGFVRYWNDTAKVPY     411
B.cereus chiB           NNGQYQPCKPGSDGKLASKGTWDDYSTGDTGVYDYGDLAANYVNKNGFVRYWNDTAKVPY     409
                           : : .       . *   .           :  *  .*:*:**: ***** ****:
B.circulans chi41       LYNAENGNFITYDDEQSFGHKTDFIKANGLSGAMFWDFSGDS---------NPTLLNKLA     443
B.licheniformis TP      LYNAENGNFITYDDEQSFGHKTDFIKANGLSGAMFWDFSGDS---------NRTLLNKLA     443
B.subtilis              LYNAENGNFITYDDEESYGYKTDLIQSNGLSGAMFWDFSGDS---------NQTLLNKLA     441
B.circulans chi1        LYNASNKRFISYDDAESIGHKTAYIKSKGLGGAMFWELSGDR---------NKTLQNKLK     448
B.circulans A1          LYNASNKRFISYDDAESVGYKTAYIKSKGLGGAMFWELSGDR---------NKTLQNKLK     448
B.thuringiensis         LYNATTGTFISYDDNESMKYKTDYIKTKGLSGAMFWELSGDCRTSPKYSCSGPKLLDTLV     471
B.cereus chiB           LYNATTGTFISYDDNESMKYKTDYIKTKGLNGAMFWELSGDCRTSPKYSCSGPKLLDTLV     469
                        **** .  **:*** :*  :**  *:::**.*****::***          . .* :.*
```

```
B.circulans chi41      AEFKFCTR-------------------------------------------------     451
B.licheniformis TP     ADLDFAPDGGNPEPPS-SAPVNVRVTGKTATSVSLAWDAASSGTNITEYVVSF-ESRSIS     501
B.subtilis             ADLGFAPGGGNPEPPS-SAPDNLRVTEKTATSISLAWDAPSDGANIAEYVLSY-EGGAVS     499
B.circulans chi1       SDLSTGGTVPPTDTTAPSVPGNARSTGVTASSVTLAWNASTDNVGVTGYTVYNGTSLVTS     508
B.circulans A1         ADLPTGGTVPPVDTTAPSVPGNARSTGVTANSVTLAWNASTDNVGVTGYNVYNGANLATS     508
B.thuringiensis        KEL-LGGPINQKDTEPPTNVKNIVVTNKNSNSVQLNWTASTDNVGVTEYEITAGEEK-WS     529
B.cereus chiB          KEL-LGGPISQKDTEPPTNVKNIVVTNKNSNSVQLNWTVSTDNVGVTEYEITAGEEK-WS     527
                        ::

B.circulans chi41      ----TEAIRNRLHPHLWNVLVIRKNCY-----------------------KCQPGVGCA     483
B.licheniformis TP     VKETSAEIGNLNRGTAYSFTVSAKDADGEL---------------------HTGPTVKVT     540
B.subtilis             VKDTSATIGQLKPNTTYSFTVSAKDADGKL---------------------HTGPTIEAA     538
B.circulans chi1       VTGTTATISGLAPGTSYTFTVKAKDAAGNLSAASNSLTVSTTVQPGGDTQAPTVPTNLTS     568
B.circulans A1         VTGTTATISGLTAGTSYTFTIKAKDAAGNLSAASNAVTVSTTAQPGGDTQAPTAPTNLAS     568
B.thuringiensis        TTTNSITIKNLKPNTEYTFSIIAKDAAGNK---------------------SQPTALTVK     568
B.cereus chiB          TTTNSITIKNLKPNTEYKFSVIAKDAAGNK---------------------SQPTALTVK     566
                           :  *       :.. :  *:.                           .

B.circulans chi41      E------------QRNKHYGIC-RVI---------------------------------     496
B.licheniformis TP     TNSDQACSYDEWKETNAYTGGE-RVAFNGKVYEAKWWTKGDRRINPVNGAYGGWSEAANN     599
B.subtilis             TNSDQTCGYNEWKDTAVYTGGD-RVVFNGKVYEAKWWTKGEQPDQ--AGESGVWKLIGDC     595
B.circulans chi1       TAKTSSTITLSWAASTDNVGVIGYEVYNGTALVTTVSGTSATVTGLTADTSYTFTVKAKD     628
B.circulans A1         TAQTTSSITLSWTASTDNVGVTGYDVYNGTALATTVTGTTATISGLAADTSYTFTVKAKD     628
B.thuringiensis        TDEANTTPPDGNGTATFSVTSNWGSGYNFSIIIKNNGTTPIKNWKLEFDYSGNLTQVWDS     628
B.cereus chiB          TDEANTTPPDGNGTATFSVTSNWGSGYNFSIIIKNNGTTPIKNWKLEFDYSGNLTQVWDS     626


B.circulans chi41      ------------------------------------------------------------
B.licheniformis TP     RKSNG--------------------------------------------------------     604
B.subtilis             K--------------------------------------------------------     596
B.circulans chi1       AAGNLSAASSALTVKTEVGTTNPGVSAWQANTAYVVGQLVTYNGKTYKCLQSHTFLTGWE     688
B.circulans A1         AAGNVSAASNAVSVKTAAETTNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWE     688
B.thuringiensis        KISSKTNNHYVITNAGWNGEIPSGGSITIGGAG---------TGNPAELLNAVISEN---     676
B.cereus chiB          KISSKTNNHYVITNAGWNGEIPPGGSITIGGAG---------TGNPAELLNAVISEN---     674


B.circulans chi41      ----------------------------
B.licheniformis TP     ----------------------------
B.subtilis             ----------------------------
B.circulans chi1       PSNVAALWELQPYIPVKDDTSDKLGSVFM      717
B.circulans A1         PSNVPALWQLQ------------------      699
B.thuringiensis        ----------------------------
B.cereus chiB          ----------------------------
```

Figure 10 Amino acids sequences alignment of chitinase gene from *B.circulans* chi41, *B.licheniformis* TP, *B.subtilis*, *B.circulans* chi1, *B.circulans* A1, *B.thuringiensis* and *B.cereus* chiB.

The asterisks indicated identical residues, colon indicated that very similar residues and dot indicated similar residues. The blue colour highlight indicated amino acids used to design primer.

Amino acid sequence, which used for designs primer;

NYAFADICW (E/N) G

[Asn Tyr Ala Phe Ala Asp Ile Cys Trp (Glu/Asn) Gly]

Codon usage of *bacillus* group (*B.circulans* chi41, *B.licheniformis* TP, *B.subtilis*, *B.circulans* chi1, *B.circulans* A1, *B.thuringiensis* and *B.cereus* chiB)

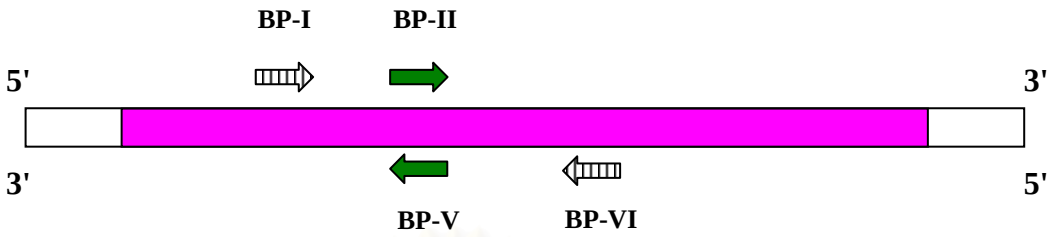
Asn	Tyr	Ala	Phe	Ala	Asp	Ile	Cys	Trp	Glu / Asn	Gly
AAT	TAT	GCT	TTT	GCT	GAT	ATT	TGT	TGG	GAA	GGT
AAC	TAC	GCA	TTC	GCA	GAC	ATC	TGC		GAG	GGC
		GCG		GCG		ATA			AAT	GGA
									AAC	GGG

Nucleotide sequence of primer (5'⟶3')

AAY TAY GCD TTY GCD GAY ATH TGY TGG RAN GG

Abbreviations ; **Y** = C,T **R** = A,G **D** = A,G,T
H = A,C,T **N** = A,C,G,T

Figure 11 Flow chart for degenerated primer designed for amplified partial chitinase gene.



Forward primers ;

BP-I ; 5′ NYAFADICW(E/N)G 3′

5' AAYTAYGCDTTYGCDGAYATHTGYTGGRANGG 3' **(32 mers)**

BP-II; 5′ FDGVDLDWEY 3′

5' TTYGAYGGNGTNGAYYTNGAYTGGGARTA 3' **(29 mers)**

Reverse primers ;

BP-V ; 5′ FDGVDLDWEY 3′

5' RTAYTCCCARTCNARRTCNACNCCRTCRAA 3' **(30 mers)**

BP-VI; 5′ NIMTYDFNGGW 3′

5' CCANCCNCCRTTRAARTCRTANGTCATGATRTT 3' **(33 mers)**

Abbreviations ; Y = C,T R = A,G D = A,G,T
H = A,C,T N = A,C,G,T

Figure 12 First set of primers designed for amplified partial chitinase gene

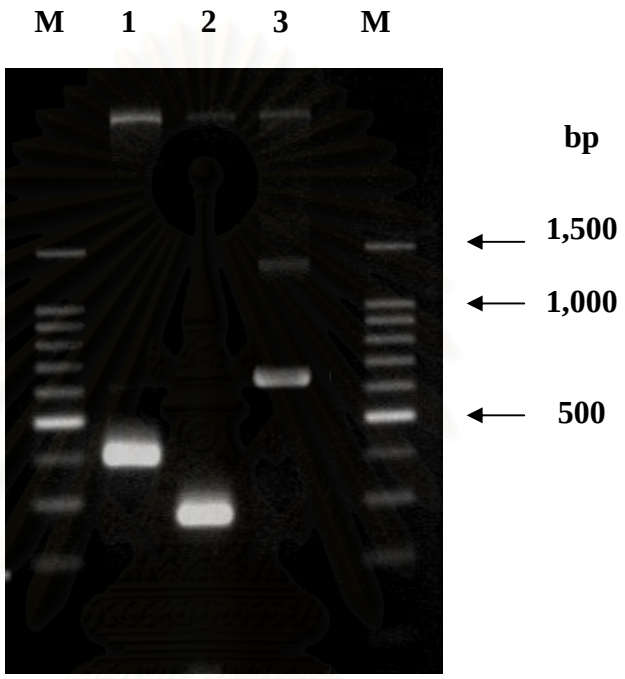


Figure 13 Partial chitinase gene fragments from PCR

Lane M = 100 bp marker,

Lane 1 = PCR products when using primer BP-I incorporating BP-V,

Lane 2 = PCR products when using primer BP-II incorporating BP-VI

Lane 3 = PCR products when using primer BP-I incorporating BP-VI

(All primers were using genomic DNA of *Bacillus licheniformis* PR-1 as template)

Sequence 635 BP; 178 A; 142 C; 133 T; 182 G; 0 other

```
TAACTATGCG TTTGCGGATA TTTGTTGGGA AGGAAGGCAT GGGAACCCTG ATCCGACAGG  60
CCCCAATCCT CAAACGTGGT CATGCCAGGA TGAAAACGGA GTAGTCGACG CGCCAAATGG 120
AACAATCGTG ATGGGCGATC CCTGGATTGA CGCACAAAAG GCAAATCCCG GGGATGTCTG 180
GGATGAACCG ATCCGCGGCA ACTTTAAACA ATTGTTGAAG CTGAAAAAGA GCCACCCTCA 240
TTTGAAAACG TTCATATCGG TCGGGGGGTG GACTTGGTCT AACCGCTTTT CAGATGTCGC 300
GGCAGATCCT GCGGCAAGGG AGAATTTCGC CGCTTCGGCC GTTGAGTTTT TAAGGAAATA 360
CGGGTTTGAC GGGGTCGATC TTGACTGGGA ATATCCGGTC AGCGGAGGAT TGCCGGGGAA 420
CAGCACACGT CCGGAAGATA AAAGAAACTA CACGCTGCTC CTGCAAGAGG TGCGCAAAAA 480
ACTTGACGCT GCAGAAGCAA AAGACGGCAA GGAATACTTG CTGACGATCG CATCCGGCGC 540
AAGTCCCGAT TATGTAAGCA ACACTGAGCT CGATAAAATC GCTCAAACCG TGGATTGGAT 600
TAACATCATG ACCTATGACT TCAATGGGGG TTGGA                             635
```

BLASTN 2.1.3 [Apr-11-2001]

RID: 990094116-16454-7662

Query=(635 letters)

Database: nt 861,799 sequences; 3,247,139,289 total letters

Distribution of 27 Blast Hits on the Query Sequence

Sequences producing significant alignments:	Score (bits)	E Value
gi|1845336|gb|U71214.1|BLU71214 Bacillus licheniformis TP c...	965	0.0
gi|6683783|gb|AF154827.1|AF154827 Bacillus circulans chitin...	959	0.0
gi|3193264|gb|AF069131.1|AF069131 Bacillus subtilis chitina...	474	e-131

Sequence 211 aa; 18 A; 2 C; 19 G; 11 T; 161 other;

```
NYAFADICWE GRHGNPDPTG PNPQTWSCQD ENGVVDAPNG TIVMGDPWID AQKANPGDVW  60
DEPIRGNFKQ LLKLKKSHPH LKTFISVGGW TWSNRFSDVA ADPAARENFA ASAVEFLRKY 120
GFDGVDLDWE YPVSGGLPGN STRPEDKRNY TLLLQEVRKK LDAAEAKDGK EYLLTIASGA 180
SPDYVSNTEL DKIAQTVDWI NIMTYDFNGGW                                 211
```

BLASTP 2.1.3 [Apr-11-2001]

RID: 990093224-8841-26794

Query= (211 letters)

Database: nr 689,446 sequences; 217,131,788 total letters

Distribution of 334 Blast Hits on the Query Sequence

Sequences producing significant alignments:	Score (bits)	E Value
gi|1845337|gb|AAB47847.1| (U71214) chitinase [Bacillus lich...	386	e-106
gi|6683784|gb|AAF23368.1|AF154827_1 (AF154827) chitinase [B...	376	e-103
gi|3193265|gb|AAC23715.1| (AF069131) chitinase [Bacillus su...	369	e-101

Figure 14 Nucleotide sequence of 635 bp and BLAST results of nucleotide and amino acid sequence partial chitinase gene

The sequence submitted to BLAST and the first three sequences with the highest scores are shown.

Partial characterization of crude chitinase from *B. licheniformis* PR-1

Enzyme production

The activity of crude chitinase from cultured medium was determination as described in chapter II. The level of chitinase activity in the culture medium assayed during a 8-day cultivation period. *B. licheniformis* PR-1 chitinase activity was detected after 2 days of cultivation

When 0.02% colloidal chitin minimum medium with 0.05% yeast extract was used, level of chitinase activity was ranging from 2 to 20 mU/mL and the highest chitinase activity found at eighth day (Figure 15.A).

When 0.02% colloidal chitin minimum medium with 0.25% yeast extract was used, level of chitinase activity was ranging from 3 to 36 mU/mL and the highest chitinase activity found at the fifth day. The activity decreased drastically often the 5^{th} day (Figure 15.B).

Optimum pH

The optimum pH of crude chitinase was measured in 0.1 M buffer at pH range of 3-10. Crude chitinase showed highest chitinase activity in citrate buffer pH 5.0. Reletive activities at different pH were shown in Figure 16.

Optimum Temperature

The optimum temperature of crude chitinase was measured in pH 5.0 at the range of 30-80 °C. Crude chitinase showed highest chitinase activity at 70 °C. Reletive activities at different temperature were shown in Figure 17.

Substrate Specificity

The activity of chitinase was examined at pH 5.0, 70 °C on chitin and its related compounds, chitinase was able to hydrolyze colloidal chitin (from shrimp shell) very well followed by powder chitin, 80% deactylation chitosan, flake chitin and regenerated chitin, respectively (Figure 18).

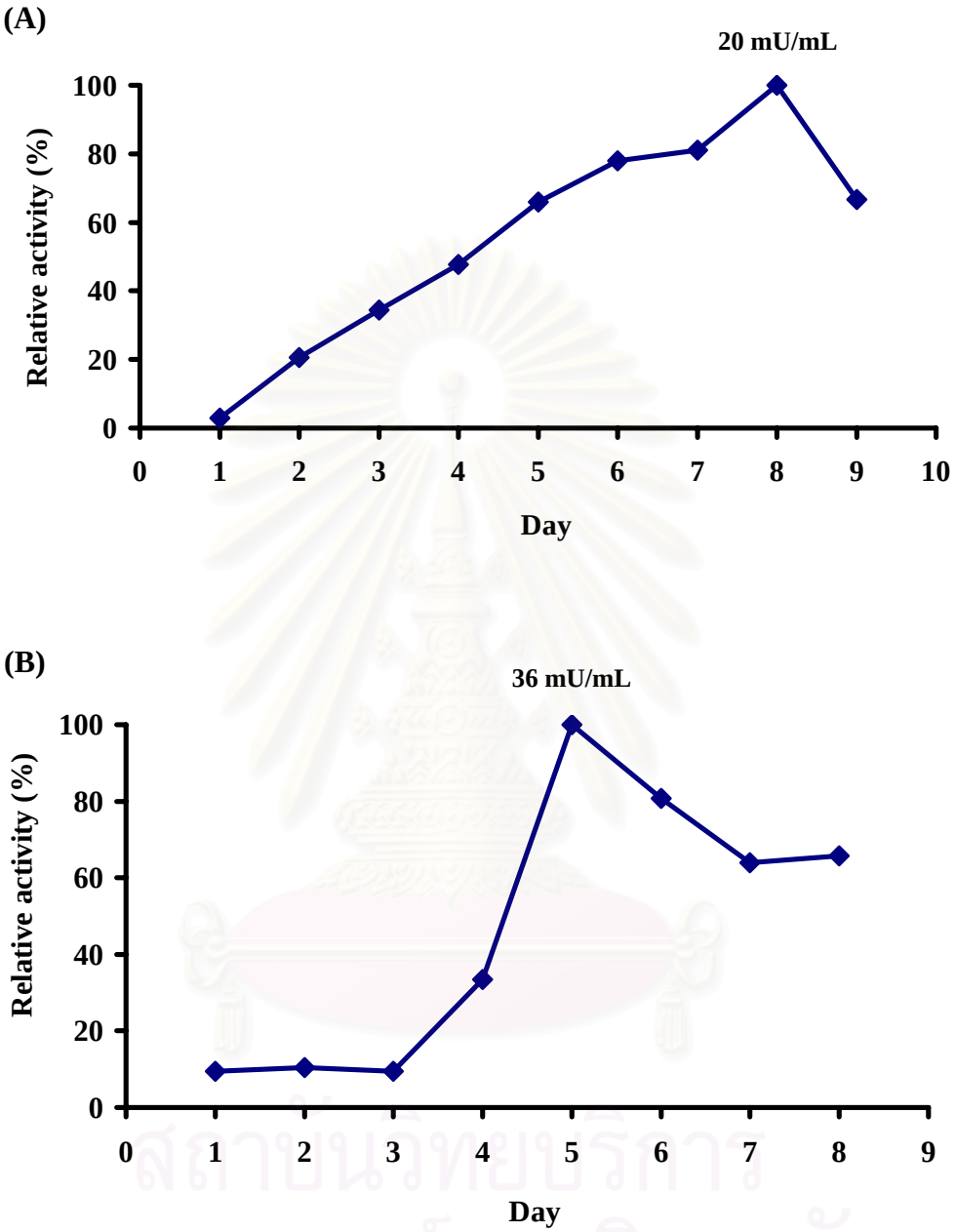


Figure 15 Profile of chitinase production from *Bacillus licheniformis* PR-1 by colourimetric method using colloidal chitin as a sustrate

(A) = Profile of chitinase production from *B. licheniformis* PR-1 in 0.05 % yeast extract colloidal chitin minimum medium.

(B) = Profile of chitinase production from *B. licheniformis* PR-1 in 0.25 % yeast extract colloidal chitin minimum medium.

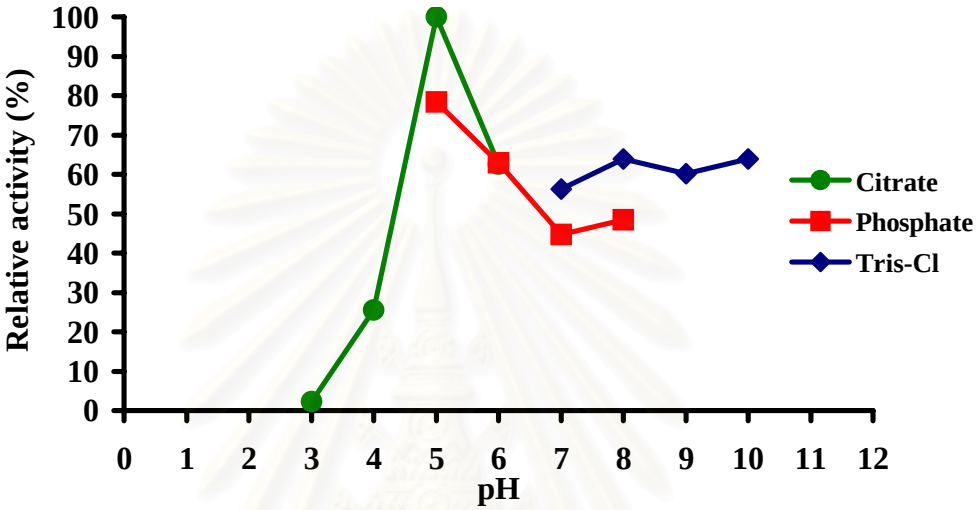


Figure 16 Effect of pH on crude chitinase from *Bacillus licheniformis* PR-1 by colourimetric method

Enzymes activities were measured at pH range from 3-10, using colloidal chitn as a substrate.

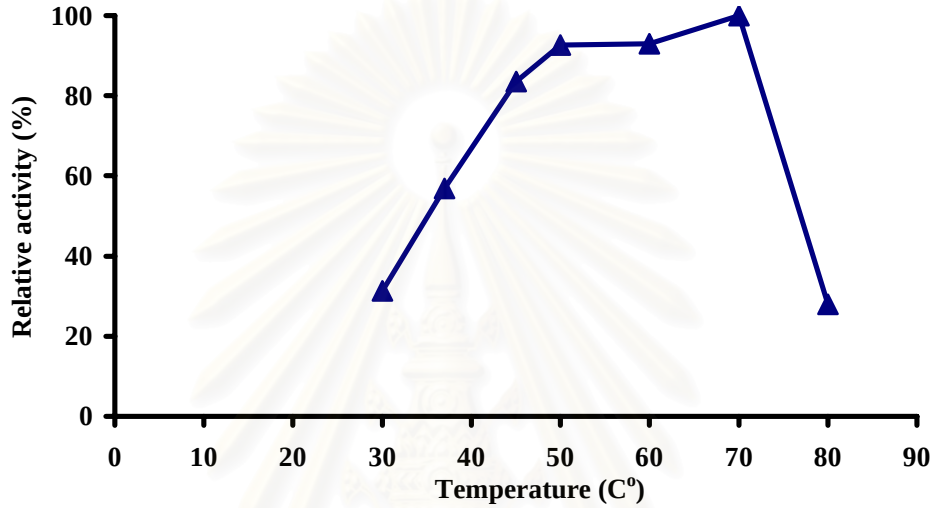


Figure 17 Effect of temperature on crude chitinase from *Bacillus licheniformis* PR-1 by colourimetric method

Enzymes activities were measure at 30, 37, 45, 50, 60, 70 and 80 ^{o}C in citrate buffer pH 5.0 using colloidal chitin as a substrate.

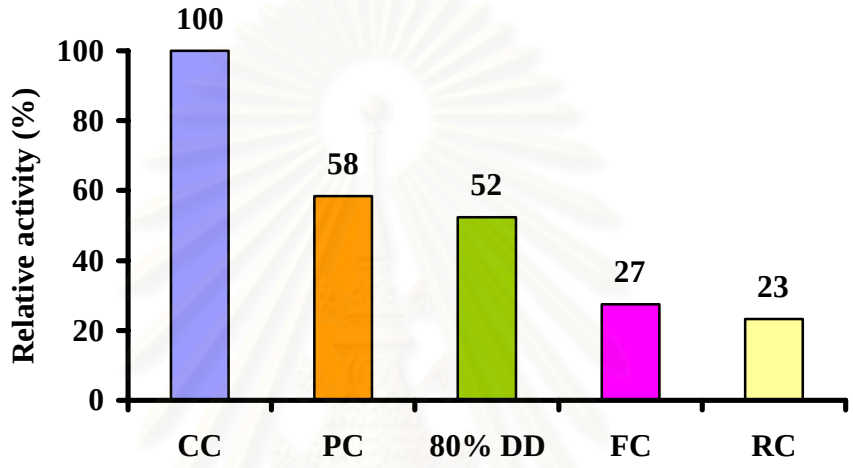


Figure 18 Substrate specificity of crude chitinase from *Bacillus licheniformis* PR-1 by colourimetric method

Enzymes activities were measure at 70 ^{o}C in citrate buffer pH 5.0 using colloidal chitin (CC), powder chitin (PC), 80% deactylase chitosan (80% DD), flake chitin (FC) and regenerated chitn (RC) as substrates.

Estimation of molecular weight of chitinase by SDS-PAGE

A concentrated crude chitinase from culture supernatant gave at least 3 chitinolytic bands with calculated molecular mass of 70, 65 and 58 kDa, respectively (Figure 19).

Detection of products produced by chitinase from *B. licheniformis* PR-1

Hydrolutic products produced by chitinase from *B. licheniformis* PR-1 were separated by HPLC (Figure 20). The major products was $(GlcNAc)_2$, with some GlcNAc.

Cloning of chitinase gene

Library construction and recombinant DNA construction

DNA fragments containing chitinase gene were prepared by partial *PstI* digestion, fragments approximately 2-6 kb in length were collected to construct DNA library in pBluescript SK^- cut with *Pst*I, dephosphorelated by Calf Intestinal Alkaline Phosphatase (CIAP).

Detection of chitinase gene (phenotype screening)

After transformation, 2 from 8,000 transformants gave clear zone around the colonies on screening medium after cultivation for 5 days (Figure 21). Suggesting that the cells may contain a plasmid carrying a chitinase gene. The plasmid were extracted and retransformed into other *E.coli* strains to demonstrate that the chitinase activity seen was from the gene fragment on the plasmid only clone I gave clear zone in other hosts (Figure 30)

Induction and repression of chitinase synthesis

The clone I was streaked on plate with absence of IPTG, gave clear zone around the colonies on both plates with or without IPTG (Figure 22).

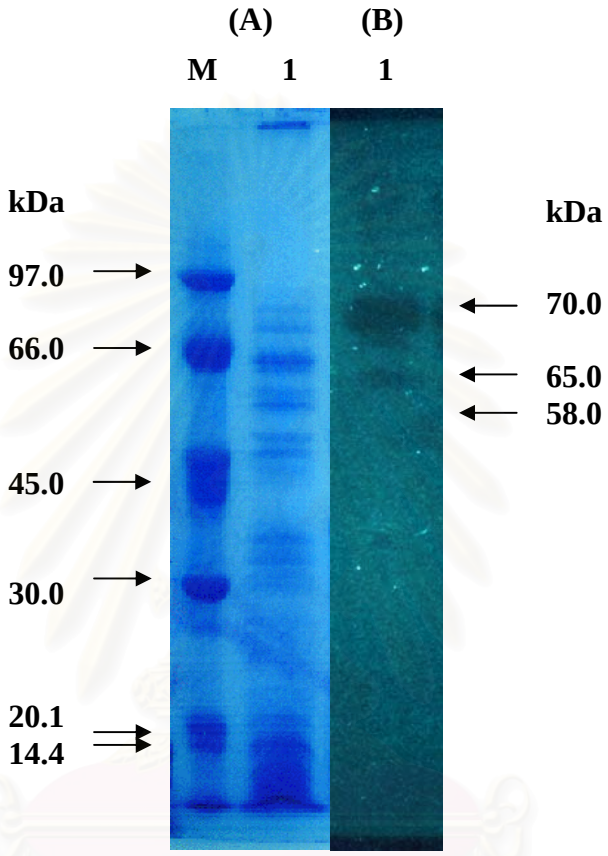


Figure 19 SDS-PAGE and activity staining of crude chitinase in 10 % gel

Panel (A): Protein stain

Lane M = Molecular weight standard marker with molecular weight of 97.0, 66.0, 45.0, 30.0, 20.1 and 14.4 kDa, respectively

Lane 1 = Crude chitinase from *B. licheniformis* PR-1

Panel (B): Activity stain

Lane 1 = Crude chitinase from *B. licheniformis* PR-1

Mapping of recombination plasmid containing chitinase Gene

The size of the insert fragment from clone I was determined by cutting with *Pst*I. We found an insert fragments with a total size of 5 kb in length. The results of single and double digested with various restriction enzymes shown in Figure 23. Restriction map of plasmid is shown in Figure 24.

Orientation of *chi*65 gene detected by PCR

Primers FPSK$^-$, BP-I, BPVII, BP-VI and BP-VIII were used for determining the orientation of *chi*65 gene in pBluescript SK$^-$. The result demonstrated that *chi*65 gene was in a reverse orientation compared to the *lac*Z gene in pBS/SK$^-$ (Figure 24) thus schematic of *chi*65 map is shown in Figure 25.

Construction of pPRChi65/PS

After mapping, 7 kb fragments from pPRChi65 were cut with *Sal*I removing the 2 kb fragment. The result clone pPRChi65/PS retains chitinase activity. Restriction maps of both clones are shown in Figure 25.

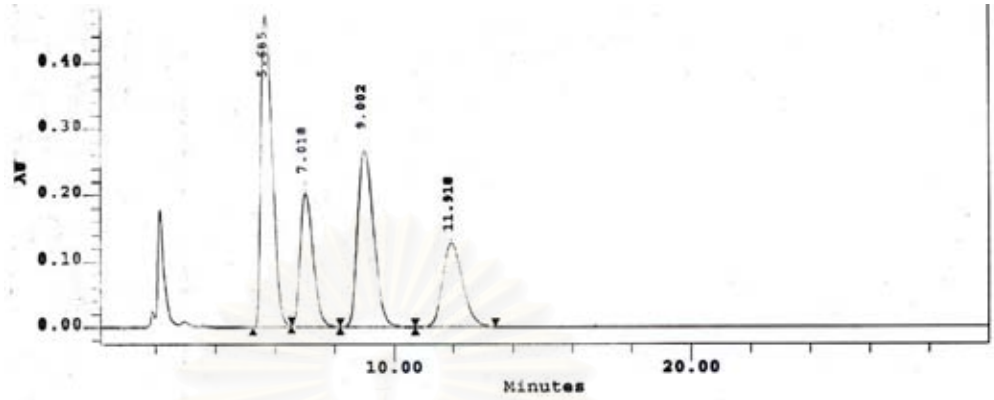
DNA Sequencing

Primers BP-II, BP-IX and M13 universal primers were used for sequencing by automate sequencer at Bioservice unit (BSU), Thailand. Nucleotide sequencing of *chi*65 gene showed in Figure 26.

Chitinase gene amplification

Sample bacteria were screened for chitinase production on CCMM plate (Figure 26). Primer BP-I and BP-VI were then used to amplify partial chitinase gene, primer BP-F and BP-R were then used to amplify full length chitinase from *Bacillus licheniformis* SK-1, *Bacillus circulans* PP8, *Paenibacillus Compinasensis* (PT), *Bacillus ciculans* A11 (RB) and *Peanibacillus* (BT) using *Bacillus licheniformis* PR-1 as positive control. From the results we found that the primers was able to amplify chitinase gene in *B. licheniformis* PR-1, *B. licheniformis* SK-1, *B. circulans* PP8 and *Paenibacillus Compinasensis* (PT) as shown in Figure 27 corresponding with formation of clear zone on CCMM plate.

(A) **1 2 3 4**



(B) **1 2 3**

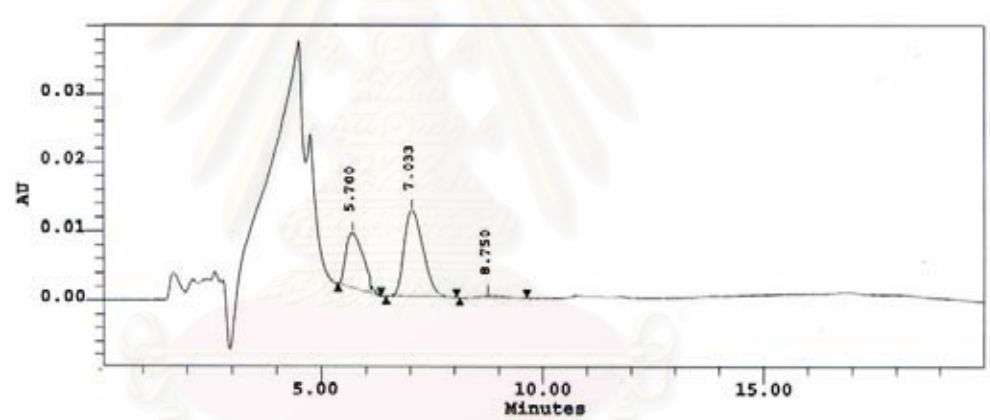


Figure 20 Hydrolytic product of crude enzyme from *B. licheniformis* PR-1

(A) = Standard chitooligosaccharide

1: Monomer of N-acetylglucosamine GlcNAc

2: Dimer of N-acetylglucosamine $(GlcNAc)_2$

3: Trimer of N-acetylglucosamine $(GlcNAc)_3$

4: Tetramer of N-acetylglucosamine $(GlcNAc)_4$

(B) = Products from 10 dilution of crude chitinase using colloidal chitin as a substrate

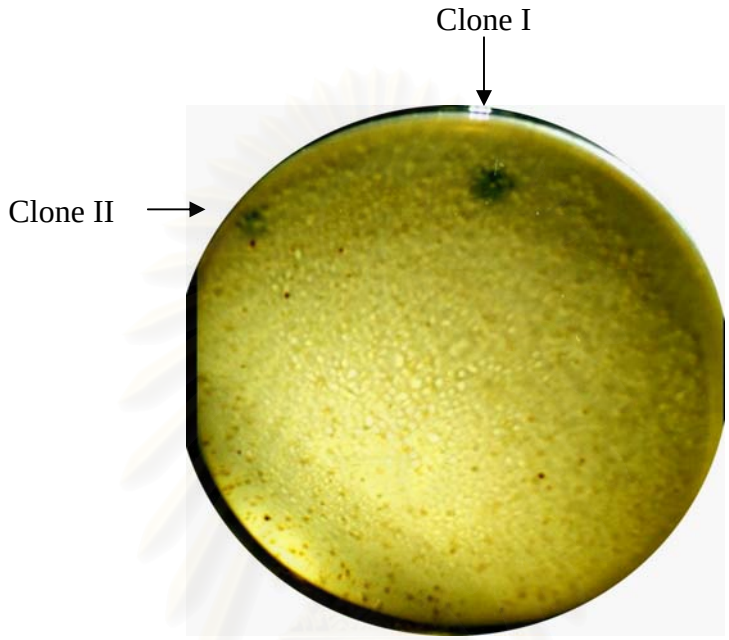


Figure 21 Clearing zone formation of transformants on screening medium plate

The clearing zone was detected on 5 days of cultivation

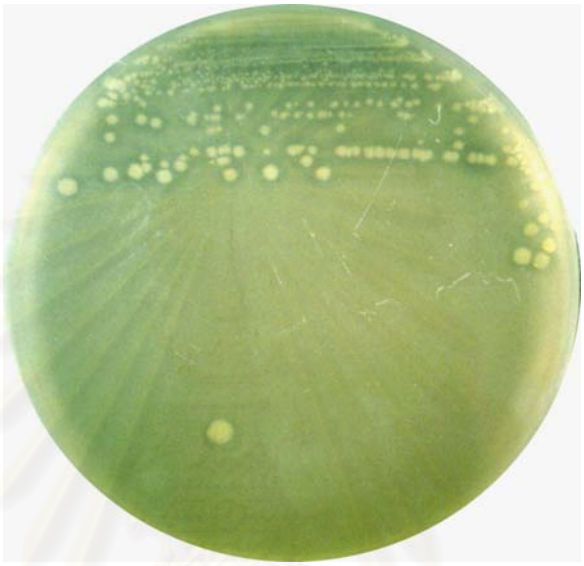


Figure 22 Clearing zone formation of clone I on media plate without IPTG. The cultured was grown for 2 days on screening medium.

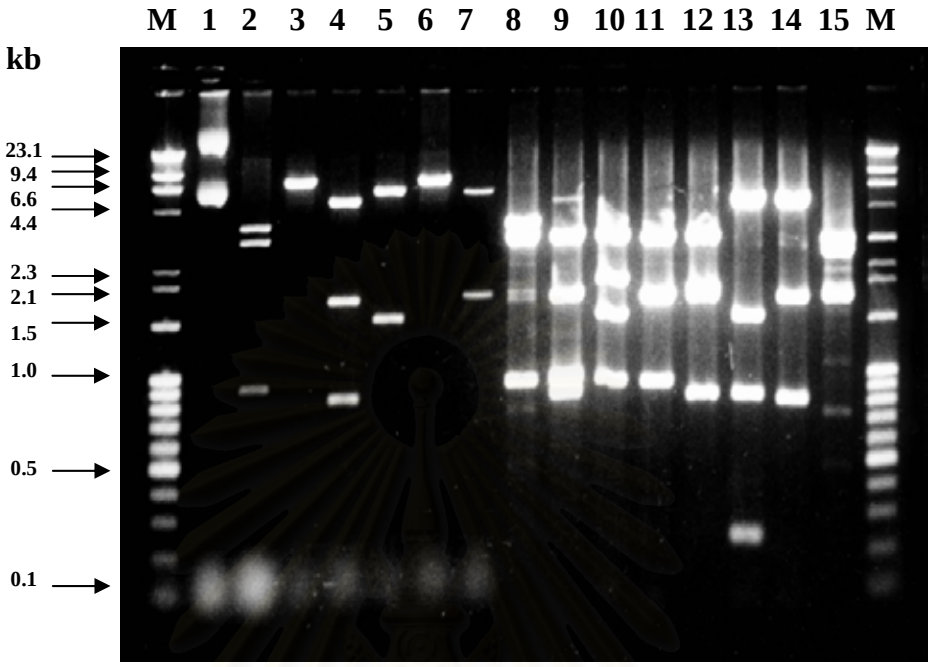


Figure 23 **Mapping of pPRChi65 from clone I with various restriction enzymes.**

Lane M = Stadard DNA marker λ/*Hin*dIII, 100 bp marker and pBluescript SK$^-$ linear form.

Lane 2-8 = Single digested with *Pst*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Not*I and *Sal*I respectively.

Lane 9-16 = Double digested with *Pst*I-*Bam*HI, *Pst*I-*Eco*RI, *Pst*I-*Hin*dIII, *Pst*I-*Sal*I, *Eco*RI-*Bam*HI, *Eco*RI-*Hin*dIII, *Eco*RI-*Sal*I and *Bam*HI-*Sal*I respectively.

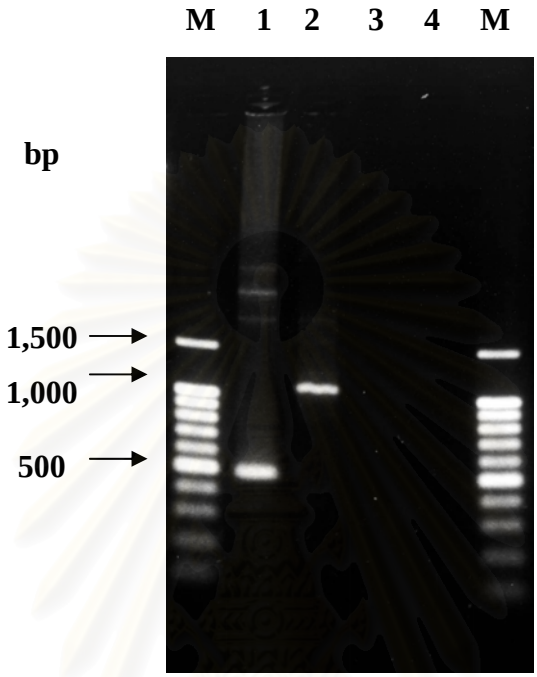


Figure 24 **Determination of the orientation of *chi*65 gene in pBS/SK$^-$**

Lane M = 100 bp marker

Lane 1 = PCR products from FPSK$^-$ corporated with BP-VII

Lane 2 = PCR products from FPSK$^-$ corporated with BP-VI

Lane 3 = PCR products from FPSK$^-$ corporated with BP-I

Lane 4 = PCR products from FPSK$^-$ corporated with BP-VIII

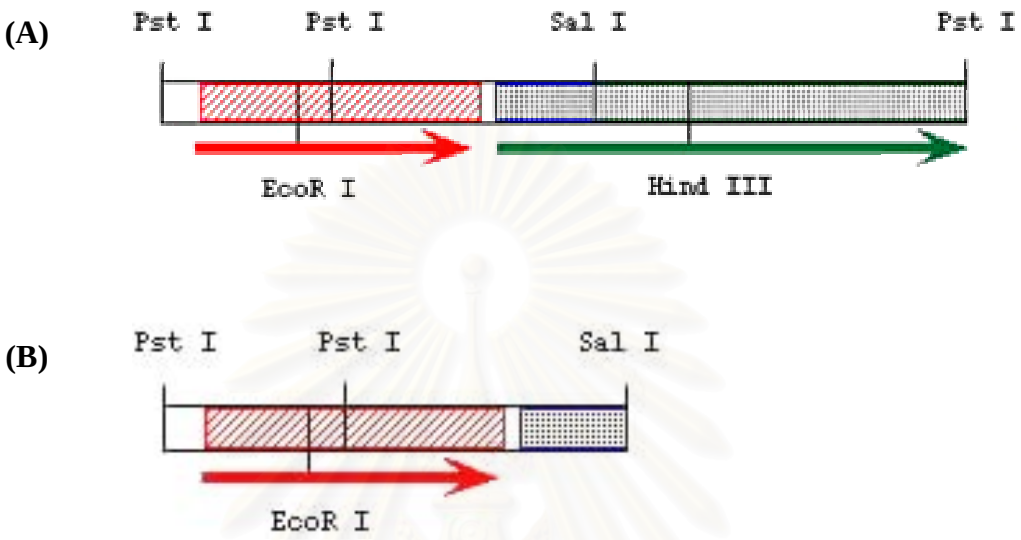


Figure 25 **Restriction map of insert fragment pPRchi65 and pPRChi65/PS**

Red arrow indicates orientation of *chi*65 gene in PBS/SK^{-}.

Green arrow indicates orientation of the partial sequence of chitodextrinase

(A) = pPRChi65 restriction map

(B) = pPRChi65/PS restriction map

```
      10         20         30         40         50         60
CTGCAGAACCCTTTTCAAACGCCAAAATTTTAATGAAACCATGCTGTCGATCTCACTTTT
      70         80         90        100        110        120
                                      -35
GTAAGCGTTTTCCCTTGTTGTCTTCAATGTATCTGCTGCTATTAGATGACAAGGAAAAAT
     130        140        150        160        170        180
-10
ATAAAACCAGCAAAAAAGGCGGTGAGGAAAAAGAGAGTTCTAGTTTCATAGCTTGCCAAA
     190        200        210        220        230        240
AAATTGCTTGTAAAGGAGATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTT
                 M  K  I  V  L  I  N  K  S  K  K  F  F  V
     250        260        270        280        290        300
TTTTCTTTCATTTTTGTTATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAA
F  S  F  I  F  V  M  M  L  S  L  S  F  V  N  G  E  V  A  K
     310        320        330        340        350        360
GCCGATTCCGGAAAAAACTATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGA
A  D  S  G  K  N  Y  K  I  I  G  Y  Y  P  S  W  G  A  Y  G
     370        380        390        400        410        420
AGGGATTTTCAAGTTTGGGATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTT
R  D  F  Q  V  W  D  M  D  V  S  K  V  S  H  I  N  Y  A  F
     430        440        450        460        470        480
GCTGATATTTGCTGGGAGGGAAGGCATGGGAACCCTGATCCGACAGGCCCCAATCCTCAA
A  D  I  C  W  E  G  R  H  G  N  P  D  P  T  G  P  N  P  Q
     490        500        510        520        530        540
ACGTGGTCATGCCAGGATGAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATG
T  W  S  C  Q  D  E  N  G  V  I  D  A  P  N  G  T  I  V  M
     550        560        570        580        590        600
GGCGATCCCTGGATTGACGCACAAAAGGCAAATCCCGGGGATGTCTGGGATGAACCGATC
G  D  P  W  I  D  A  Q  K  A  N  P  G  D  V  W  D  E  P  I
     610        620        630        640        650        660
CGCGGCAACTTTAAACAATTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTC
R  G  N  F  K  Q  L  L  K  L  K  K  S  H  P  H  L  K  T  F
     670        680        690        700        710        720
ATATCGGTCGGGGGGTGGACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCG
I  S  V  G  G  W  T  W  S  N  R  F  S  D  V  A  A  D  P  A
     730        740        750        760        770        780
GCAAGGGAGAATTCCGCCGCTTCGCCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGG
A  R  E  N  S  A  A  S  P  V  E  F  L  R  K  Y  G  F  D  G
     790        800        810        820        830        840
GTCGATCTTGACTGGGAATATCCGGTCAGCGGAGGATTGCCGGGGAACAGCACACGTCCG
V  D  L  D  W  E  Y  P  V  S  G  G  L  P  G  N  S  T  R  P
     850        860        870        880        890        900
GAAGATAAAAGAAACTACACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTGACGCTGCA
E  D  K  R  N  Y  T  L  L  L  Q  E  V  R  K  K  L  D  A  A
     910        920        930        940        950        960
GAAGCAAAAGACGGCAAGGAATACTTGCTGACGATCGCATCCGGCGCAAGTCCCGATTAT
E  A  K  D  G  K  E  Y  L  L  T  I  A  S  G  A  S  P  D  Y
     970        980        990       1000       1010       1020
GTAAGCAACACTGAGCTCGATAAAATCGCTCAAACCGTGGATTGGATTAACATTATGACC
V  S  N  T  E  L  D  K  I  A  Q  T  V  D  W  I  N  I  M  T
    1030       1040       1050       1060       1070       1080
TATGACTTTAATGGCGGATGGCAAAGCATAAGCGCCCATAATGCACCGCTGTTCTATGAT
Y  D  F  N  G  G  W  Q  S  I  S  A  H  N  A  P  L  F  Y  D
    1090       1100       1110       1120       1130       1140
CCAAAAGCGAAAGAAGCAGGCGTTCCAAACGCTGAGACCTACAATATTGAAAACACTGTG
P  K  A  K  E  A  G  V  P  N  A  E  T  Y  N  I  E  N  T  V
    1150       1160       1170       1180       1190       1200
AAACGCTACAAGGAAGCCGGTGTCAAGGGTGACAAATTAGTGCTTGGAACACCGTTCTAC
K  R  Y  K  E  A  G  V  K  G  D  K  L  V  L  G  T  P  F  Y
    1210       1220       1230       1240       1250       1260
GGAAGGGCTGGAGCGGTTGTGAATCCAGGGGGCACGGAGAATATCAGAAATGCGGACCGG
G  R  A  G  A  V  V  N  P  G  G  T  E  N  I  R  N  A  D  R
    1270       1280       1290       1300       1310       1320
CTAAAGAAGGGACATGGGAAAAGGGCGTATTCGATTTTTCAGATCTTGAAAGGAACCTAT
L  K  K  G  H  G  K  R  A  Y  S  I  F  Q  I  L  K  G  T  Y
```

```
      1330       1340       1350       1360       1370       1380
GTGAATCAAAACGGCTATAAAAGGTATTGGAACGATCAAGCAAAAGTGCCGTTTTTGTAT
V  N  Q  N  G  Y  K  R  Y  W  N  D  Q  A  K  V  P  F  L  Y
      1390       1400       1410       1420       1430       1440
AATGCGGAAAATGGCAATTTCATCACTTATGATGATGAACAATCATTCGGCCACAAAACG
N  A  E  N  G  N  F  I  T  Y  D  D  E  Q  S  F  G  H  K  T
      1450       1460       1470       1480       1490       1500
GATTTTATTAAAGCAAACGGATTAAGCGGAGCAATGTTCTGGGATTTCAGCGGCGATTCC
D  F  I  K  A  N  G  L  S  G  A  M  F  W  D  F  S  G  D  S
      1510       1520       1530       1540       1550       1560
AATCGGACGCTTCTCAATAAATTGGCAGCCGATTTAGATTTTGCACCGGACGGAGGCAAT
N  R  T  L  L  N  K  L  A  A  D  L  D  F  A  P  D  G  G  N
      1570       1580       1590       1600       1610       1620
CCGGAGCCGCCTTCATCGGCACCTGTGAATGTGCGTGTAACCGGAAAAACTGCTACAAGT
P  E  P  P  S  S  A  P  V  N  V  R  V  T  G  K  T  A  T  S
      1630       1640       1650       1660       1670       1680
GTCAGCCTGGCGTGGGATGCGCCGAGCAGCGGAGCAAACATTGCGGAATATGTCGTGTCA
V  S  L  A  W  D  A  P  S  S  G  A  N  I  A  E  Y  V  V  S
      1690       1700       1710       1720       1730       1740
TTTGAAAACCGGTCGATATCTGTAAAAGAAACATCAGCGGAAATAGGCGGCTTGAAGCCG
F  E  N  R  S  I  S  V  K  E  T  S  A  E  I  G  G  L  K  P
      1750       1760       1770       1780       1790       1800
GGTACGGCCTACTCATTTACTGTTTCAGCAAAGGATGCGGATGGAAAGCTCCATGCCGGA
G  T  A  Y  S  F  T  V  S  A  K  D  A  D  G  K  L  H  A  G
      1810       1820       1830       1840       1850       1860
CCAACGGTAGAGGTCACGACGAATTCTGACCAAGCCTGTTCATATGACGAATGGAAAGAG
P  T  V  E  V  T  T  N  S  D  Q  A  C  S  Y  D  E  W  K  E
      1870       1880       1890       1900       1910       1920
ACGAGCGCATACACAGGCGGAGAGCGGGTTGCATTTAACGGAAAAGTGTATGAAGCGAAA
T  S  A  Y  T  G  G  E  R  V  A  F  N  G  K  V  Y  E  A  K
      1930       1940       1950       1960       1970       1980
TGGTGGACGAAAGGCGACCGGCTGATCAATCCGGTGAATGGGGCGTATGGCGGCTGATCG
W  W  T  K  G  D  R  L  I  N  P  V  N  G  A  Y  G  G  *
      1990       2000       2010       2020       2030       2040
GAGGCTGCGAATAAGAGAAAGTCAAATGGATAGAAAACGATAAAGAGAGATTTGGGGAAC
      2050       2060       2070       2080       2090       2100
AGCTTCTCACGTCTTCTCTTTATGGACAAAGGAGTCTGAGTAAACATGAAGAAAGCCGCT
      2110       2120       2130       2140       2150       2160
TCATCTTTTTTATCTTGTATGCTGCTCCTCGCGCTTTTTATCCCGAACCGGCAGATTTCC
      2170       2180       2190       2200       2210       2220
GCAGAGACTTCAAGCGAAAGACCGGACTGCCGTCCGGAAGGGCTATGGGACTCGGGCGTT
      2230       2240       2250       2260       2270       2280
GAACATGTGCCGTATTGCGATGTATACGACAAGGACGGCCGTGAAAAGCTGGCCAATCAG
      2290       2300       2310       2320       2330       2340
TTAGACCGGAGAATCATCGGTTACTTTACGAGCTGGCGTACGGGAAAAGGAAATCAAGAT
      2350       2360       2370       2380       2390       2400
CGTTATTTAGTAAGCGATATCCCCTGGAAGTACTTGAGCCACATTAATTATGCGTTTGCC
      2410       2420       2430       2440       2450       2460
CACATCGGGGAAGATCATCGGATTTCAGTCGGAGAAGAGGCAGATGAGAACAACCCGTCC
      2470       2480       2490       2500       2510       2520
ATCGGGATGACTTGGCCGGAGCATCCCGATGTAAAAATGGATCAAACCTTGCCGTATAAG
      2530       2540       2550       2560       2570       2580
GGGCATTTTAACCTTATCCATCAATATAAAGATAGATACCCGGATGTGAAGGTTTTGGCC
      2590       2600       2610       2620
GCTGTCGGCGGTTGGGCCGAAACCGGCGGCTATGTCGAC
```

Figure 26 **Nucleotide sequencing of *chi*65 gene**

*Chi*65 gene is typed in blue colour. CHI65 protein is typed in pink colour. Start and stop codon are typed in red colour. The position –10 and –35 are typed in green colour and underlined. Ribosomal binding site is underline.

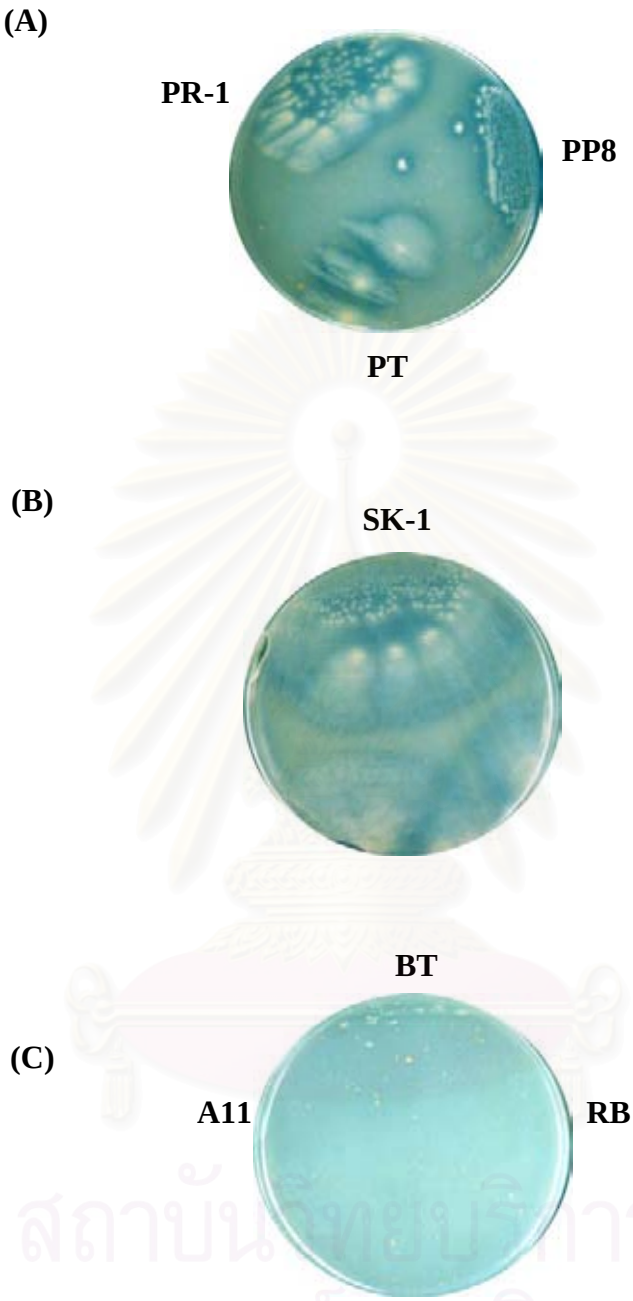


Figure 27 Chitinase activity on CCMM screening for chitinase activity

(A) = *B. licheniformis* PR-1 (PR-1), *Bacillus circulans* PP8 (PP8) and *Paenibacillus Compinasensis* (PT)

(B) = *B. licheniformis* SK-1 (SK-1)

(C) = *B. ciculans* A11 (A11), and *Peanibacillus* (BT)

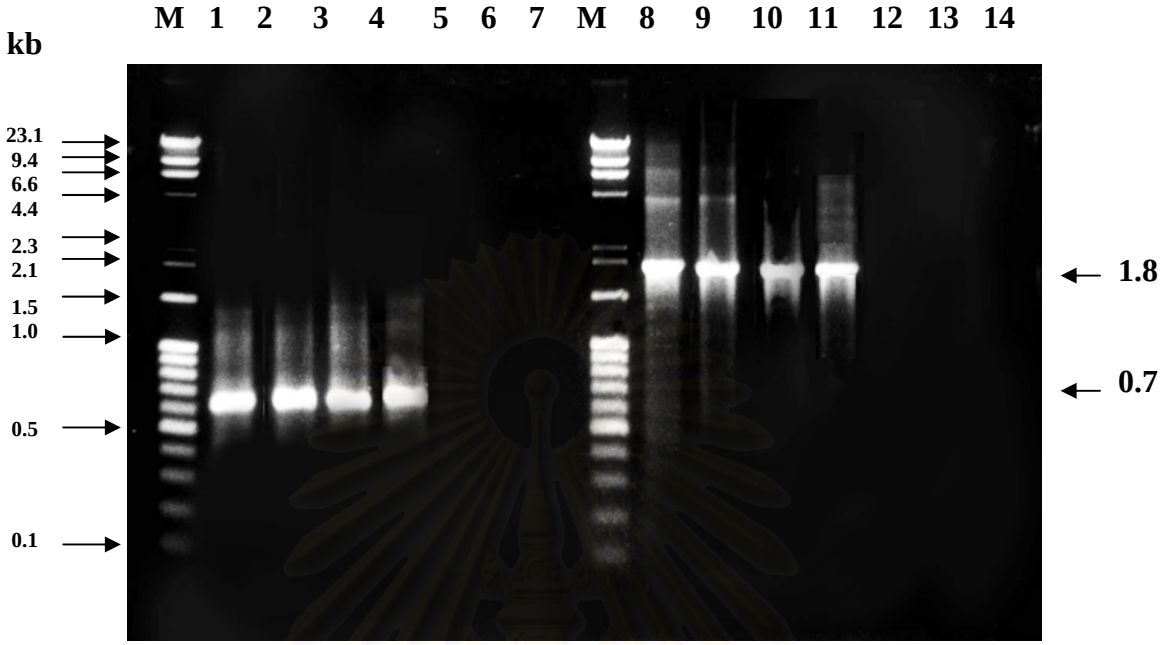


Figure 28 Amplification of full length chitinase gene from *B. licheniformis* PR-1 and related species

Lane M = Standard DNA marker λ/*Hin*dIII, 100 bp ladder

Lane 1,8 = PCR products from *B. licheniformis* PR-1

Lane 2,9 = PCR products from *Bacillus circulans* PP8

Lane 3,10 = PCR products from *Paenibacillus Compinasensis* (PT)

Lane 4,11 = PCR products from *B. licheniformis* SK-1 (SK-1)

Lane 5,12 = PCR products from *B. ciculans* A11

Lane 6,13 = PCR products from *Peanibacillus* (BT)

Lane 7,14 = PCR products from *Peanibacillus* (RB)

Regulation in another host strains and preliminary study of effects of various supplements in chitinase production.

Plasmid pPRChi65 in *E.coli* XL-1 blue, DH5α and JM109 cells and pPRChi65/PS in DH5α cells produced clear zone on CCMM plates containing 0.25 and 0.5 % yeast extract (Figure 29). JM109 produce the largest clear zone followed by XL-1 blue and DH5α cells.

Plasmid pPRChi65 in XL-1 blue, DH5α and JM109 cells and pPRChi65/PS in DH5α cells produced clear zone on LB plates containing colloidal chitin and CCMM plate with glucose, but in LB plates containing colloidal chitin and glucose all strains had no clear zone (Figure 30). We observed larger clear zone in DH5α cells in the presence of glucose, and reduction in the size of clear zone in XL-1 blue cells (Panel B, Figure 30).

Analysis of *Chi*65 gene

Homology search of *Chi*65 gene using BLAST program

Analysis of the sequence by BLAST program to search for homology of *Chi*65 gene to proteins in the Genbank. The amino acid comparison indicated that *Chi*65 is 89 % similarity to chitinase from *B. licheniformis* TP-1 followed by 79 % similarity to chitinase from *B. subtilis*.

Genetic Information Processing Software For Windows

GENETYX-WIN version 3.1 was used to analyse *Chi*65 gene. We found two open reading frame (ORF), one ORF was 1,779 bp, coding for 593 amino acids, which corresponded to 65,100 Da with calculated isoelectric point of 5.84. The other one was a partial gene sequence of chitodextrinase.

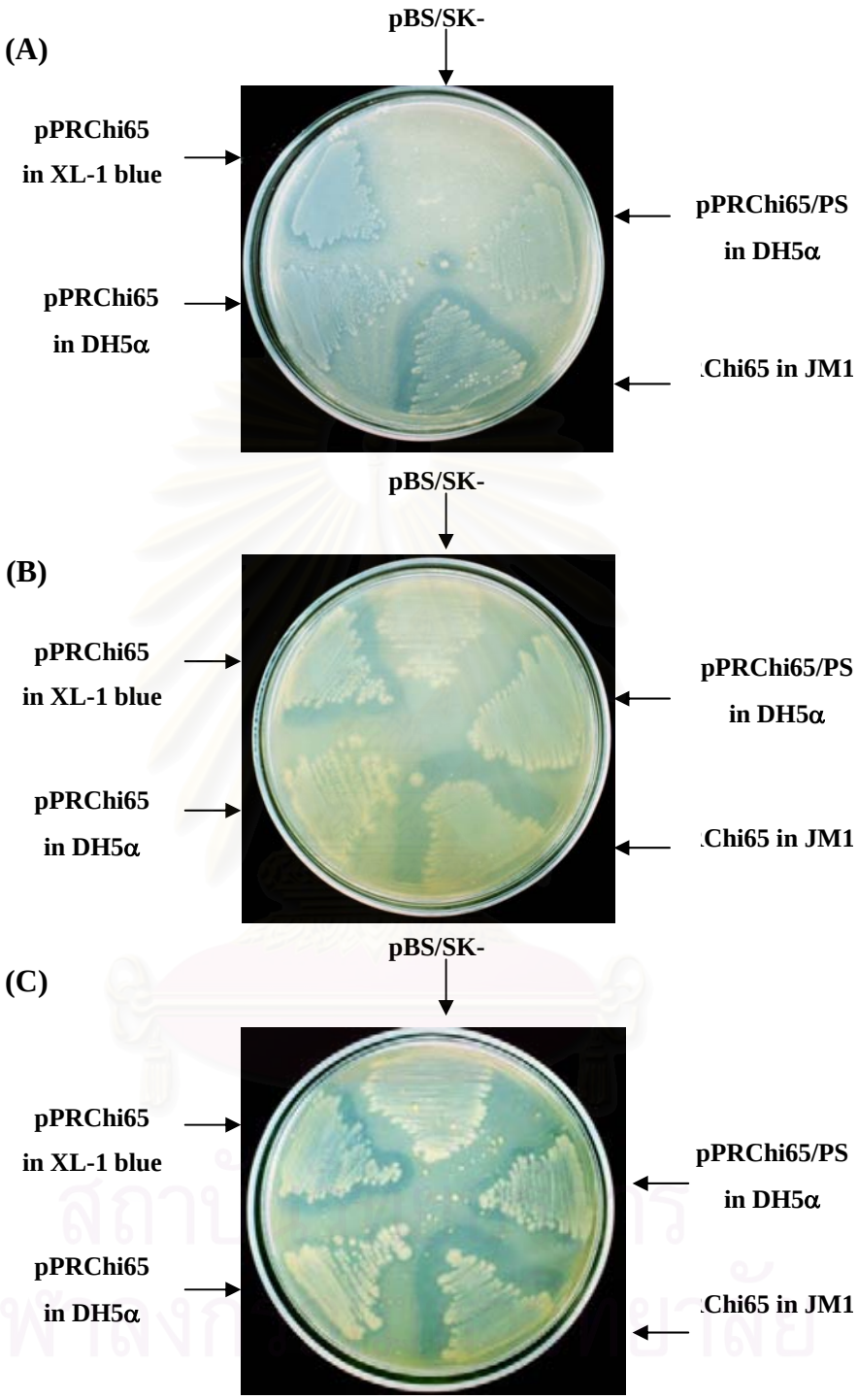


Figure 29 **Induction of chitinase synthesis**

All plates were cultivated for 6 days.

(A) = CCMM with 0.05 % yeast extract

(B) = CCMM with 0.25 % yeast extract

(C) = CCMM with 0.5 % yeast extract

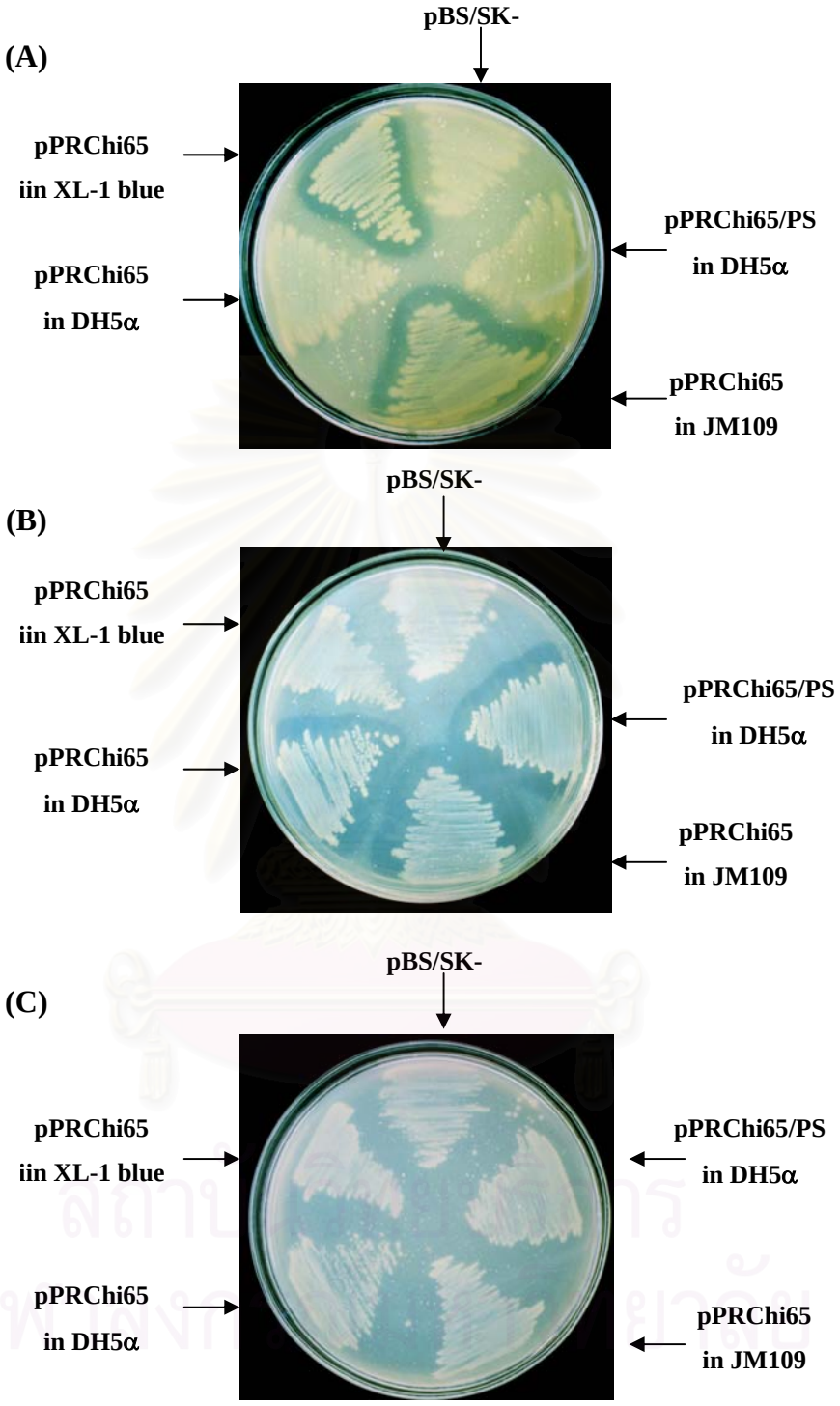


Figure 30 **Induction and repression of chitinase synthesis**

All plates were cultivated for 6 days.

(A) = LB with 0.5% yeast extract an colloidal chitin

(B) = CCMM with 0.05% yeast extract and glucose

(C) = LB with 0.5% yeast extract, colloidal chitin and glucose

```
[GENETYX-WIN : Amino Acid Composition]
Date             : 2002.03.28
Filename         :
Sequence Size    : 592
Sequence Position: 1 - 592

hydrophobic:   291( 49.16)
neutral    :   130( 21.96)
hydrophilic:   171( 28.89)
other      :     0(  0.00)

[ hydrophobic residues ]
Gly(G)     57(  9.63%) Ala(A)     53(  8.95%) Val(V)     40(  6.76%)
Leu(L)     32(  5.41%)
Ile(I)     27(  4.56%) Met(M)      7(  1.18%) Phe(F)     27(  4.56%)
Trp(W)     17(  2.87%)
Pro(P)     31(  5.24%)

[ neutral residues ]
Ser(S)     42(  7.09%) Thr(T)     31(  5.24%) Asn(N)     41(  6.93%)
Gln(Q)     13(  2.20%)
Cys(C)      3(  0.51%)

[ hydrophilic residues ]
Asp(D)     42(  7.09%) Glu(E)     29(  4.90%) Lys(K)     45(  7.60%)
His(H)      8(  1.35%)
Arg(R)     20(  3.38%) Tyr(Y)     27(  4.56%)

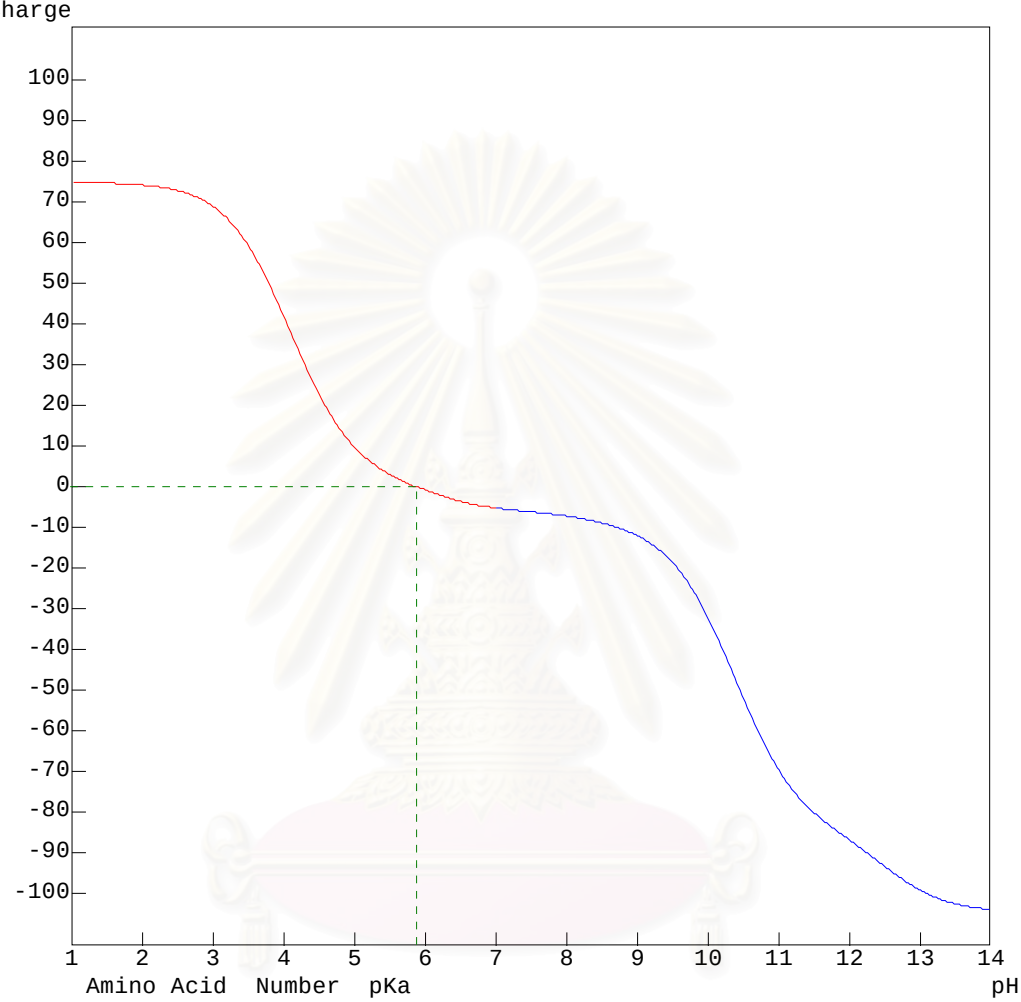
[ other residues ]
Asx(B)      0(  0.00%) Glx(Z)      0(  0.00%) Xaa(X)      0(  0.00%)
???(?)      0(  0.00%)

Average Molecular Weight = 65163.35
Monoisotopic Molecular Weight = 65124.2201
```

Figure 31 **Amino acid composition and molecular weight estimation of CHI65 by GENETYX v.3.1**

```
[GENETYX-WIN : Caluculate isoelectric point]
Date              : 2002.03.26
Filename          : Sequence8
Sequence size     : 594
Sequence position : 1 - 594

Charge
```



Amino Acid	Number	pKa
Arg (R)	20	12.5
His (H)	8	6.0
Lys (K)	45	10.5
Asp (D)	42	3.9
Cys (C)	3	8.3
Glu (E)	29	4.3
Tyr (Y)	27	10.1

```
N-terminal Met (M)  9.3
C-terminal  (
Isoelectric point: 5.84
```

Figure 32 Calculated isoelectric point of CHI65 by GENETYX v.3.1

Partial characterization of crude chitinase from *E.coli* containing pPRChi65

Enzyme production

The activity of crude chitinase from cultured medium was determined. In CCMM with 0.25 % yeast extract broth only *E.coli* JM109 showed detectable chitinase production, the level of maximum chitinase activity was 75 mU/mL found at the seventh day of cultivation (Figure 33).

Optimum pH of pPRChi65

The optimum pH of crude chitinase was measured in 0.1 M buffer at pH range 3-10. Crude chitinase showed highest chitinase activity in citrate buffer, pH 5.0. Relative activities at different pHs were shown in Figure 34.

Optimum temperature of pPRChi65

The optimum temperature of crude chitinase was determined at pH 5.0 in the range of 30-80 °C. Crude chitinase showed highest chitinase activity at 60 °C. Relative activities at different pHs were shown in Figure 35.

Substrate specificity of pPRChi65

The activity of chitinase was examined at pH 5.0, 60 °C on chitin and its related compounds, chitinase was able to hydrolyze colloidal chitin very well followed by 80 % DD, powder chitin, flake chitin and regenerated chitin, respectively (Figure 36).

Determination of molecular weight of CHI65 using SDS-PAGE

On a 10 % SDS-PAGE chitinase activity stained gel, showed 3 bands containing chitinase activity with the molecular weight of 70, 65 and 58 kDa, which was similar to *B. licheniformis* PR-1 (Figure 37).

Detection of hydrolytic products produced by chitinase from pPRChi65 and pPRChi65/PS

Hydrolytic products produced by chitinase from pPRChi65 and pPRChi65/PS were seperated by HPLC (Figure 38), we found a mixture of products, $(GlcNAc)_2$ and GlcNAc, the same result as in *B. licheniformis* PR-1.

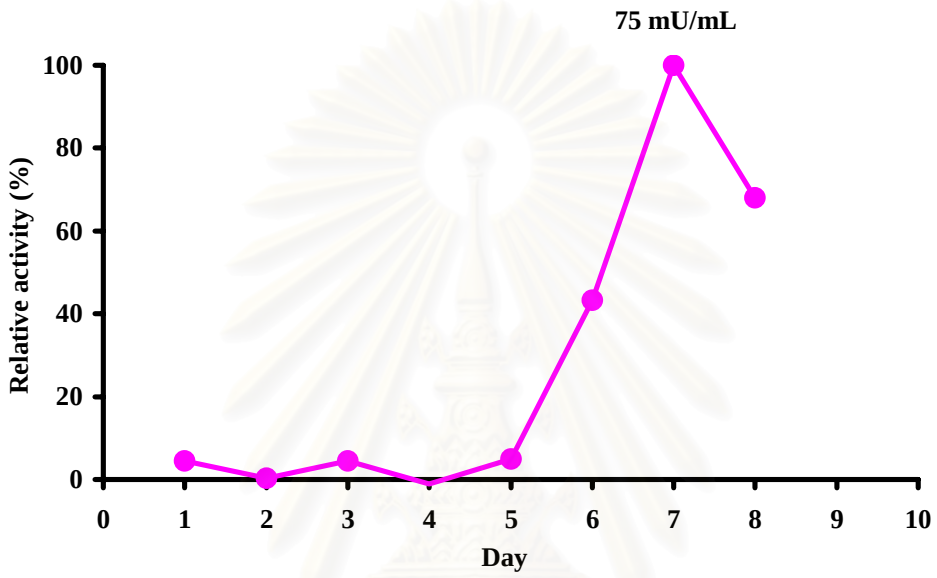


Figure 33 **Chitinase production from *E.coli* JM109 containing pPRChi65 by colourimetric method using colloidal chitin as sustrate**

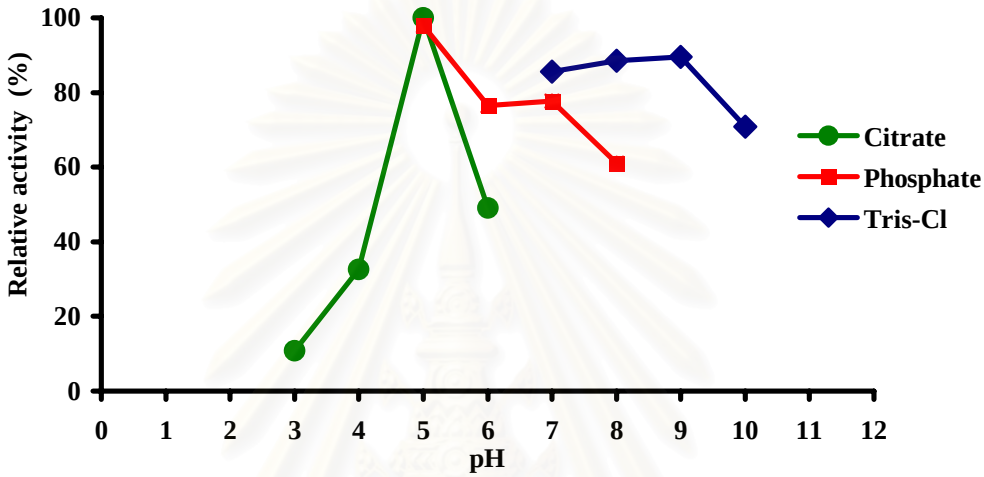


Figure 34 Effect of pH of crude chitinase from *E.coli* JM109 containing pPRChi65 by colourimetric method

Enzyme activities were measured at pH range from 3-10, using colloidal chitn as substrate.

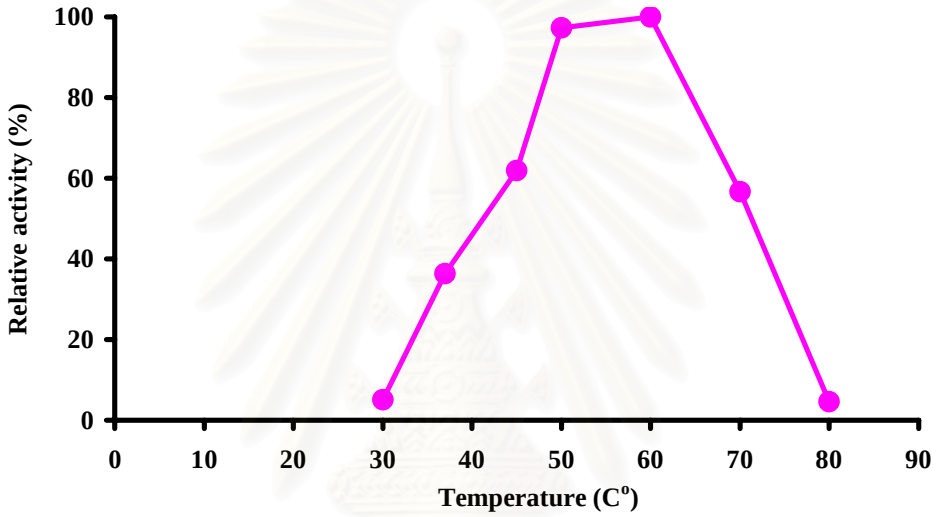


Figure 35 Optimum temperature of crude chitinase from *E.coli* JM109 containing pPRChi65 by colourimetric method

Enzyme activities were measured at 30, 37, 45, 50, 60, 70 and 80 ^{o}C in citrate buffer pH 5.0 using colloidal chitin as substrate.

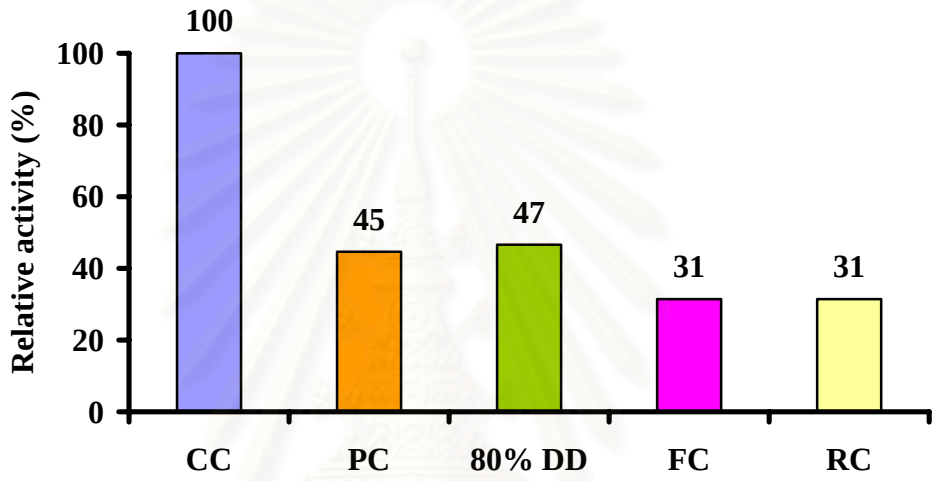


Figure 36 Substrate specificity of crude chitinase from *E.coli* JM109 containing pPRChi65 by colourimetric method

Enzyme activities were measured at 60 ^{o}C in citrate buffer pH 5.0 using colloidal chitin (CC), powder chitin (PC), 80% Deactylase chitosan (80% DD), flake chitin (FC) and regenerated chitn (RC) as substrate.

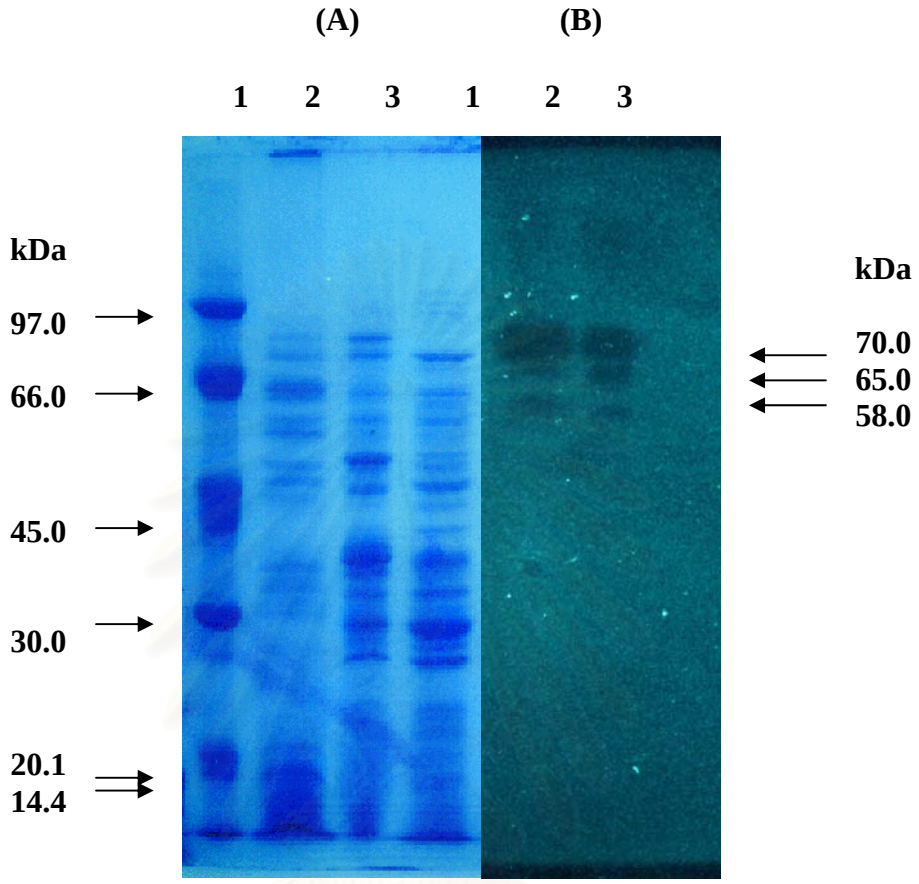


Figure 37 Determination of molecular weight of CHI65

Panel (A): Protein stain

Lane M = Molecular weight standard marker with molecular weight of 97.0, 66.0, 45.0, 30.0, 20.1 and 14.4 kDa, respectively

Lane 1 = Crude chitinase from *B. licheniformis* PR-1

Lane 2 = Crude chitinase from *E. coli* JM109 contained pPRChi65

Lane 3 = Crude protein from *E. coli* JM109 contained pBS/SK$^-$

Panel (B): Activity stain

Lane 1 = Activity of crude chitinase from *B. licheniformis* PR-1

Lane 2 = Activity of crude chitinase from *E. coli* JM109 contained pPRChi65

Lane 3 = Activity of crude protein from *E. coli* JM109 contained pBS/SK$^-$

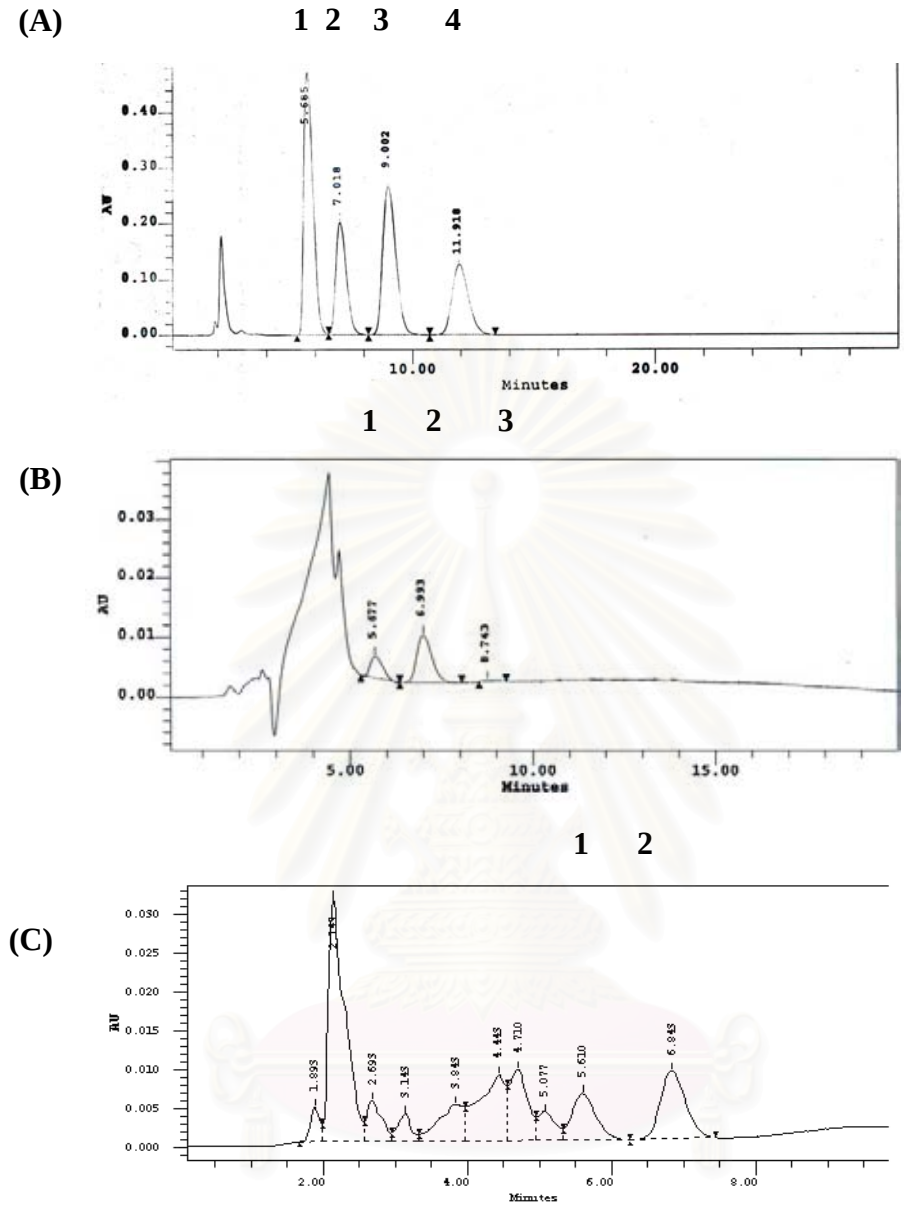


Figure 38 Chromatograph of HPLC

(A) = Standard chitooligosaccharide

1: Monomer of N-acetylglucosamine GlcNAc

2: Dimer of N-acetylglucosamine $(GlcNAc)_2$

3: Trimer of N-acetylglucosamine $(GlcNAc)_3$

4: Tetramer of N-acetylglucosamine $(GlcNAc)_4$

(B) = Products from crude chitinase from pPRChi65 using colloidal chitin as a substrate

(C) = Products from crude chitinase from pPRChi65/PS using colloidal chitin as a substrate

CHAPTER IV

DISCUSSION

Primer for generated partial chitinase gene.

PCR products amplified by designed primers from chitinase family 18. Primers BP-I, II, V and VI resulted in fragments 400, 280 and 700 bp in length. After sequencing, the 700 bp PCR product and analyze by BLAST program in Genbank (Altschul *et al*., 1997), 635 bp in length was found, which was a partial chitinase gene. This indicated that the primers we designed were specific for family 18 chitinases in *Bacillus licheniformis* PR-1.

Characterization of crude enzymes from *Bacillus licheniformis* PR-1

Enzyme production

In general when the bacteria was cultured in minimum medium, more cells should produce more enzyme. Our attempt to increase enzyme production by increasing of yeast extract, resulted in different enzyme production profiles of chitinase activity from *B. licheniformis* PR-1.

Cells grown in CCMM with 0.05 % yeast extract showed increasing enzyme production continuously from second day to eighth day (2-21mU/mL) of cultivation. In CCMM with 0.25 % yeast extract, more enzyme production was observed and chitinase activity maximized (36 mU/mL) on fifth days of cultivation.

These results demonstrated that quantity of supplement had effected with enzyme production of these bacteria.

Optimum pH and temperature of chitinase activity

After crude enzyme from *Bacillus licheniformis* PR-1 was assayed, we found that this enzyme worked in a wide pH and temperature range, 4-10 and 30-80 °C, respectively. The highest chitinase activity was observed in citrate buffer pH 5.0. The observed optimum pH of *B. licheniformis* PR-1 was similar with *Aeromonas hydrophilia* H-2330 (Hiraga *et al*., 1997), Chitinase A1 from *B. circulans* WL-12 (Wanatabe *et al*., 1990), *B. licheniformis* X-7u chitinases (Takayanagi., 1991), *B. licheniformis* B-6839 chitinases (*Lesya et al*.,1996) *Streptomyces erythraeus* (Hara

et al., 1989) and Chitinase A from *Serratia marcescens* QMB1466 (Roberta *et al*., 1982). However, our enzyme was more stable in basidic buffers, we observed more than 60% of relative activity.

The optimum temperature of crude enzyme was 70 ^{o}C. When compared with chitinase from the other organisms, it was similar to *B. licheniformis* X-7u chitinases (Takayanagi., 1991), *B. licheniformis* B-6839 chitinases (*Lesya et al*.,1996), which work at high temperature. Thus enzyme CHI65 from *B. licheniformis* PR-1 is a thermostable enzyme.

Substrate specificity

The crude enzyme had the highest hydrolytic activity on colloidal chitin (100%), followed by powder chitin (58%), 80 % DD chitosan (52%), flake chitin (27%) and regenerated chitin (23%), respectively. This experiment indicated that crude chitinase works well on amorphous substrate, colloidal chitin, better than crystalline substrates, powder chitin and flake chitin. The low activity on chitosan indicated that this enzyme was able to hydrolyzed only β-1,4 glycosidic bond between N-acetylglucosamine not glucosamine, suggesting the lack of chitosanase activity.

Estimate of molecular weight of protein's with chitinolytic activity in crude enzyme by SDS-PAGE

On 10 % SDS-PAGE stained for proteins with chitinolytic activity in the crude enzyme. We observed three bands with chitinolytic activity, with the molecular weight of 70, 65 and 58 kDa. The lower molecular weight bands could be hydrolytic product form the 70 kDa, to 65 and 58 kDa protein.

Detection of hydrolytic products produced by chitinase

That HPLC analysis of the digestive products, showed a mixture of products, $(GlcNAc)_2$ and GlcNAc. This result indicated that there might be more than one enzyme in crude enzyme from *B. licheniformis* PR-1 with chitinolytic activity. A chitinase which produces $(GlcNAc)_2$ and N-acetylhexosaminidase which produces (GlcNAc) or it may be a single chitinase that can produce a mixture of the two products.

Cloning of chitinase gene

Two positive clones with chitinolytic activity had been isolated from 8,000 transformants. Both showed clear zone on the screening medium. The positive clones I and II contained plasmids with 5 and 3 kb inserted fragments, respectively.

When plasmids were extracted from the original clone and retransformed into other host strains, such as DH5α and JM109. Chitinase activity was detected in all strains of *E. coli*, with only the plasmid with 5 kb inserted fragment, from clone I (pPRChi65). This indicated that the pPRChi65 contains chitinase gene.

We detected chitinase activity either in absence or presence of IPTG on screening medium. This suggested that pPRChi65 had its own promoter and we have successfully cloned chitinase gene.

When pPRChi65 was cut with *Sal*I removing 2 kb fragment (pPRChi65/PS) and retransformed into DH5α, we found that pPRChi65/PS retains chitinase activity demonstrating that pPRChi65/PS still contains the full length gene.

In the next steps, we cultured all strains containing pPRChi65 in various medium (CCMM, 0.25 and 0.5 % yeast extract CCMM, CCMM with glucose, LB with 0.02 % (dry wt.) colloidal chitin and LB with 0.02 % (dry wt.) colloidal chitin including glucose) by using *E.coli* containing pBS/SK^- as negative control.

We found clear zone from pPRChi65 around colonies in almost all medium plates excepted in LB with colloidal chitin where DH5α strain could not produced clear zone. However in CCMM with glucose are observed an induction of *Chi*65 in DH5α cells and repression in XL-1 blue cells.

In LB with colloidal chitin including glucose, we did not detect chitinase expression in any host cells. These results demonstrated that promoter in *Chi*65

properly work in *E.coli*, but in rich medium could suppress in DH5α strain and rich medium with glucose can suppress promoter of *Chi*65 in all host strains.

Analysis of *Chi*65 gene

Two open reading frame (ORF) were found in the inserted fragment from pPRChi65/PS, one ORF of 1,779 bp encoding 593 amino acids, which corresponded to 65,100 Da with isoelectric point of 5.84 (GENTYX-WIN version 3.1, Japan) and another ORF was partial gene of chitodextrinase.

Characterization of *Chi*65

Enzyme production from *E.coli* JM109 containing pPRChi65

In CCMM with 0.25 % yeast extract broth, only in *E.coli* JM109 host, chitinase activity could be detected with a maximum activity of 75 mU/mL, on the seventh days of cultivation. This result suggests that in broth DH5α and XL-1 blue host strains could not produce CHI65or produced only trace amount of enzyme.

Optimum pH and temperature of *Chi*65

From the result of enzyme production, crude chitinase was collected at day 7 and observed chitinase activity in various pH and temperature. The highest chitinase activity of *Chi*65 was in citrate buffer pH 5.0. The optimum temperature of cloned enzyme was 60 ^{o}C. Both results indicated that the characteristic of chitinase from pPRChi65 is almost the same as chitinase from *B. licheniformis* PR-1.

Substrate specificity

The activity of chitinase was assayed at pH 5.0, 60 °C, the crude enzyme had the highest hydrolytic activity on colloidal chitin (100%), followed by 80 % DD (45%), powder chitin (47%), flake chitin and regenerated chitin (both 31%).

This experiment indicated that crude chitinase from *Chi*65 works better on amorphous substrate than crystalline substrate.

And crude chitinase from *Chi*65 works better on amorphous substrate than chitosan. Indicating this enzyme was able to hydrolyzed only β-1,4 glycosidic bond between GlcNAc not glucosamine similar to chitinase from *B. licheniformis* PR-1.

Estimate molecular weight of chitinase from *chi*65

On a 10 % SDS-PAGE chitinase activities stained gel, shown 3 bands had chitinase activity with the molecular weight of 70, 65 and 58 kDa which is the same as in *B. licheniformis* PR-1. It indicated that we had successfully cloned chitinase gene from *B. licheniformis* PR-1. The smaller proteins with chitinolytic activity may result from a protiolytic cleavage of the 70 kDa protein. This result was similar with chitinase from *B. licheniformis* PR-1. Tantimavanich. *et al* succesfully cloned chitinase gene from *Bacillus licheniformis* TP-1, and they also found 3 activity bands 68, 62 and 50 kDa. They also suggested that the 62 and 50 kDa protein was generated from the 68 kDa protein.

Detection of product produced by chitinase from pPRChi65 and pPRChi60/PS by HPLC

Analysis of the hydrolytic product of pPRChi65 and pPRChi65/PS by HPLC, showed a mixture products of $(GlcNAc)_2$ and GlcNAc same as the hydrolytic product of in *B. licheniformis* PR-1.

CHAPTER V

CONCLUSION

Bacillus licheniformis PR-1, isolated from soil, showed the ability to produce chitinase when grown on colloidal chitin minimum medium. *Bacillus licheniformis* PR-1 produced chitinase to a detectable level at second day of cultivation and increased to reach the maximum activity on the 7^{th} and 5^{th} day when grown in CCMM with 0.05 and 0.25% yeast extract, respectively.

Sequence alignment and primer design resulted in primers BP-I, II, V, VI, VII, VIII, IX, BP-F and BP-R. Primer BP-F and BP-R were able to amplify full length chitinase gene from *B. licheniformis* SK-1, *B. circulans* PP8, *Paenibacillus Compinasensis* (PT), *B. ciculans* A11 (RB) and *Peanibacillus* (BT). That suggested all designed primers were specific for familly 18 chitinases among *Bacillus spp*.

Crude chitinase was characterized, optimum pH and temperature of these chitinase was pH 5.0 in citrate buffer and 70 ^{o}C, respectively. Crude chitinase hydrolyzed colloidal chitin (100%) the best followed by powder chitin (58%), 80 % DD chitosan (52%), flake chitin (27%) and regenerated chitin (23%), respectively.

Products from cloned enzyme analysed by HPLC, mixture products of chitobiose $(GlcNAc)_2$ and (GlcNAc) while the $(GlcNAc)_2$ was a major products.

After SDS-PAGE and activity staining of crude enzymes, three bands with chitinolytic activity was observed. The estimate molecular weights of chitinase species were 70, 65 and 58 kDa.

Shotgun cloning results demonstrated that two transformants from 8,000 colonies showed clear zones with plasmid containing inserted fragment 5 and 3 kb, respectively. Only the plasmid with the 5 kb pPRChi65 inserted fragment showed chitinase activity when retransformed. Orientation of *Chi*65 gene was opposite to *lac*Z gene in pBS/SK^{-} indicating that the expression of *Chi*65 was regulated from an endogenous promoter. *Chi*65 had the highest chitinase activity expression in *E.coli* JM109 and promotor of *Chi*65 could be suppress when cells are grown in rich medium with glucose. Crude chitinase produced from pPRChi65 was characterized. Optimum pH and temperature was in citrate buffer pH 5.0 and at 60 ^{o}C, respectively.

Two kb deletion of pPRChi65 by *Sal*I digestion, resulted in pPRChi65/PS, which also produces chitinase. This demonstrated that pPRChi65/PS still contains the full length gene. Full length insert of pPRChi65 contains two ORF, one is from position 199 to 1977 total 1,779 bp encoding for a 593 amino acids protein, with a calculated molecular weight 65,100 Da and an isoelectric point is 5.84 (predicted by GENTYX-WIN version 3.1). The other ORF is codes for partial sequence of chitodextrinase.

The amino acid sequence comparison indicated *Chi*65 is 89 % similar to chitinase from *B. licheniformis* TP-1 followed by 79 % chitinase from *B. subtilis*.

The optimum pH and temperature of the cloned enzyme was in citrate buffer pH 5.0 and at 60 ^{o}C, respectively. Crude chitinase can hydrolyse colloidal chitin (100%) the best followed by 80 % DD (45%), powder chitin (47%), flake chitin and regenerated chitin (both 31%), suggesting that it is an exochitinase.

After SDS-PAGE and activity staining of crude enzyme of the recombinant clone, three types of chitinase were detected. The molecular weights of the proteins were approximately 70, 65 and 50 kDa, which is identical to chitinase produced in the culture medium of *B. licheniformis* PR-1, suggesting we had successfully cloned chitinase gene from *B. licheniformis* PR-1.

Analysis of the hydrolyzed products of the cloned enzyme by HPLC, found a mixture products of $(GlcNAc)_2$ and GlcNAc from both pPRChi65 and pPRChi65/PS, suggesting these enzyme produced two types of products and hydrolytic products came from only chitinolytic activity from chitinase not from partial chitodextrinase gene.

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APPENDICES

APPENDIX A

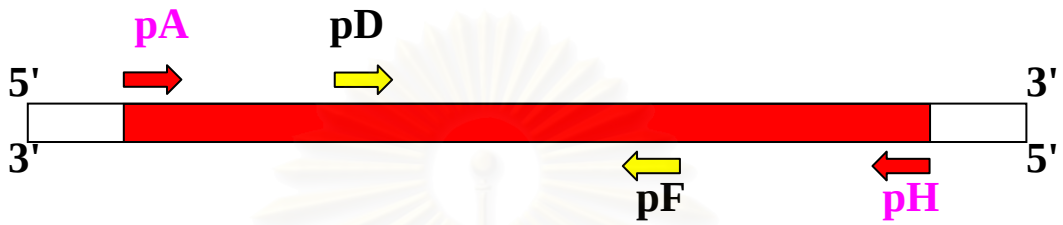
Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Whistler and BeMiller (1962) with some modification. A 40 grams of crab shell chitin (Sigma) was hydrolysed by adding 400 mL of conc. HCl (12M) and stir for 6 hours at 0 °C with magnetic stirrer. Then stir for a while at 37 °C. The chitin hydrolysate was deprotinated by filtrate into 4,000 mL of chilled distilled water. The milk-like mixture was kept standing overnight at 4°C. The colloidal chitin was collected by centrifugation at 8,000 rpm for 15 min, then resuspended with distilled water to wash the pellet. The pellets were washed until the pH is between 5-7.

The colloidal chitin was resuspended in steriled distilled water containing 0.02% (w/v) sodium azide and kept at 4 °C. Take 1 mL to determine wet weight and dry weight. The colloidal chitin solution can be kept at this condition for at least 2 years.

APPENDIX B

Condition and primers for 16S RNA amplification

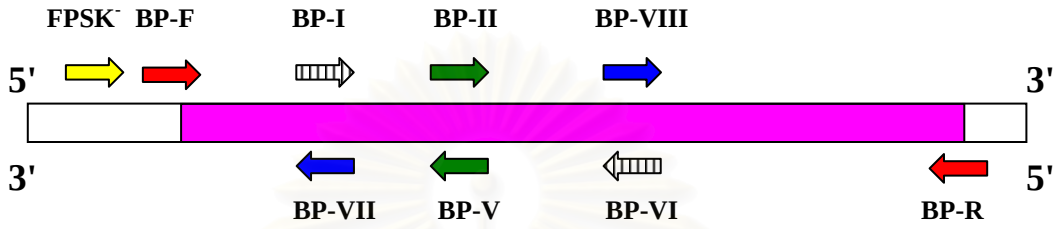


Concentration of ingredients ; In **1** reaction consist of :

Template (25 ng/µl)	16	µL	(1 µg)
Primer pA (1 pmol/µl)	10	µL	(10 pmol)
Primer pH' (1 pmol/µl)	10	µL	(10 pmol)
10x buffer	10	µL	(1x)
dNTPs (2.0 mM/µl)	10	µL	(20 mM)
$MgCl_2$ (25 mM/µl)	8	µL	(200 mM)
Tag (2 u/µl)	1.25	µL	(2.5 u)
Ultrapure autoclaved water	46.75	µL	
Total	100	µL	

APPENDIX C

Condition and primers for chitinase gene amplification



Concentration of ingredients ; In **1** reaction consist of :

Template (50 ng/µl)	1	µL	(1 µg)
Primer (1 pmol/µl)	10	µL	(10 pmol)
Primer (1 pmol/µl)	10	µL	(10 pmol)
10x buffer	2.5	µL	(1x)
dNTPs (2.0 mM/µl)	2	µL	(20 mM)
$MgCl_2$ (25 mM/µl)	1.6	µL	(200 mM)
Tag (2 u/µl)	0.25	µL	(2.5 u)
Ultrapure autoclaved water	12.15	µL	
Total	25	µL	

APPENDIX D

Preparation for polyacrylamide gel electrophoresis

1) Stock reagent

30% Acrylamide, 0.8% bis-acrylamide stock solution

Acrylamide	30.0	g
N,N'-methylene-bis-acrylamide	0.8	g

Adjust volume to 100 mL with distilled water

Tris-SDS stock solution, pH 6.8

Tris (hydroxymethyl)-aminomethane	3.94	g
Sodium dodecyl sulfate (SDS)	0.2	g

Adjust pH to 6.8 with 1 N HCl and adjust volume to 100 mL with distilled water

Tris-SDS stock solution, pH 8.9

Tris (hydroxymethyl)-aminomethane	3.94	g
Sodium dodecyl sulfate (SDS)	0.2	g

Adjust pH to 8.9 with 1 N HCl and adjust volume to 100 mL with distilled water

Ammonium persulphate solution (freshly prepared)

Ammonium persulphate	1.0	g

Dissolve in 1 mL distilled water

Sample buffer (5x)

Tris-SDS stock, pH 6.8	5.0	mL
SDS	0.4	g
Glycerol	3.0	mL
β-mercaptoethanol	1.0	mL
1% bromphenol blue	0.5	mL

Adjust volume to 10 mL with distilled water

Tris-glycine electrode buffer stock solution (5x)

Tris (hydroxymethyl)-aminomethane	3.03	g
Glycine	14.4	g
SDS	1.0	g

Adjust pH to 8.3 with 1 N HCl and adjust volume to 200 mL with distilled water

Staining solution

Dissolve 1.25 g of Coomassie brilliant blue R-250 in 500 mL of 95% methanol. Stir for one hour, add 500 mL of 15% glacial acetic acid and filter.

Destaining solution

10% glacial acetic acid and 10% methanol

2) SDS-PAGE

10% Seperating gel

30% Acrylamide stock solution	1.67	mL
Tris-SDS stock solution, pH 8.9	1.25	mL
TEMED	5	μL
10 % ammonium persulphate solution	50	μL
Distilled water	2	mL
1% glycol chitin	100	μL
Total volume	5	mL

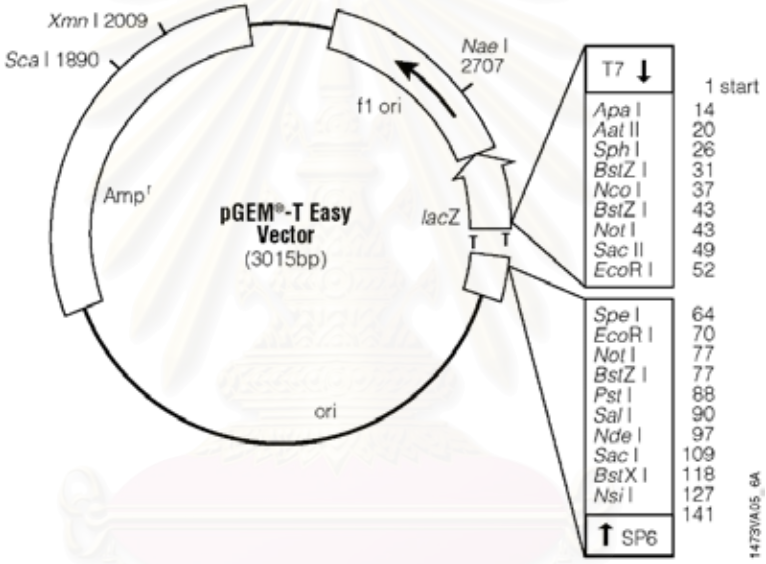
5% Seperating gel

30% Acrylamide stock solution	670	μL
Tris-SDS stock solution, pH 6.8	1	mL
TEMED	5	μL
10 % ammonium persulphate solution	30	μL
Distilled water	2.3	mL
Total volume	4	mL

Each four parts of sample was added one part of sample buffer (5x), mixed and heated 5 minutes in boiling watr before loading to the gel.

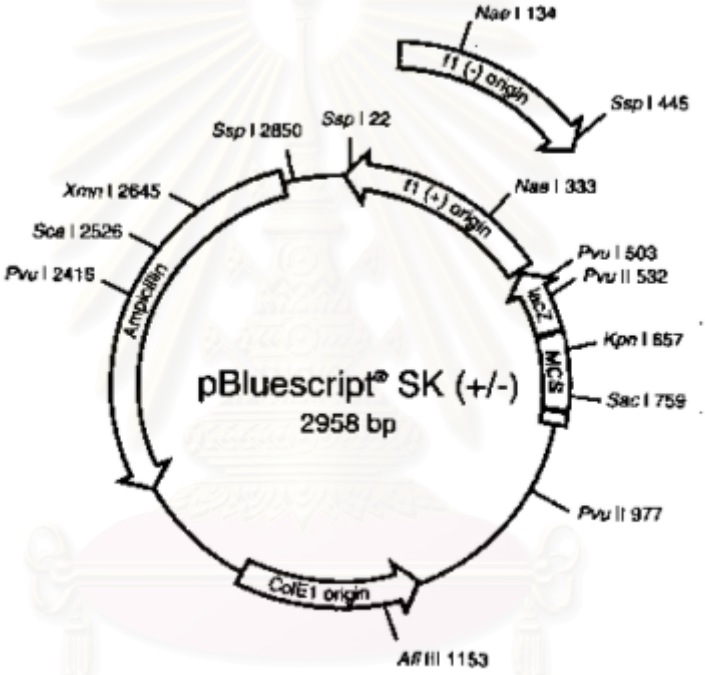
APPENDIX E

Restriction map of Plasmid pGEM®-T Easy



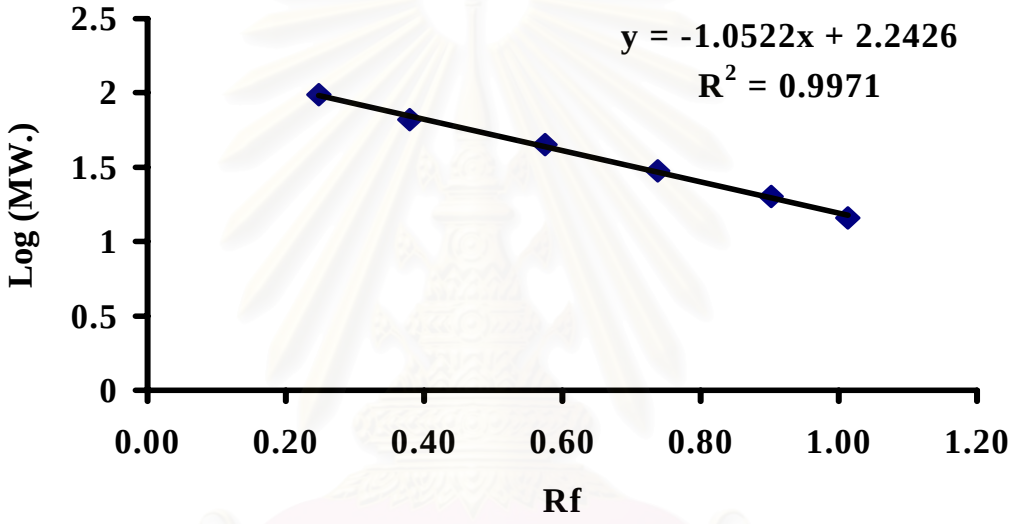
APPENDIX F

Restriction map of Plasmid pBluescript II KS$^-$



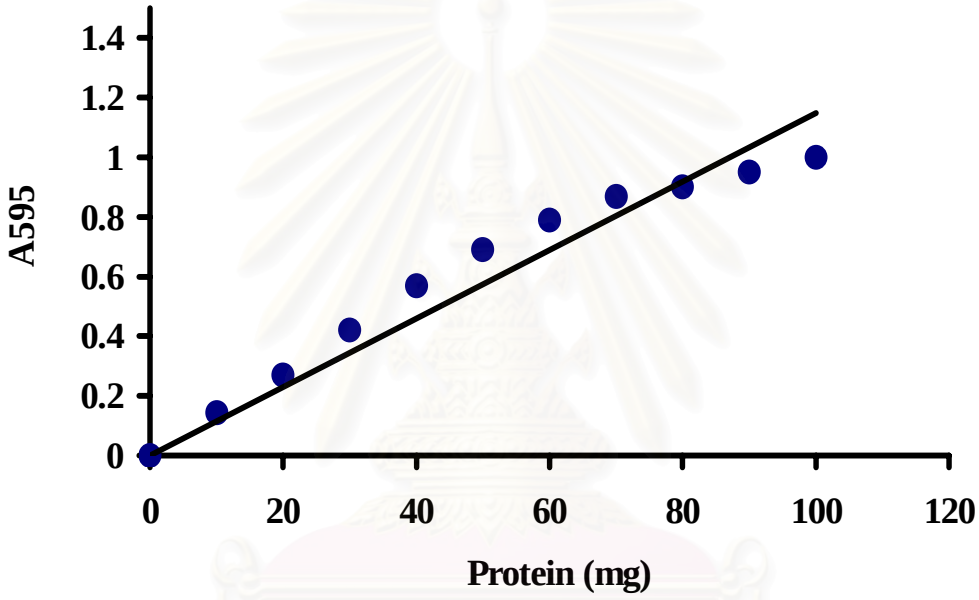
APPENDIX G

Standard curve for estimated molecular weight determination



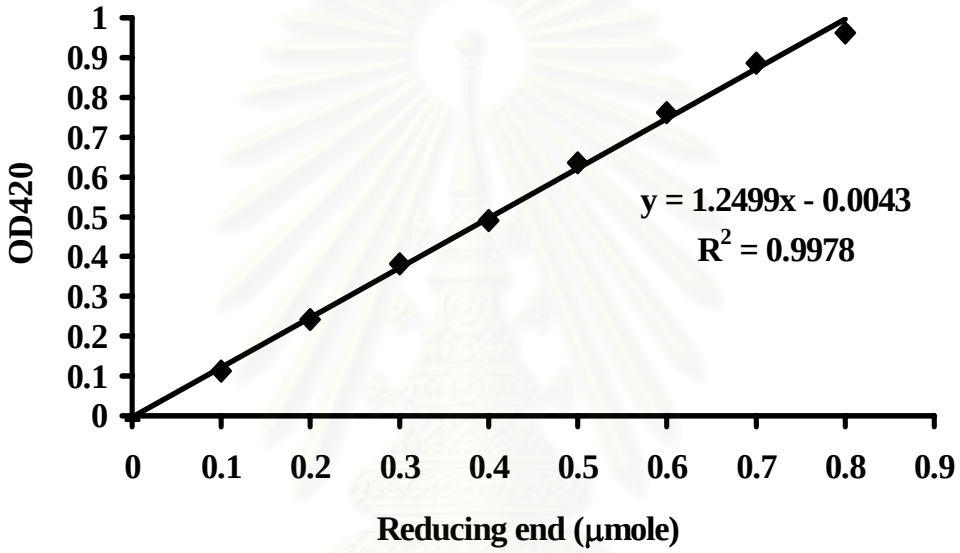
APPENDIX H

Standard curve for protein determination by Bradford's method



APPENDIX I

Standard curve for GlcNAc for colorimetric method



BIOGRAPHY

Mr. Prakarn Ruldeekulthamrong was born on December 25th 1975 in Bangkok, Thailand. He graduated with a Bachelor of Science (Health Science), Faculty of Science, Thammasat University in 1996. He was enrolled in the M.Sc.Biotechnology Program, Faculty of Science, Chulalongkorn University since 1997.