การโคลนและการหาลำดับนิวคลีโอไทด์ของยีนไคทิเนสจาก *Burkholderia cepacia* TU09

นางสาวกมลทิพย์ ขัตติยะวงศ์

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CLONING AND NUCLEOTIDE SEQUENCING OF CHITINASE GENE FROM *Burkholderia cepacia* TU09

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for the Degree of Master of Science in Biochemistry

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กมลทิพย์ ขัตติยะวงศ์: การโคลนและการหาลำดับนิวคลีโอไทด์ของยีนไคทิเนสจาก *Burkholderia cepacia* TU09 (CLONING AND NUCLEOTIDE SEQUENCING OF CHITINASE GENE FROM *Burkholderia cepacia* TU09) อ.ที่ปรึกษา: อ.ดร.รัฐ พิชญางกูร, 99 หน้า. ISBN 974-17-0681-2.

ไคทิเนส (EC 3.2.1.14) เป็นเอนไซม์ที่เร่งปฏิกิริยาการย่อยสลายไคทิน *Burkholderia cepacia* TU09 ที่แยกได้จากดินในประเทศไทย สามารถย่อย α-และ β –chitinได้ 2-acetamido-2-deoxy-D-glucose มากกว่า 80%ของผลผลิตในวันที่ 1 และ 7 ตามลำดับ นอกจากนี้ยังสามารถย่อย 45% deacetylated chitiosan และ colloidal chitinได้ดี รองลงมาคือ flake chitin และ crabs shells เมื่อทำการศึกษา SDS-PAGE พบโปรตีนที่มีไคทิเนสแอคติวิตีอย่างน้อย 4 แถบที่มีขนาดต่างๆ คือ 40, 50, 60 และ 90 kDa ตามลำดับ สภาวะความเป็นกรด-ด่าง และ อุณหภูมิที่เหมาะสมให้ไคทิเนสมีแอคติวิตีสูงสุด คือ 5.0/8.0 และ 37°C/55°C ตามลำดับ เมื่อนำโครโมโซมัลดีเอ็นเอมาตัดแบบไม่สมบูรณ์ แล้วทำ shot gun cloning เข้าสู่ *E. coli* JM109 โดยใช้ pBluescriptSK$^-$ เป็นดีเอ็นเอพาหะ พบหนึ่งโคโลนีจาก 7,000 โคโลนี ที่สามารถเกิดวงใสบนอาหารเลี้ยงเชื้อแบบแข็งที่มี colloidal chitin อยู่โดยทรานสฟอร์แมนท์มี pBluescriptSK$^-$ ที่มีชิ้นดีเอ็นเอขนาด 2.8 kb แทรกอยู่ pKKChi60 เมื่อนำมาหาลำดับนิวคลีโอไทด์ พบ open reading frame ขนาด 1689 bp เมื่อนำมาแปลรหัสเป็นกรดอะมิโนได้กรดอะมิโน 564 ตัว ซึ่งมีขนาด 60,942 Da โดยกรดอะมิโนที่ได้มีความคล้ายคลึงกับ ChiAของ *Serratia marcescens* นอกจากนี้ยังทำการศึกษาสมบัติบางประการของ Chi60 เมื่อนำ crude enzyme มาทำการศึกษาด้วย SDS-PAGE และย้อมสีแอคติวิตีพบแถบโปรตีนที่มีไคทิเนสแอคติวิตีขนาด 60 kDa จากอาหารเลี้ยงเชื้อ และจากเซลล์ที่ได้จากการเลี้ยงทรานสฟอร์แมนท์ที่มี pKKChi60 อยู่ สภาวะที่เหมาะสมที่ Chi60 มีแอคติวิตีมากที่สุดอยู่ระหว่างค่าความเป็นกรด-ด่าง 4.0-6.0 และอุณหภูมิ 50-60°C ตามลำดับ ส่วนการศึกษาการย่อยไคทินของ Chi60 พบว่า สามารถย่อย crystalline chitin (powdered chitin, α-chitin crab shells) พบว่ามีค่าการย่อยสัมพัทธ์เป็น 50% และ 70% ของแอคติวิตีในการย่อย soluble chitin และ amorphous chitin ตามลำดับ และเมื่อทำการศึกษาผลของการย่อย colloidal chitin โดย HPLC พบว่า Chi60 สามารถย่อย colloidal chitin ได้ *N, N′*diacetyl chitobiose เป็นส่วนใหญ่

ภาควิชา ..... **ลายมือชื่อนิสิต** .....

สาขาวิชา ..... **ลายมือชื่ออาจารย์ที่ปรึกษา**.....

ปีการศึกษา..... **ลายมือชื่ออาจารย์ที่ปรึกษาร่วม** .....-.....

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KEY WORD: CHITINASE / *Burkholderia cepacia* / CLONING

KAMONTIP KUTTIYAWONG: CLONING AND NUCLEOTIDE SEQUENCING OF CHITINASE GENE FROM *Burkholderia cepacia* TU09 THESIS ADVISOR: RATH PICHYANKURA, Ph. D., 99pp. ISBN 974-17-0681-2.

Chitinase (EC3.2.1.14) is an enzyme that catalyzes the degradation of chitin. *Burkholderia cepacia* TU09, isolated from soil in Thailand, is capable of producing chitinase. Chitinase from *B. cepacia* TU09 produced mostly 2-acetamido-2-deoxy-D-glucose from β-chitin and α–chitin, over 85% yield was produced within 1 day and 7 days, respectively. Crude chitinase was able to hydrolyze 45% deacetylated chitiosan colloidal chitin well, followed by flake chitin and crab shells. After SDS-PAGE and activity staining of crude enzyme, at least 4 bands of chitinase activity, molecular weight 40, 50, 60 and 90 kDa were detected. The pH and temperature optimum of the crude enzyme was 5.0/8.0 and 37°C/55°C, respectively. Chromosomal DNA of *B.cepacia* was partially cut and shotgun cloned into *E.coli*, JM109. Clones harboring chitinase gene were detected by the formation of clear-zone around the colony on agar plate containing colloidal chitin. One out of seven thousand colonies was found to produce clear-zone. The positive clone contained a plasmid with 2.8 kb insert fragment, pKK*Chi60*. DNA sequencing analysis revealed that pKK*Chi60* contained chitinase gene with an open reading frame of 1,689 bp, *Chi60*. *Chi60* encodes for a protein with 563 amino acid residues, and a deduced molecular weight of 60,942 Da. The amino acid sequence of *Chi60* exhibited over 90% homology to chitinase A of *Serratia marcescens*. After SDS-PAGE and activity staining of culture medium from the *E.coli* tranformant, a single band of chitinase activity, molecular weight 60 kDa was observed. Subsequently, partial characterization of Chi60 demonstrated that the optimum pH and temperature was between 4.0–6.0 and 50–60°C, respectively. The relative hydrolytic activity of Chi60 for crystalline chitin (α–chitin crab shells) were 50% and 70% of the activity observed on soluble chitin and amorphous chitin substrate, respectively. Analyses of the degradation product by HPLC showed that the cloned enzyme, Chi60, produces *N, N′*-diacetylchitobiose from chitin.

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

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

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ABBREVIATIONS

A	Absorbance
bp	Base pair
°C	Degree celcius
CCMM	Colloidal chitin minmum medium
DNA	Deoxyribonucleic acid
g	Gram
GlcNAc	*N*-acetyl-D-glucosamine
hr	Hour
kb	Kilobase
kDa	Kilodalton
L	Litre
M	Molar
mL	Mililitre
mg	Miligram
ng	Nanogram
µg	Microgram
µL	Microlitre
min	Minute
rpm	Revolution per minute

CHAPTER I

INTRODUCTION

Chitin

Chitin is an insoluble linear β-1,4-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc) (Figure 1.1). It is a common constituent of fungal cell walls, exoskeletons of insects, arachnids and many other groups of invertebrates, shells of crustaceans, and extracellular polymers of some bacteria. Chitin is the second most abundant biopolymer, after cellulose, which is a strong indication of its importance in nature. It is chemically similar to cellulose, a polymer of β-1, 4- linked glucose and its derivatives. Chitosan is a copolymer of β-1, 4- linked glucosamine and *N*-acetylglucosamine (Figure1.1). Chitin in nature is a polymer of *N*-acetylglucosamine, which are arranged in 3 crystalline structural forms, an antiparallel (α-chitin), parallel (β-chitin) and mixture between antiparallel and parallel form (γ-chitin) depending on the origin (Figure 1.2). Most chitins, including those from crustaceans, insects, and fungi, are in the α form. β-chitin has been identified in the spines of polychaete *Aphrodite*, pen of the squid *Loligo*, tubes of *Pogonophora*, and spines of certain marine diatoms. The third form, γ-chitin, has been reported from the stomach lining of *Loligo*.

In recent years significant research has been directed toward the use of chitin and chitin derivatives in many fields such as effluent water treatment and drug delivery (Brine, 1984). For industrial applications, chitin and its deacetylated form, chitosan, have been used to produce high value products such as cosmetics, drug carrier, food additives, semi-permeable membranes and pharmaceutical products (Table1.1) (Shahidi *et al.*, 1999; Riccardo and Martin, 1997 and Goosen, 1997).

Chitinase

Chitinase (EC 3.2.1.14) is a glycosyl hydrolase that catalyzes the hydrolytic degradation of chitin. Chitinases are found in a wide variety of organisms that possess chitin as well as bacteria, plants, and vertebrates. The roles of chitinases in these organisms are diverse (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. Bacteria produce chitinases to digest chitin and utilize it as carbon and energy source. Production of chitinases by higher plants is a part of the defense mechanisms against fungal pathogens (Boller, 1985).

Based on their amino acid sequences and mechanism of action, chitinases are grouped into two distinct 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 of higher plants. The crystal structures of these chitinases, e.g., chitinase B from *Serratia marcescens* (Perrakis *et al*. 1994) (Figure 1.3), hevamine from *Hevea brasiliensis* (Termisscha van Scheltirga *et al.*, 1994), endo-β- N-acetyl-glucosaminidase F_1 from *Flavobacterium meningosepticum* (Roey *et al*., 1994), and endo-β- N-acetyl-glucosaminidase H from *Streptomyces plicatus* (Rao *et al.*, 1995), reveal a α/β 8 barrel fold that is common to family 18 enzymes.

Family 19 chitinases are bilobal structures with an ancient core structure of α-helices and three stranded β-sheets. These chitinases include not only chitinase from plants in classses I, II, and IV, but also a bacterial chitinases, *Streptomyces griseus* HUT 6307 chitinases C (Ohno *et al.*, 1996). The crystal structure of the family 19 chitinases shows a completely different fold, which remotely resembles that of lysozyme (Tews *et al*., 1996) (Figure 1.3).

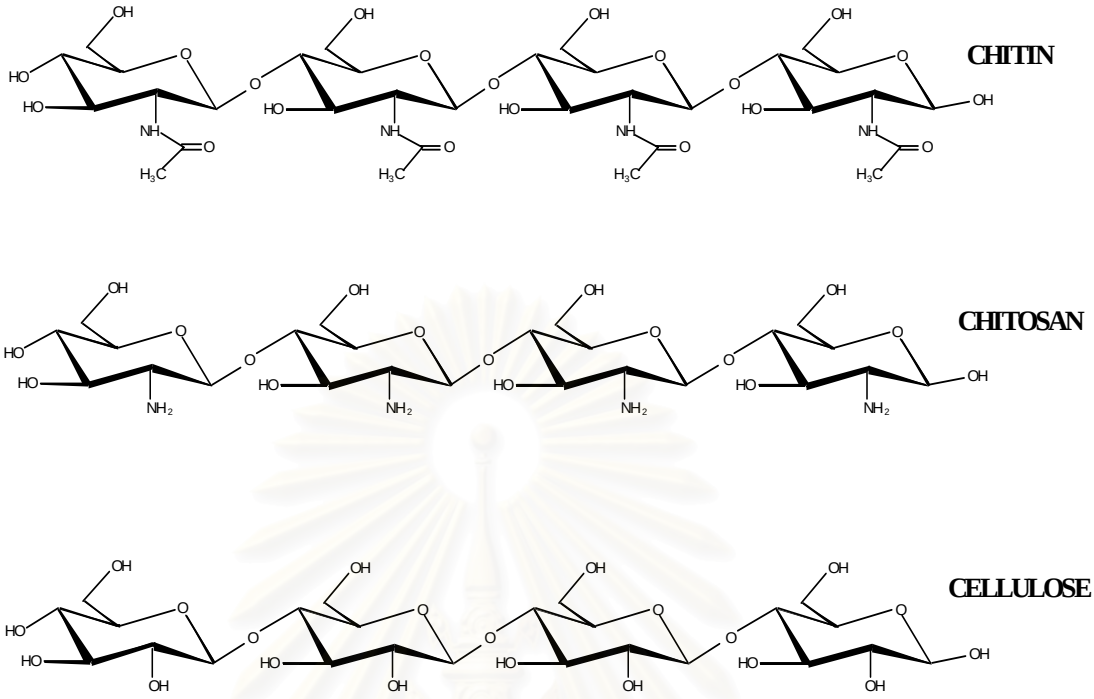


Figure 1.1 The structure of chitin, chitosan and cellulose.

A. α- Chitin **B. β- Chitin** **C. γ- Chitin**

Figure 1.2 Model of α-Chitin, β-Chitin and γ-Chitin. The direction of poly N-acetyl-D-glucosamine chain is marked with an arrow.

Table 1.1 Current practical uses of chitin, chitosan and their derivatives

Area of Application	Example
Agriculture	- Plant seed coating
	- Fertilizer
Food Industry	- Color stabilization
	- Emulsifying agent
	- Clarification and deacidification of fruits and beverages
	- Dietary fibre
	- Fiber-optic sensor for determination ethanol beverages and organic acid
Medical	- Dressing materials for the burns and skin lesions of humans and animals
	- Wound - dressings
	- Artificial limbs
	- Carrier-drug conjugates
Wastewater Treatment	- Recovery of metal ions, pesticides and phenols
	- Removal of dye
Cosmetics	- Moisturizer
	- Face, Hand and Body creams
Paper	- Surface Treatment
	- Photographic Paper
Biotechnology	- Enzyme immobilization
	- Chromatography
	- Ultrafiltration membranes

A.

B.

Figure 1.3 The ribbon drawing of a chitinase Family 18 and 19. The ribbon drawing of chitinase, (A) family 19 (barley chitinase) and (B) family 18 (chitinase B from *Serratia marcescens*) displayed by Rasmol 2.6.

Mechanisms of chitinases

Chitinases act by hydrolytically cleaving the β(1,4)-glycosidic linkages between GlcNAc residues. In general, this hydrolysis can occur in one of the two ways, either with retention of anomeric configuration in the product or with inversion.

Extensive studies of the mechanism of lysozyme show that glycoside hydrolysis requires two acid residues (Glu 36 and Asp 52), one of which is protonated. The consensus view of the mechanism (Scheme I, Figure 1.4) 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 similarity with hen lyzoyme, suggesting an analogous double displacement mechanism (Hart *et al*., 1995). However, subsequent analysis of the anomeric products for family 19 chitinases shows 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 lysozyme. 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.

The substrate binding cleft of barley chitinase has been hypothesized to contain at least six sugar binding subsites labeled A-F, from the non reducing end (Hart *et al*., 1995). The hydrolytic profile for hexasaccharides by barley chitinase suggests the preferred binding of substrates may be at sites B-G (Hollis *et al*., 1997), that is, hexasaccharides are cleaved into two

trisaccharides. This binding mode, together with the catalytic residues, is shown in scheme II, figure 1.4. Two carboxylates were hypothesized to be responsible for catalysis, Glu67 as a catalytic acid and Glu89 as a 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 by Fukamizo *et al*. 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 fashion. As indicated in Figure 3.5, 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 mechanism.

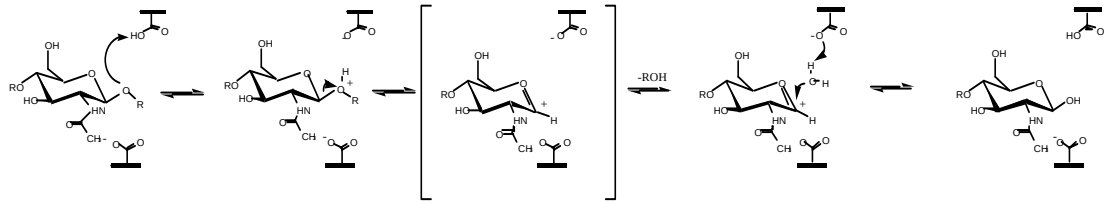
The earlier proposed catalytic mechanism (shown in figure 1.4, Scheme III) invoked a substrate assistance mechanism (Brameld *et al*., 1998). That is, the N-acetyl group at position 2 for the scissile sugar may itself facilitate the reaction via formation of a transient oxazolinium intermediate (Terwisscha *et al*., 1995 and Prouillard *et al*., 1997). Chitinase A from *Serratia*

marcescens has 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 is 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, *Bacillus circulans* chitinase, to a Gln was reported to essentially eliminate chitinase activity (Watanabe *et al*., 1993). The likehood of proton transfer in these reactions primarily depends on the distance between the proton donor and acceptor. These results indicated that Glu315 is the proton donor and the proton acceptor is the β- (1, 4) - glycosidic oxygen between sugar residues -1 and +1, the 2 residues spaning the active site where cleavage occurs.

The conformation of the sugar in the active site is also important for catalysis. It is evident that the 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 from the glycosidic oxygen and occasionally much closer when a direct hydrogen bond is made. From this evidence we may concluded that 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 active sites chitinase A of *Serratia marcescens.*

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

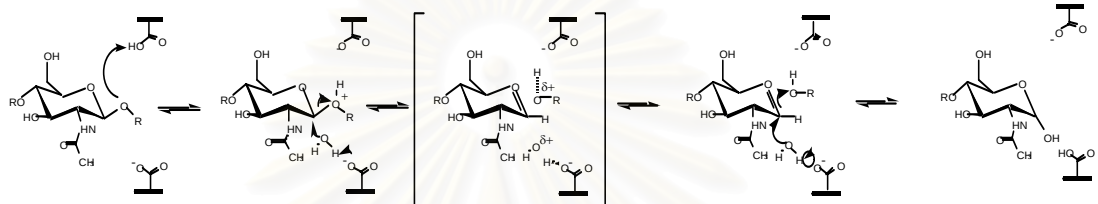
Scheme I



R = GlcNAc

Oxocarbenium TS

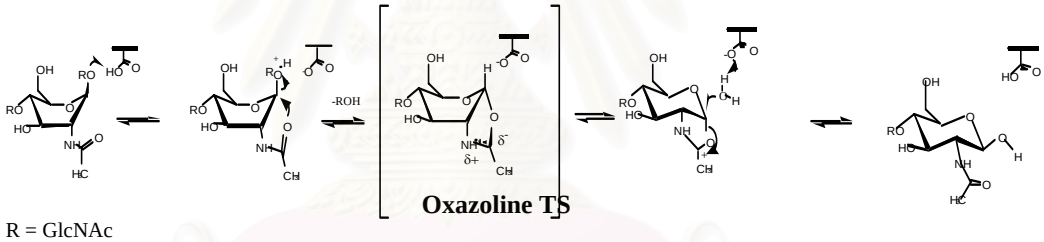
Scheme II



R = GlcNAc

Oxocarbenium TS

Scheme III



R = GlcNAc

Oxazoline TS

Scheme IV

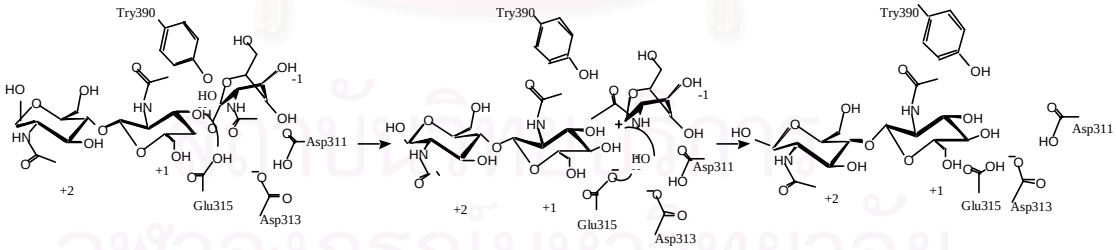


Figure 1.4 Chitinase mechanism Mechanisms of lysozyme (scheme I), family 19 chitinase(scheme II) and family 18 chitinase (scheme III, IV).

In this mechanism, we will not observe an oxazoline ring intermediate, the acetamido group of -1 sugar comes close to O5 atom in a way that could allow a modified “substrate assisted” reaction, shown in figure 1.4, scheme IV.

Biotechnological applications of chitinase

Practical applications of chitinase include its use 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 chitio-oligosaccharides as biologically active substances (Usui *et al*., 1990).

Chitinases are reported to dissolve cell walls of various fungi, a property that has been used for the generation of fungal protoplasts (Anjani Kumari and Panda, 1992 and 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). An enzyme complex from *Bacillus circulans* WL-12 with high chitinase activity was effective in generating protoplasts from *Phaffia rhodozyme* (Johnson *et al*., 1979). A mixture of commercially available chitinase and cellulose was used to release viable protoplasts from *Caprinus macrorhizus* (Yanagi and Takebe, 1984). Chitinase from *Trichoderma harzianum* was the most efficient generator of protoplasts from different fungi (Kitomoto *et al.*, 1988 and Anjani Kumari and Panda, 1992).

Chitinase-producing organisms are used in agriculture as an effective biocontrol agent against a number of phytopathogenic fungi (Kapat *et al*., 1996 and Elad *et al*., 1982). Chitinase from *T. harzianum* was found to be active against broadest range of pathogens and soil-borne fungi (Irene *et al*., 1994). *Aeromonas caviae* controlled infection *by Rhizoctonia solani* and *Fusarium oxysporum* in cotton and *Sclerotium rolfsii* in beans by producing chitinase (Inbar and Chat, 1991). Chitinase produced by *Serratia marcescens* was effective against pathogenic fungi *S. rolfsii* (Ordentlich *et al*., 1988) 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).

Chitinase-producing organisms are effectively used in the bioconversion process to treat shellfish waste and also to obtain value-added products from such wastes (Revah-Moiseev and Carroad, 1981; Tom and Carroad, 1981 and Carroad and Tom, 1978). Thus, chitinolytic enzymes have been purified and cloned from many microorganisms, and their enzymatic properties have been investigated.

Purification, characterization and molecular cloning of chitinase

To date, various chitinases have been isolated, purified and characterized from microorganisms such as *Bacillus circulans* WL-12 (Wanatabe *et al*., 1990), *Serratia marcescens* (Nawani and Kapadnis, 2001) and an A*eromonas sp*.10S-24 (Ueda *et al*., 1992). One-step purification of chitinase has been described by Roberts and Cabib (1982) using chitin affinity chromatography that a most specific method. It was used successfully for the purification of the chitinases from various organisms. However, this method cannot always be used, as problems in the binding or the release of chitinase occurs. Brurberg *et al*. (1994) purified chitinase from *E. coli* culture carrying *chiA* gene of *S. marcescens* using single step hydrophobic interaction chromatography. In addition, the gel filtration chromatography system was developed for purification of chitinase from *S. marcescens*NK1 in one step (Nawani and Kapadnis, 2001). A single step is generally inadequate for obtaining pure protein. Additional steps such as gel filtration may be used. The research on the purification of chitinases is summarized in Table 1.2. 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 are active at a wide pH range of 4.0-8.0 and a temperature range of 40-55 °C.

Several genes encoding chitinases were cloned and sequenced such as Chitinase A1 of *Bacillus circulans* WL-12(Wanatabe *et al*., 1990), ChitinaseA, B and C of *Serratia marcescens* 2170 (Watanabe *et al*., 1997 and Suzuki *et al*., 1999), an A*eromonas caviae* (Sitrit *et al*., 1995) and an *Alteromonas sp.* strain O-7 (Tsujibo *et al*., 1992). A summary of the research on cloning of microbial chitinase genes is given in Table 1.3.

In this study, chitinase from bacteria was chosen because bacterial chitinase is a simple cultivation, highly quantity of enzyme production and not difficult to purify enzyme. I chose to study *Burkholderia cepacia* because it was an active producer of chitinase. It is one of bacteria which secrete chitinase into culture medium and shows a visible clear zone when grown on colloidal chitin medium plate.

Burkholderia cepacia

Burkholderia cepacia is a gram-negative bacterium that is currently attracting considerable attention for its extraordinary versatility as a plant pathogen, saprophyte, biocontrol agent, bioremediation agent and human pathogen. Formerly known as *Pseudomonas cepacia*, this bacterium was first described in 1950 as the cause of sour skin of anions by Cornell University plant pathologist Walter Burkholder (Burkholder, 1950). *P. cepacia* was later renamed *Burkholderia cepacia* (Yabuuchi *et al*., 1992) and transferred to the beta subdivision of the proteobacteria (Olsen *et al*., 1994). *B. cepacia* is naturally abundant in soil, water and on plant surfaces (McArthur *et al*., 1988). It is distinctive in its ability to metabolize a broad range of organic compounds as carbon and energy source, an attribute which has spurred the development of *B. cepacia* for use in bioremediation of soil and ground water contaminated with chlorinated hydrocarbons (Krumme *et al*., 1993) and herbicides

Table 1.2 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					Tsujibo *et al*., 1992
Chitinase A	70,000	8.0	50	3.9	
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 B1	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 1.2 (Continued)

Source Organism	Molecular weight	Optimum pH	Optimum temp(°C)	pI	Reference
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
Streptomyces thermoviolaceus OPC-250					Tsujibo *et al*., 2000
Chi30	30,000	4.0	60	3.8	
Streptomyces RC1071	70,0 00	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

Table 1.2 (Continued)

Source Organism	Molecular weight	Optimum pH	Optimum temp(°C)	pI	Reference
Serratia marcescens QMB1466					Roberta *et al*., 1982
Chitinase A	58,000	4.0-7.0	N.D.	6.4	
Xanthomonas sp. strain AK					Yamaoka *et al*., 1999
Chitinase A	64,000	4.0	35	N.D.	
Chitinase B	48,0 00	6.0	0	N.D.	

Table 1.3 Molecular cloning of chitinase genes

Source Organism	Host	Vector	DNA insert sizes (kb)	ORF (kb)	Reference
Aphanocladium album	*E. coli*	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. coli*	pBluesccriptIISK$^-$	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. coli*	pKK223-3	4.0	2.1	Wanatabe *et al*., 1990
Chitinase C	*E. coli*	pUC19	2.8	1.5	Wanatabe *et al*., 1995
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 1.3 (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*	pBluescriptIISK	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

(Sangodkar *et al.*, 1988). *B. cepacia* has also been the focus of considerable research by plant pathologists who have shown it to be an effective biocontrol agent against soil borne (Cartwringht and Benson, 1995 and Mao *et al*., 1997), foliar (Joy and Parke, 1994) and post-harvest diseases (Janisiewicz *et al.*, 1991 and Smilanick *et al.*, 1993). Many strain of *B. cepacia* produce one or more antibiotics actively against a broad range of plant pathogenic fungi (Rosales *et al.*, 1995). These antibiotics appear, in many cases, to be important for disease suppression. Biocontrol with *B. cepacia* can be an effective substitute for chemical pesticides. However some strains can cause fatal lung infection of individals with cystic fibrosis (CF), an inherited disease that impairs lung functions and makes them susceptible for opportunistic bacteria infections.

Three *B. cepacia* types, Wisconsin strains are registered by the U.S. EPA for use as microbial pesticides (biological control agents). The products include Blue Circle and Deny (Stine Microbial Products). Other strains of *B. cepacia* are currently being considered by EPA for experimental use permits or registration.

B. cepacia's capacity to propagate as an environment microbe and as an opportunistic pathogen may be due to its possession of large (more than four times that of *H. influenzae*, two times that of *E. coli*, and half as large as *P. aeruginosa*) complex and variable genome. The genome contains numerous insertion sequences and is divided into one to four circular replicons (Cheng and Lessie, 1994). A few other bacterial species of agricultural and medical importance also have multiple chromosomes, such as *Brucella melitensis, B. abortus, B. suis, B. ovis, Rhodobacter sphaeroides* and *Agrobacterium tumefaciens* (Allardet-Servent *et al.*, 1993; Suwanto and Kaplan, 1989 and Michaux *et al*., 1993). This unusual genomic arrangement may account for *B. cepacia*'s nutritional versatility. Such a division of genomic content would allow for high levels of homologous and illegitimate recombination. The resultant chromosomal rearrangements and associations could provide a basis for spontaneous "pulse" evolutionary spurts, such as that seen from soil to the

CF lung, suited for rapid adaptation to radical changes in environmental growth conditions.

In this study, chitinase gene from *Burkholderia cepacia* TU09 was clone and nucleotide sequence was determined. In addition, the properties of chitinase from the culture supernatant of *B. cepacia* and the transformant carrying one of the chitinase genes, Chi60 was described.

CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

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

Autopipette: Pipetteman, Gilson, France

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

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

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

Incubator: Model 1H-100, Gallenkamp, England

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

Incubator, waterbath: Model M20S, Lauda, Germany

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

pH meter: Model PHM95, Radiometer Copenhegen, Denmark

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

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

Thermolyne dri-bath: Sybron corporation, U.S.A.

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

Water bath

2.2 Chemicals

Acrylamide: Merck, U.S.A.

Agarose: GIBCOBRL, U.S.A.

Aqua sorb: Fluka, Switzerland

Ammonium persulphate: Sigma, U.S.A.

Ampicilin: Sigma, U.S.A.

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

β-mercaptoethanol: Fluka, Switzerland

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

Bovine serum albumin: Sigma, U.S.A.

Bromophenol blue: Merck, U.S.A.

Chloroform: BDH, EnglandCongo Red: Sigma, U.S.A.

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

Dialysis Tube: 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 *Hind* III: GIBCOBRL, U.S.A.

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

Ethidium bromide: Sigma, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

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.

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

Magnesium sulphate-7-hydrate: BDH, England

Methanol: Merck, Germany

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

NNN′N′- Tetramethyl-1,2-diaminoethane: Carlo Erba Reagenti,

Italy

Phenol: BHD, England

Potassium ferricyanide: BDH, England

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

Qiaquick Gel 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: Boehringer Mannheim Gmbtt, Germany

Sodium hydroxide: Merck, Germany

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

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

TritonX-100: Merck, Germany

Tryptone: DIFCO, U.S.A.

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

Yeast extract: DIFCO, U.S.A.

2.3 Enzymes and Restriction Enzymes

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.

Calf Intestine Alkaline Dephosphorylase (CIAP): New England BioLabs, Inc., U.S.A.

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

2.4 Bacterial Strains

Burkholderia cepacia TU09, isolated from Bangkok, Thailand, South-East Asian soil.

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 (Dower, 1990) 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)] (Dower, 1990) 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 (Dower, 1990) was purchased from GIBCOBRL, U. S.A.

2.5 Media Preparation

2.5.1 Luria-Bertani (LB) medium (Maniatis *et al*., 1982)

LB consisted of 1.0% tryptone, 0.5% yeast extract and 1.0% NaCl. pH was adjusted to pH 7.2 with NaOH. For solid medium, 2% agar was added. Medium was steriled by autoclaving at 121°C for 15 minutes.

2.5.2 Colloidal chitin minimum medium (CCMM)

Medium for cultivation and enzyme production from *B. cepacia* TU09 contained 0.2% colloidal chitin (wet weight), 0.05% yeast extract, 0.1% $(NH_4)SO_4$, 0.03% $MgSO_4.7H_2O$, 0.6% KH_2PO_4 and 1% K_2HPO_4 with pH 7.2. For solid medium, 1.5 - 2% agar was added. Medium was sterilized as described above.

2.6 Cultivation of Bacteria

2.6.1 Starter inoculum

A colony of *Burkholderia cepacia* TU09 was grown in 2 mL of LB medium at 30°C and a colony of *E.coli* carrying pBSSK $^{-}$ and its derivatives were grown of LB medium containing 100 μg/mL ampicillin at 37°C for 12-16 hours.

2.6.2 Culture conditions

Starter *Burkholderia cepacia* TU09 was diluted 1:100 into 100 mL of CCMM containing 100 μg/mL ampicillin in 250 mL Erlenmeyer flask. The culture was incubated at 30°C for chitinase production and was grown in LB medium containing 100 μg/mL ampicillin for chromosomal DNA extraction. Cells were collected by centrifugation at 5,000 rpm for 20 minutes at 4°C. For chitinase production, culture broth with crude chitinase enzyme was concentrated by aqua sorb and then dialyzed with 10 mM phosphate buffer (pH 7.0). Concentrated crude chitinase enzyme was kept at 4°C for characterization.

Culture condition of *E.coli* contained chitinase gene (*Chi60*), was the same as described above, but it was cultivated at 37°C.

2.7 Enzyme Assay

Chitinase activity was determined by modified Schale's method (Imoto, 1971), which was the determination of liberated reducing sugar from hydrolysis.

2.7.1 Determination of chitinase activity by measuring reducing sugar

Chitinase activity was assayed by measuring reducing sugar (Imoto and Yagishita, 1971) produced from a mixture containing 1 mg/mL of colloidal chitin and the desired amount of enzyme in 0.1 M buffer (1.5mL). The mixture was incubated at 37°C for 30 minutes. The reaction was stopped by adding 2.0 mL color reagent (made by dissolving 0.5 g of potassium ferric cyanide in 1 liter of 1.5 M Na_2CO_3) and heat to 100°C for 15 minutes. Small particles were removed from the mixture by centrifugation at 10,000 rpm for 10 minutes. The absorbance of the sample (A1) at 420 nm was measured by a spectrophotometer versus water. A blank value (A0) was obtained when denatured enzyme was used instead of the enzyme in the reaction. The different between A0 and A1 was used to estimate the amount of N-acetylglucosamine from standard curve. One unit (U) of enzyme activity was defined as the amount of an enzyme able to liberate 1 μmol product (as N-acetylglucosamine equivalent) per minutes.

2.8 Protein Concentration Determination

Protein concentration was determined by dye binding method (Bradford, 1976), using bovine serum albumin as a standard.

Eight hundred microliter of sample was mixed with 200 μL of Bradford working solution (5x) and left for 20 minutes before measuring the absorbance at 595 nm.

Bradford working solution (5x) contains 100 mg Coomassie Brilliant Blue G-250, 50 mL of 95% ethanol, 100 mL of 85% phosphoric acid and 50 mL of distilled water.

2.9 Extraction of Chromosomal DNA from *Burkholderia cepacia* TU09

Burkholderia cepacia TU09 was grown in LB broth (5 mL) at 30°C for 24 hours. Cells were harvested in 1.5 mL microcentrifuge tube by centrifugation at 5,000 x g for 5 minutes. Bacterial pellets were resuspended in 300 μL SET buffer (20 % sucrose 25 mM Tris–HCl and 10 mM EDTA, pH 8.0) then 200μL of 10 mg/mL lysozyme was added and mixed well. The mixture was incubated at 37°C for 1 hour then 5 μL of 10 % SDS and 3 μL of 20 mg/mL proteinase K was added and mixed by multiple inversion. The mixture was further incubated at 50°C overnight. After that, 50 μL of 3M sodium acetate was added and mixed. Then, equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added. After mixing by inversion, the mixture was centrifuge at 100,000 x g for 10 minutes. The upper aqueous phase was removed and precipitated by adding 0.6 volume of isopropanol. After gently inversion, fibrous strands of DNA were spooled out and dipped in 1 mL of 70% ethanol to remove excess salt. The DNA was air-dried then resuspended in 100 μL of TE buffer (10mM Tris-HCl and 1 mM EDTA, pH 8.0). The DNA was stored at 4°C.

2.10 Recombinant DNA Techniques

All basic recombinant DNA techniques such as, plasmid preparation, CIAP treatment, ligation and dideoxynucleotide sequencing were carried out using standard protocols (Sambrook and Russell, 2001).

2.11 Library construction

The chromosomal DNA of *B. cepacia* TU09 was partially digested with *Pst*I and separated on a 0.7% agarose gel in TAE buffer (0.004M Tris-acetate and 0.001M EDTA) at 50 volts. Lamda phage DNA cut with *Hind*III (λ/*Hind*III) was used as standard size marker. The gel segment corresponding to the DNA fragment size between 2 and 9 kb was cut out, and the DNA fragments in the gel were recovered by using QIA quick Gel Extraction Kit (Qiagen, Germany).

For shotgun cloning of the 2-9 kb DNA fragments, the DNA fragments were ligated to dephosphorylated *Pst*I-digested pBluescriptSK$^-$ and a portion of the ligation mixture was electrotransformed into host cell *E. coli* JM109, the method described by Dower *et al*.(1998), using a Gene Pulser and controller set at 25 μF capacitor, 2.5 kV and 200 Ω (Competent cells were prepared for electrotransformation (Dower, 1988)). After transformantion the cells were incubated at 37 °C for 1 hour in 1 mL of LB medium. The cells were spread onto the LB agar plate containing 100 μg/mL ampicillin, 40 μg/mL iso-1-thio-β-D-galactopyranoside (IPTG) and 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) per plate. Cells were grown at 37°C overnight. Transformants with inserts, white colonies were screened for plasmid harboring chitinase gene by phenotype screening described below.

2.12 Detection of Chitinase Gene (Phenotype screening)

Transformants were selected on LB agar plates containing 100 μg/mL ampicillin, 40 μg/mL IPTG and 40 μg/mL X-gal, final concentration. Cells were incubated at 37°C overnight. Then, white colonies were transferred in two replicates, onto LB agar plate containing ampicillin, IPTG and X-gal (concentration as mention before) and CCMM plate containing 100 μg/mL

ampicillin, 40 μg/mL IPTG, 0.5% yeast extract and 0.02% (dry wet) colloidal chitin. The LB agar plates were incubated at 37°C overnight and stored at 4°C. The CCMM plates were incubated at same temperature for 14 days, then the transformants carrying chitinase gene were detected by the formation of a clear zone around the colonies. Chitinase gene was grown on colloidal chitin plate with IPTG and without IPTG for 10 days. Then, the plasmid was extracted and retransformed to the other hosts (JM109, XL-1Blue and DH5α).

2.13 Mapping of recombination plasmid containing Chitinase Gene

The map of plasmid fragment containing chitinase gene was constructed from single and double digested pattern of the plasmid with chitinase gene digested with various restriction enzymes (*Bam*HI, *Cla*I, *Eco*RI, *Hind*III, *Kpn*I, *Nde*I, *Not*I, *Pst*I, *Sal*I, *Sma*I, *Spe*I, *Xba*I and *Xho*I), using the conditions recommended by the manufacturer (New England Biolab and Amersham Life Science). Digestion products were separated on 0.7, 1.2 and 1.8 % agarose gel operating at 50 volts in TAE buffer. The standard λ/*Hind*III and 100 bp marker was used.

2.14 Analysis of Chitinase Gene

Using the mapping result, various restriction fragments of pKKChi60 were subcloned for sequencing. A 1,000 bp *Sal*I-*Kpn*I fragment, 370 bp *Kpn*I-*Eco*RI fragment, 400 bp *Eco*RI-*Pst*I fragment and 700 bp *Sal*I fragment from pKKChi60 were subcloned into pBluescriptSK⁻. The nucleotide sequence was obtained by PCR cycle sequencing and LICOR automated sequencer. Then the deduced amino acid sequences from translation of the DNA sequences were aligned with the protein sequences in Genbank database using program clustal W (Thompson *et al*., 1994).

2.15 Characterization of Crude Enzyme

2.15.1 Chitinase production of Chi60

Transformant harboring chitinase gene (pKKChi60) was cultivated into minimum medium containing 0.2% (wet weight) colloidal chitin, 0.5% yeast extract and 100 μg/mL ampicillin, LB medium containing 0.2% colloidal chitin and 100 μg/mL ampicillin, and LB medium containing 100 μg/mL ampicillin at 37°C with 250 rpm rotary shaking for 7 days. Everyday, crude chitinase in cultured medium was collected and assayed for chitinase activity.

2.15.2 Optimum pH

Chitinase activity was measured at different pH values by assay method as described above using colloidal chitin as the substrate. The pH of reaction mixtures was controlled using the following buffer, 0.1M sodium citrate buffer (pH 3-6), 0.1M phosphate buffer (pH 6-8) and 0.1M Tris-HCl buffer (pH 8-10), final concentration.

2.15.3 Optimum temperature

The effect of temperature on enzyme activity was determined by incubating the reaction mixtures at different temperatures ranging from 30 - 80°C, and assaying the enzyme by measuring reducing sugar at pH 5.0 for cloned enzyme and crude enzyme from *B. cepacia* TU09.

2.15.4 Substrate specificity

The activity of chitinase was assayed on chitin and chitin-related compounds, including flake chitin, powder chitin, colloidal chitin, 45%deacetylated chitosan and 80%deacetylated chitosan. The activity was determined by measuring reducing sugar produced from a reaction mixture composed of 10 mg/mL of crystalline chitin substrate, 1 mg/mL of amorphous chitin substrate or 0.1 mg/mL soluble substrates and 0.075 U of enzyme from *B. cepacia* TU09 and Chi60 in 0.1M citrate buffer pH 5.0 for crude enzyme from *B. cepacia* TU09 and Chi60. The mixture was incubated at 37°C.

2.15.5 Estimation of molecular weight

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% or 12.5% acrylamide gel containing 0.001% (w/v) glycol chitin. Sample solution of enzyme were denatured by heating at 100°C for 5 minutes in 1x sample loading dye containing 15% (w/v) sucrose, 2.5 % (w/v) SDS, 125 mM Tris – HCl (pH 6.7), 15% (v/v) β-mercaptoethanol and 0.01% (w/v) Bromophenol blue. The gels were run using the Davis system. Prestained proteins marker, broad rang 6-175 kDa, was used. After electrophoresis, proteins were stained with 0.25 % Coomassie Brilliant Blue R-250 at room temperature for one hours then destaining with a mixture of 10 % (v/v) acetic acid and 25 % (v/v) methanol.

The protein band containing chitinase activity after SDS-PAGE was detected by incubating gel overnight at 37°C with reciprocal shaking in 100 mM sodium citrate buffer (pH 5.0) containing 1 % (v/v) Triton X-100. The gel was then stained with 0.01 % (w/v) Fluorescent Brightener 28 in 500 mM Tris-

HCl (pH 8.9) and destained with distilled water. Lytic zones in the gel were visualized under UV light.

2.15.6 Analyses of the degradation products of Chitinase

Crude enzyme was used to digest colloidal chitin, regenerated chitin and fine powder chitin. The reaction mixture contains 20 mg/mL of each substrate and 0.54U/mL of enzyme. The buffer used McIlvain pH 5.0 and all reactions were incubated at 37°C. The enzyme was dissolved or diluted into 1.0 mL of appropriate buffer then added half (500 μL) at beginning then the other half after 12 hours of incubation time. The product at the end of 24 hours was boiled for 15 minutes and centrifuge at 3,000 rpm in a bench-top centrifuge for 10 minutes. Then 300 μL of the supernatant was withdrawn and mixed with 557 μL of acetonitrile, then filtered through a 0.45 micron filter. The product was then analyzed by HPLC. The condition used was; Shodex Asahipak NH3P column, mobile phase 300 mL water: 700 mL acetonitrile, flow rate 1.0 mL/min.

The conditions use to analyzed the products of chitinase were the same for both crude and cloned enzyme.

2.15.7 Induction and repression of chitinase gene

Transformant carrying chitinase gene was grown on various medium plates such as 0.2% colloidal chitin minimum medium containing 100 μg/mL ampicillin either in the presence or absence of IPTG, 0.2% colloidal chitin minimum medium containing 100 μg/mL ampicillin and 0.5% glucose, LB medium containing 100 μg/mL ampicillin and 0.2% colloidal chitin and LB medium containing 100 μg/mL ampicillin, 0.2% colloidal chitin and 0.5% glucose. All plates were incubated at 37°C for 1-14 days, and chitinase activity

was observed by the formation of clearing zone around the colonies on the plate.

2.15.8 pH stability of Chi60

The effect of pH on enzyme stability at 4°C for different time, 0-72 hours and assaying the enzyme by measuring reducing sugar at 37°C, in 0.1M citrate buffer pH 5.0. The enzyme was incubated in buffer pH 3-10 at 4°C. A sample was removed every 24 hours to assay for the residual activity.

CHAPTER III

RESULTS

Several chitinases from bacteria have been cloned and expressed in *E. coli* (Brurberg *et al*., 1996 and Chernin *et al*., 1997). Furthermore, the structure of many chitinases have been elucidated (Perrakis, 1994, Roey *et al*., 1994, Rao *et al*., 1995, Hollis *et al*., 2000, and Yannis *et al*., 2001). *Burkholderia cepacia*, a rod shaped gram negative bacteria was isolated from soil in Bangkok, Thailand. It is one of the most efficient bacteria for degradation of chitin that we have characterized in our laboratory (Figure3.5).

In this study, I will report the cloning, nucleotide sequencing and partial characterization of a gene encoding one of the chitinase gene from *Burkholderia cepacia* TU09. The partial characterization of the crude enzyme from the native strain is also described.

Characterization of crude chitinase from *Burkholderia cepacia* TU09

Optimum pH

At the standard assay temperature of 37°C, the effect of pH on the enzyme activity is shown in Figure3.6. The optimum pH of chitinase was at pH 5.0/9.0. The enzyme retained more than 50% activity in a wide pH range of 4.0 to 10.0.

Optimum temperature

The effect of temperature on enzyme activity was determined at pH 5.0, in the range of 30-80°C. The optimum temperature for chitinase activity was

A.

B.

Figure3.5 ***Burkholderia cepacia*** **TU09** Clear zone on colloidal chitin minimum medium plate from *Burkholderia cepacia* TU09. The plate was incubated at 30°C for 3 days (A) and stained with 0.1% Congo red solution (B).

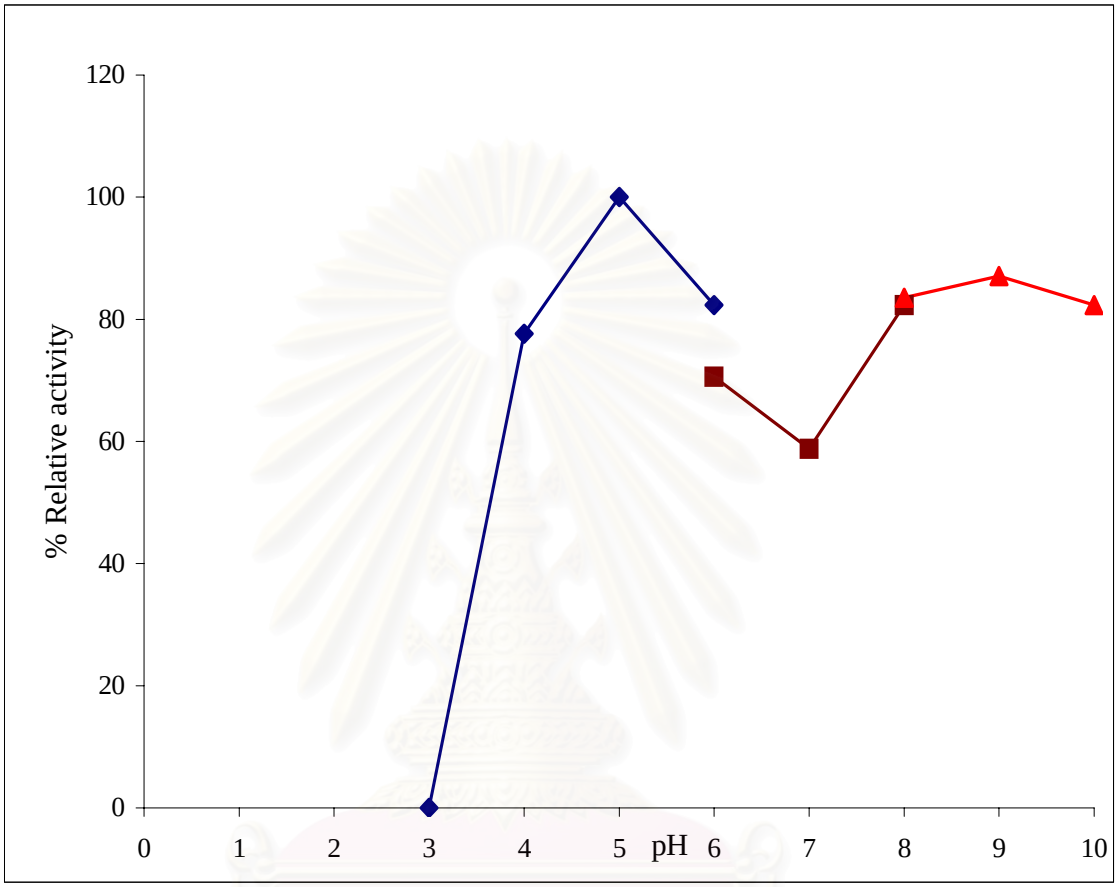


Figure3.6 Optimum pH of *B. cepacia* TU09 Effect of pH on crude chitinase activity. Chitinase activity was measured at pH range from 3-10 by measuring reducing sugar produced when colloidal chitin was used as substrate.

Buffer : pH 3-6, 0.1 M citrate buffer (♦)

: pH 6-8, 0.1 M phosphate buffer (■)

: pH 8-10, 0.1 M Tris-HCl buffer (▲)

37/55°C, as seen in Figure3.7. The enzyme retained more than 60% activity in temperature range of 30 to 60°C.

Substrate specificity of chitinase from *B. cepacia* TU09

The hydrolysis activity of chitinase on chitin and chitin-related substrates were determined at pH 5.0, 37°C. Crude enzyme has high activity towards 45% deacetylated chitosan, colloidal chitin, powder chitin, 80% deacetylated chitosan and flake chitin (Figure3.8).

Determination of proteins with chitinase activity from *B. cepacia* TU09

The extracellular proteins produced in minimum medium containing 1% colloidal chitin were analyzed by SDS-PAGE (Figure3.9). The observed molecular weight of chitinase activity bands were 40, 50, 60 and 90 kDa.

Hydrolytic products of crude enzyme from *B. cepacia* TU09

When colloidal chitin was hydrolyzed by the crude enzyme from *B. cepacia* at 37°C, GlcNAc was the major hydrolysis product, with small amounts of $(GlcNAc)_2$ and $(GlcNAc)_3$ (Figure3.10).

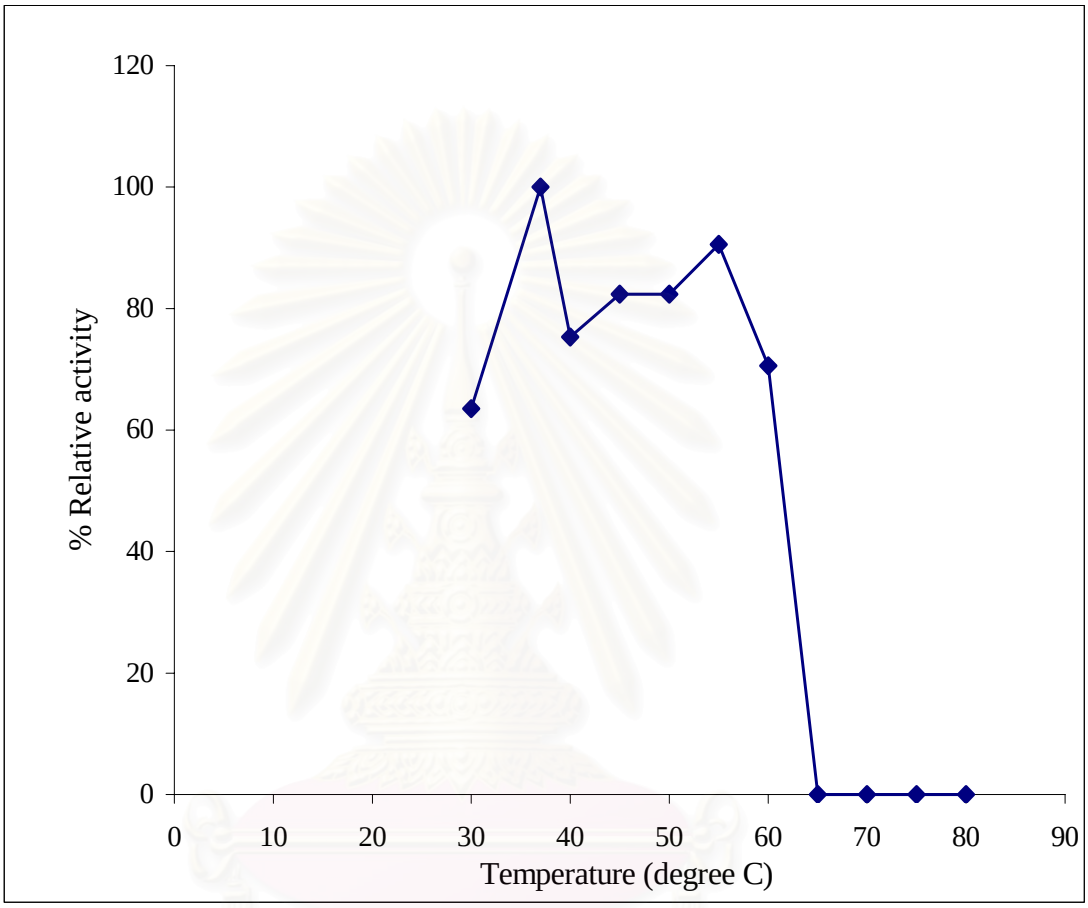


Figure 3.7 Optimum temperature of *B. cepacia* TU09 Effect of temperature on crude chitinase activity. Chitinase activity was measured at temperature range 30-80ºC in 0.1 citrate buffer pH 7.0 by measuring reducing sugar produced when colloidal chitin was used as substrate.

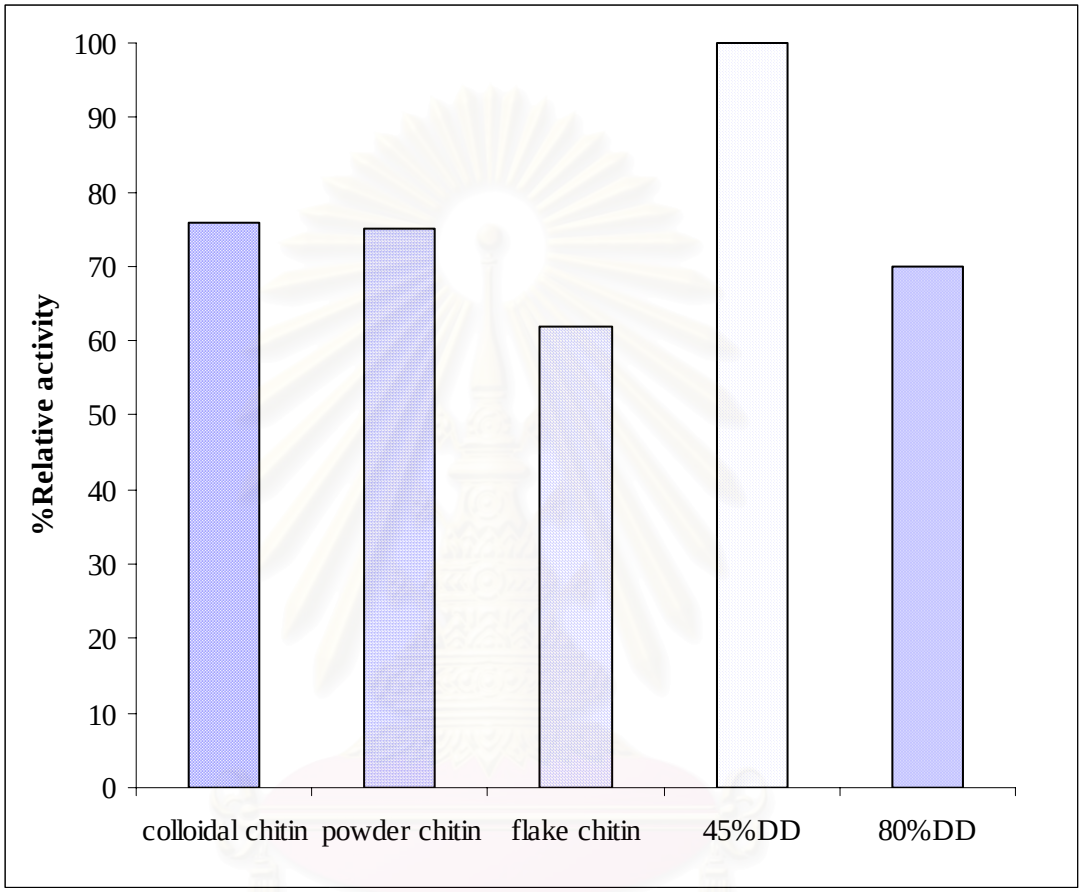


Figure3.8 Substrate specificity of crude enzyme from *Burkholderia cepacia* TU09 Chitinase activity was measured at 37°C in 0.1M citrate buffer pH 7.0 when colloidal chitin, powder chitin, flake chitin, 45% and 80% deacetylated chitosan were used as substrate.

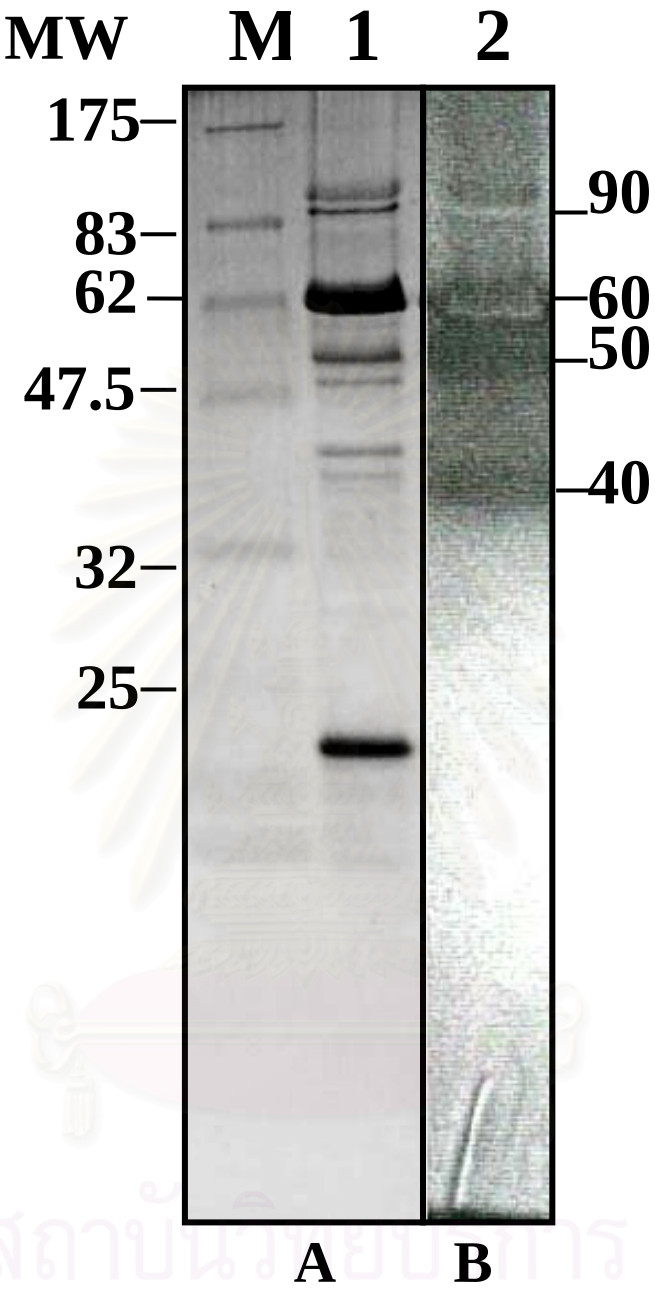


Figure 3.9 Determination of proteins with chitinase activity from culture medium of *B. cepacia* TU09 grown in CCMM The medium was removed after 2 days and the crude enzyme was collected by centrifugation.

panel A: Protein staining by Coomassie Brilliant Blue R-250

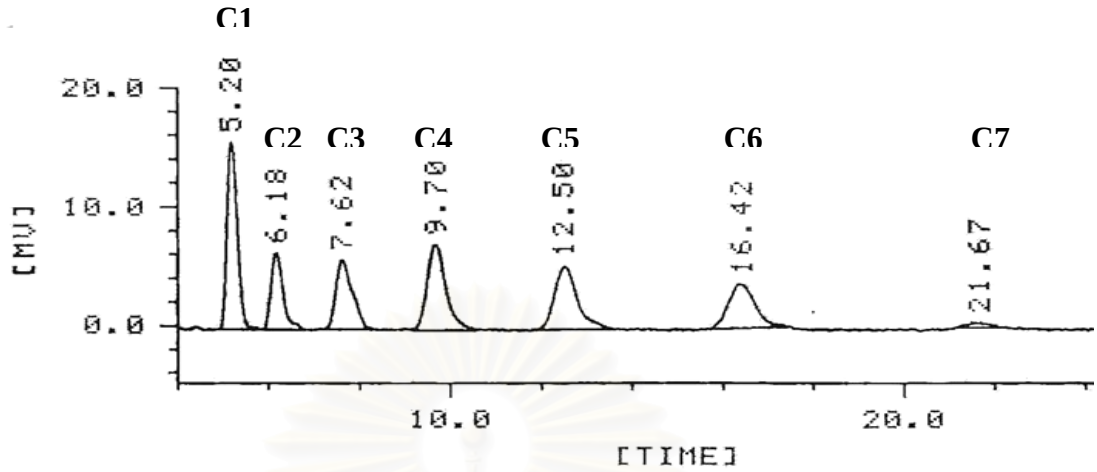
Lane M protein marker

Lane 1 crude protein in *B. cepacia* TU09' s culture medium

panel B: Chitinase activity staining

Lane 2 protein bands containing chitinase activity

A.



B.

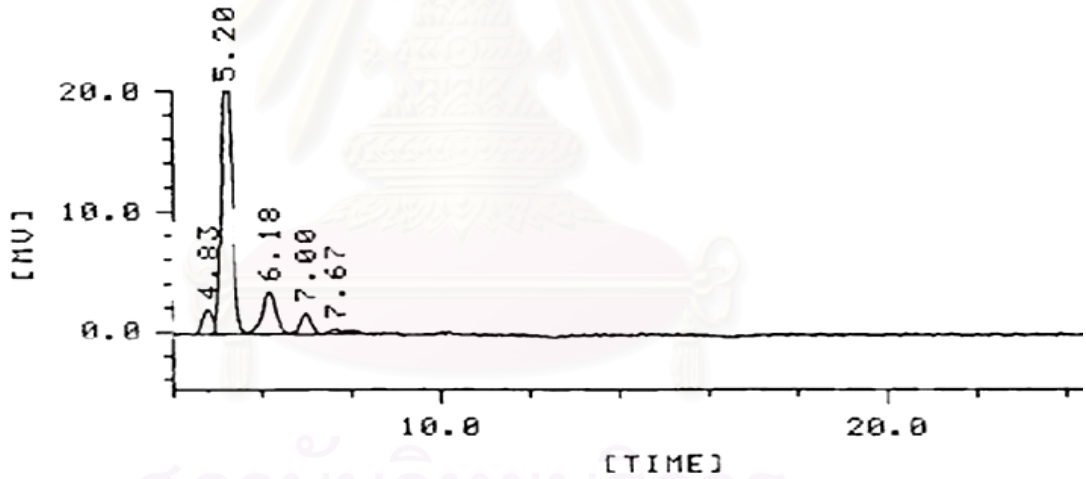


Figure3.10 Analysis of degradation product of crude enzyme from *B. cepacia* TU09 HPLC chromatogram of colloidal chitin hydrolyzed with crude chitinase at 37°C for 24 hours.

A. N-acetyl-chito-oligosaccharides standards: C1 GlcNAc, C2 $(GlcNAc)_2$, C3 $(GlcNAc)_3$, C4 $(GlcNAc)_4$, C5 $(GlcNAc)_5$, C6 $(GlcNAc)_6$ and C7 $(GlcNAc)_7$.

B Products of crude chitinase when colloidal chitin was used as substrate.

Cloning and characterization of chitinase gene from *Burkholderia cepacia* TU09

Cloning

One of the genes encoding chitinase from *B. cepacia* TU09 was cloned and sequenced. Partially *Pst*I-digested fragments of chromosomal DNA of *B. cepacia* TU09 were ligated with dephosphorylated *Pst*I-digested pBSSK$^-$ and transformed into *E. coli* JM109. Transformants carrying chitinase gene was selected by the formation of clear zone around the colonies on CCMM plate. After screening 8,000 transformant colonies, one chitinase-positive transformant was obtained (Figure 3.11). The positive clone contained a plasmid with a 2.8 kb inserted fragment, designated pKKChi60. The plasmid was retransformed into other hosts which include *E. coli* JM109, *E. coli* XL-1Blue and *E. coli* DH5α (Figure 3.12).

After retransformation of pKKChi60 into the other strains of *E. coli*, clear zone were detected in all hosts, XL-1Blue and DH5α. The expression of *Chi60* was better in XL-1Blue and DH5α than the original JM109 cells. Moreover, JM109 harboring the vector, pBSSK$^-$ and the pKKChi60 was grown on CCMM plate containing ampicillin with and with out IPTG to study the effect of IPTG on *Chi60* expression. Clear zone was observed from JM109 harboring pKKChi60 either in the presence or absence of IPTG (Figure 3.13).

Mapping of recombination plasmid containing chitinase gene

Then, the restriction map was determined. Single and double digestion of pKKChi60 with various restriction enzymes was shown in Figure 3.14, 3.15, 3.16 and 3.17. From the restriction mapping, the inserted fragment in pKKChi60 was subcloned (shown in Figure 3.18 and 3.19) and sequenced.

A.

B.

Figure 3.11 JM109 harboring pKKChi60 The positive cloned harboring plasmid with 2.8 kb inserted fragment grown on CCMM plate for 7 days, (A) and stained with 0.1% Congo red solution (B).

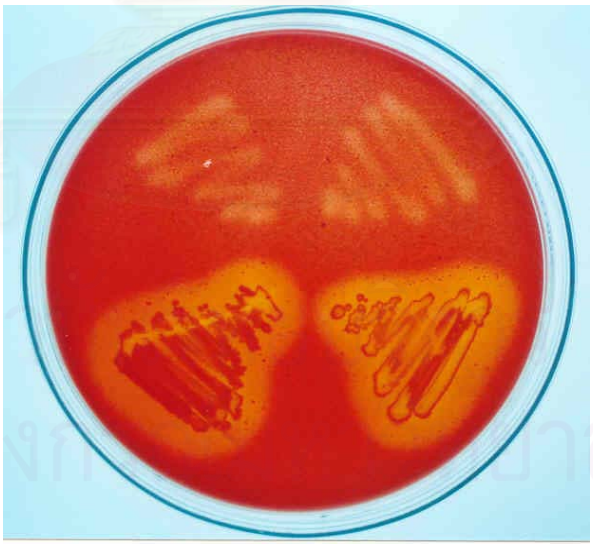
A.

JM109 **JM109retransform**

DH5α **XL-1Blue**

B.

JM109 **JM109retransform**



DH5α **XL-1Blue**

Figure 3.12 Expression of Chi60 in different *E. coli* strains different *E. coli* strains harboring pKKChi60 was grown on CCMM plate for 2 days (A) and stained with 0.1% Congo red solution (B).

A.

pKKChi60 pKKChi60

pBSSK$^-$ pBSSK$^-$

B.

pKKChi60 pKKChi60

pBSSK$^-$ pBSSK$^-$

Figure 3.13 The effect of IPTG upon chitinase production from *E. coli* JM109 carrying pKKChi60, grown on CCMM plate containing ampicillin with (A) and without IPTG (B).

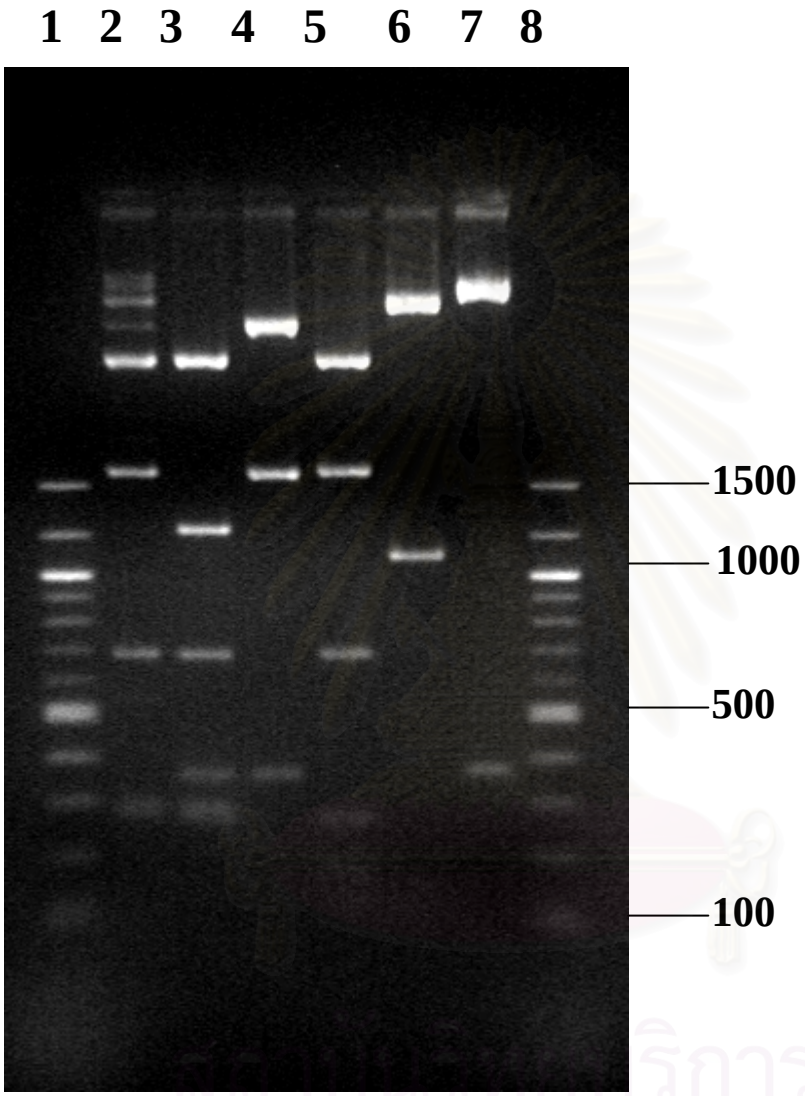


Figure 3.14 Restriction mapping of *Chi60* I 1.8% Agarose gel electrophoresis of single and double digested pKKChi60 with various restriction enzymes.

Lane1, 8 100 bp marker

Lane 2 pKKChi60/*Pst*I

Lane 3 pKKChi60/*Pst*I+*Eco*RI

Lane 4 pKKChi60/*Eco*RI+*Sal*I

Lane 5 pKKChi60/*Pst*I+*Sal*I

Lane 6 pKKChi60/*Kpn*I

Lane 7 pKKChi60/*Eco*RI

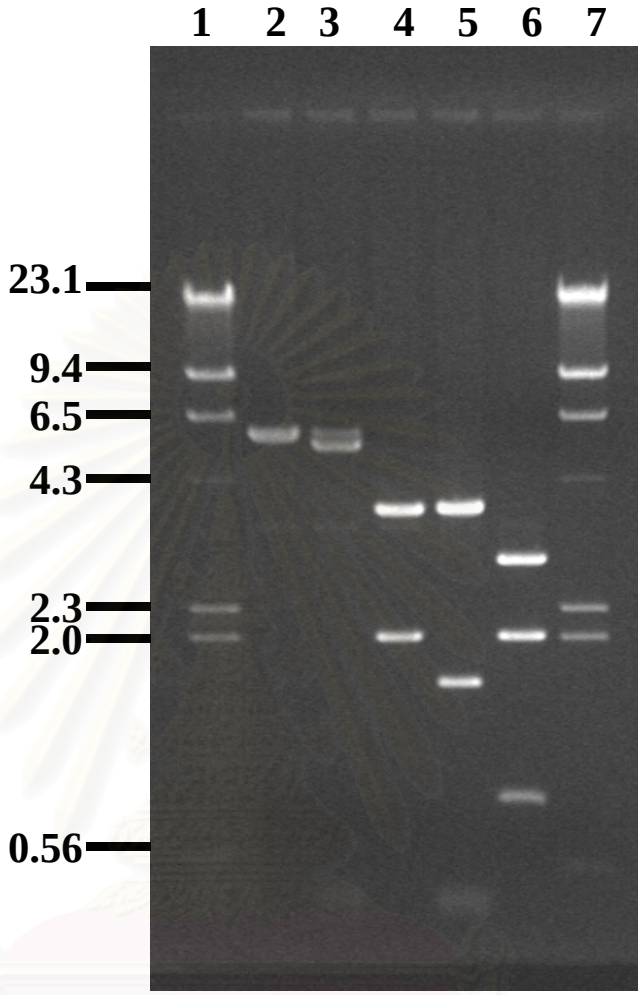


Figure 3.15 Restriction mapping of *Chi60* II 0.7% Agarose gel electrophoresis of single and double digested pKKChi60 with various restriction enzymes.

Lane1, 7 λ/*Hind*III marker
Lane 2 pKKChi60/*Cal*I
Lane 3 pKKChi60/*Eco*RI
Lane 4 pKKChi60/*Sal*I
Lane 5 pKKChi60/*Eco*RI+*Sal*I
Lane 6 pKKChi60/*Bam*HI+ *Sal*I

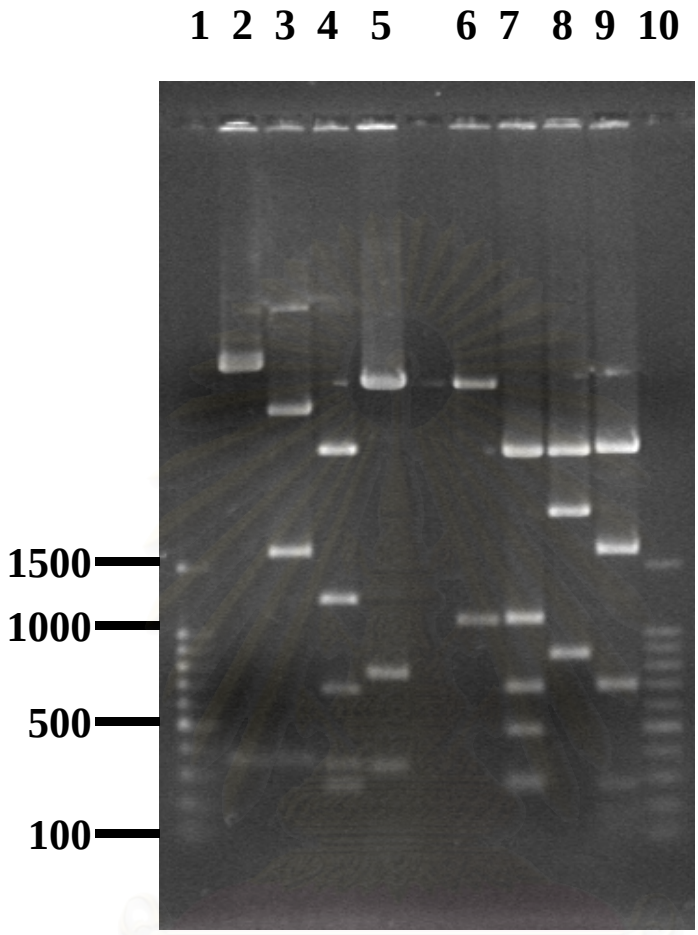


Figure 3.16 Restriction mapping of *Chi60* III 1.2% Agarose gel electrophoresis of single and double digested pKKChi60 with various restriction enzymes.

Lane1, 10 100 bp marker

Lane 2 pKKChi60/*Eco*RI

Lane 3 pKKChi60/*Eco*RI+*Sal*I

Lane 4 pKKChi60/*Eco*RI+*Pst*I

Lane 5 pKKChi60/*Eco*RI+*Kpn*I

Lane 6 pKKChi60/*Kpn*I

Lane 7 pKKChi60/*Kpn*I+*Pst*I

Lane 8 pKKChi60/*Bam*HI+*Sal*I

Lane 9 pKKChi60/*Pst*I+*Sal*I

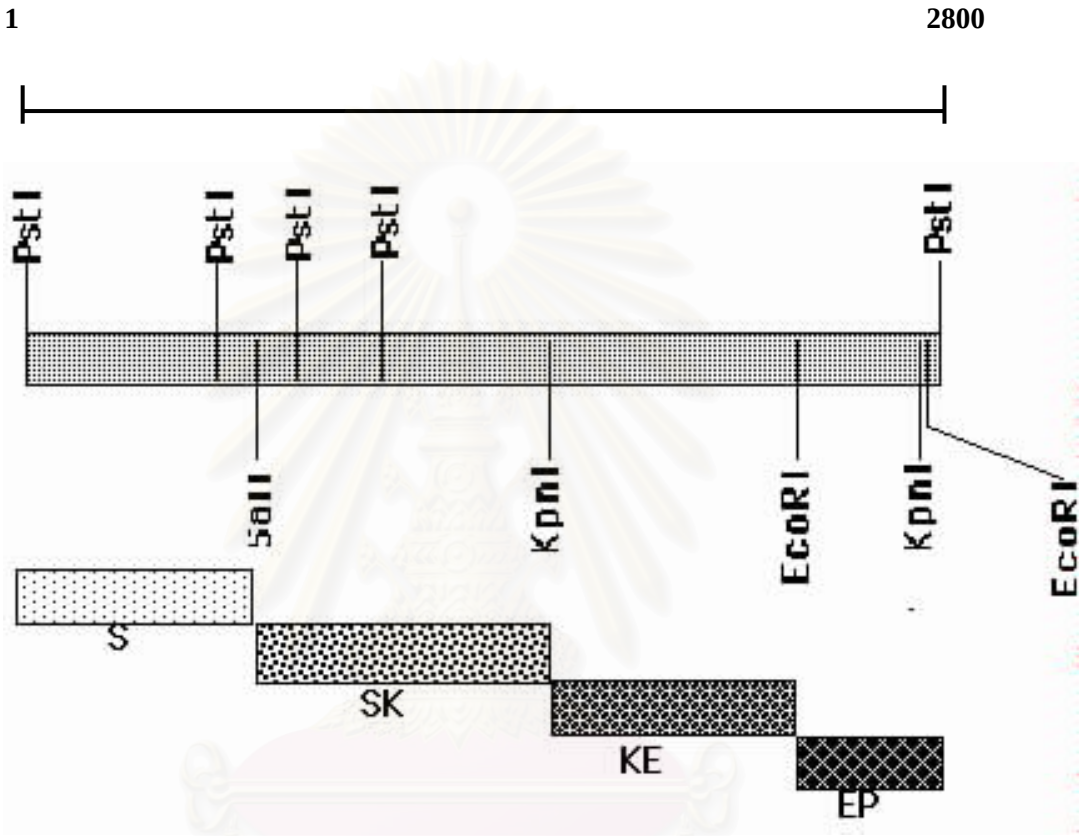


Figure 3.17 Restriction map of pKKChi60 and the subcloned DNA fragments. Abbreviation: S, 700 bp *Sal*I fragment, SK, 100bp *Sal*I-*Kpn*I fragment, KE, 370 bp *Kpn*I-*Eco*RI fragment and EP, 400 bp *Eco*RI-*Pst*I fragment.

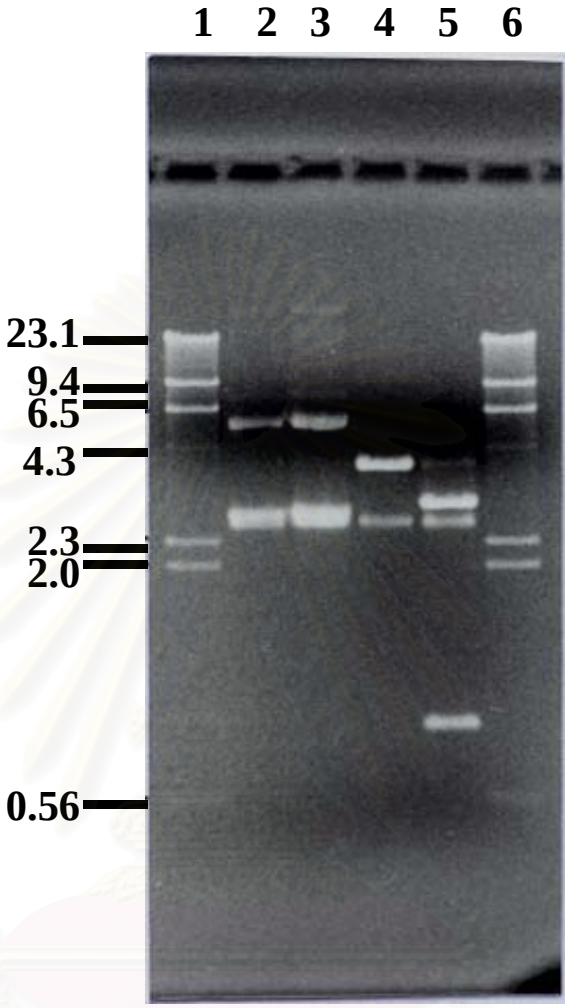


Figure 3.18 DNA fragment from *Sal*I digested pKKChi60 0.7% Agarose gel electrophoresis of pKKS that pBSSK$^-$ with inserted S fragment digested with *Sal*I.

Lane1, 6 λ/*Hind*III marker

Lane 2,3 pKK*S*

Lane 4 pKKS/*Sal*I

Lane 5 pKKSK/*Sal*I+*Bam*HI

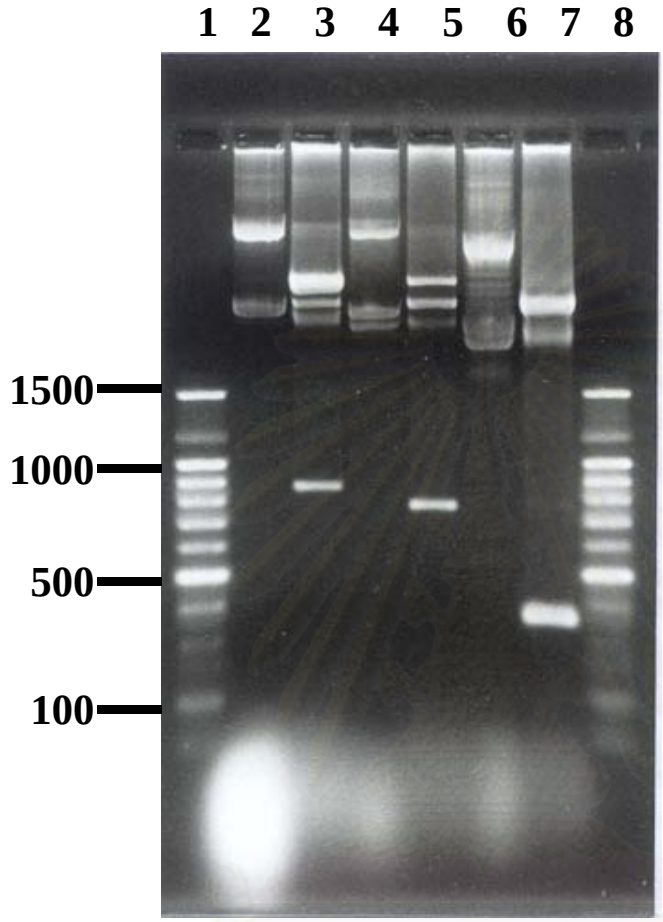


Figure 3.19 SK, KE and EP subcloned DNA fragment from pKKChi60
1.8% Agarose gel electrophoresis of pKKSK, pKKKE and pKKEP that pBSSK$^-$ with inserted SK, KE and EP fragment, respectively digested with restriction enzyme.

Lane1, 8 100 bp marker
Lane 2 pKKSK
Lane 3 pKKSK/*Sal*I+*Kpn*I
Lane 4 pKKKE
Lane 5 pKKKE/*Kpn*I+*Eco*RI
Lane 6 pKKEP
Lane 7 pKKEP/*Eco*RI+*Pst*I

Sequence 2847 BP; 662 A; 763 C; 654 T; 768 G.

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CTGCAGAGTG TGGTGCAATC CTGATAAATA TTTATCTTTC CTTAATAAAA AAATTCACTA        60
TCCATATTTG TCGTGCTTTC TTTTATTTAT ATTAAAATAA ATTCACGCTT GCTGAATAAA       120
ACCCAGTTGA TAGCGCTCTT GTTTTCACGC CTTTTTTATT TATAGTCTGA ATGTACGCGG       180
                   -35                        -10
TGGGAATGAT TATTTCGCCA CGTGGAAAGA CACTGTTGCT ATTTATTGAT TTTAATCTTC       240
GAGGATTATT GCGGAATTTT TTCGCTTCGG CAATGCATCG CGACGATTAA CTCTTTTATG       300
TTTATCCTCT CGGAATAAAG GAATCAGTTA TGCGCAAATT TAATAAACCG CTGTTGGCGC       360
TGTTGATCGG CAGCACGCTG TGTTCCGCGG CGCAGGCCGC GGCGCCGGGC AAGCCGACCA       420
TCGCCTGGGG CAATACCAAG TTCGCCATCG TTGAAGTTGA CCAGGCGGCT ACCGCTTATA       480
ATAGTTTGGT GAAGGTAAAA GATGCCGCCG ATGTTTCGGT CTCCTGGAAT TTATGGAATG       540
GCGACACCGG TACGACGGCA AAAGTTTTAT TAAATGGCAA AGAGGCGTGG AGCGGCCCGT       600
CAACCGGTTC TTCCGGTACG GCGAATTTTA AAGTCAATAA AGGCGGCCGT TATCAAATGC       660
AGGTGGCATT GTGCAATGCC GACGGCTGCA GCGCCAGCGA CGCCACCGAA ATTGTGGTGG       720
CCGACACCGA CGGCAGCCAT TTGGCGCCGT TGAAAGAGCC GCTGCTGGAA AAGAATAAAC       780
CGTATAAACA GAACTCCGGC AAAGTCGTCG GTTCTTATTT CGTCGAGTGG GGCGTTTACG       840
GGCGCAATTT CACCGTCGAC AAGATCCCGG CGCAGAACCT GACCCACCTG CTGTACGGCT       900
TTATCCCGAT CTGCGGCGGC AACGGCATCA ACGACAGCCT GAAAGAGATC GAAGGCAGCT       960
TCCAGGCGCT GCAGCGCTCC TGCCAGGGCC GCGAGGACTT CAAAGTCTCG ATCCACGATC      1020
CGTTCGCCGC GCTGCAAAAA GCGCAGAAGG GCGTTACCGC CTGGGATGAC CCCTACAAGG      1080
GCAACTTCGG CCAGCTGATG GCGCTGAAAC AGGCGCATCC TGACCTGAAA ATTCTGCCGT      1140
CGATCGGCGG CTGGACGCTG TCCGACCCGT TCTTCTTCAT GGGCGATAAG GTGAAGCGCG      1200
ATCGCTTCGT CGGTTCGGTG AAAGAGTTCC TGCAGACCTG GAAGTTCTTC GATGGCGTGG      1260
ATATCGACTG GGAGTTCCCG GGCGGCAAAG GCGCCAACCC GAACCTGGGC AGCCCGCAGG      1320
ACGGGGAAAC CTATGTGCTG CTGATGAAGG AGCTGCGGGC GATGCTGGAT CAGCTGTCGG      1380
CGGAAACCGG CCGCAAATAT GAACTGACCT CCGCCATCAG CGCCGGCAAG GACAAGATCG      1440
ATAAGGTGGC TTACAACGTT GCGCAGAACT CGATGGATCA CATCTTCCTG ATGAGCTACG      1500
ACTTCTATGG CGCCTTCGAT CTGAAGAACC TGGGGCATCA GACCGCGCTG AATGCGCCGG      1560
CCTGGAAGCC GGACACCGCT TACACCACGG TGAACGGCGT CAATGCGCTG CTGGCGCAGG      1620
GCGTCAAGCC GGGCAAGATC GTGGTCGGCA CCGCCATGTA TGGCCGCGGC TGGACCGGGG      1680
TGAACGGCTA CCAGAACAAC ATTCCGTTCA CCGGTACCGC CACTGGGCCG GTCAAAGGCA      1740
CCTGGGAGAA CGGCATCGTG GACTACCGCC AAATCGCCGG CCAGTTCATG AGCGGCGAGT      1800
GGCAGTATAC CTACGACGCC ACGGCGGAAG CGCCTTACGT GTTCAAACCT TCCACCGGCG      1860
ATCTGATCAC CTTCGACGAT GCCCGCTCGG TGCAGGCCAA AGGCAAGTAC GTGCTGGATA      1920
AGCAGCTGGG CGGCCTGTTC TCCTGGGAGA TCGACGCGGA CAACGGCGAT ATTCTCAACA      1980
GCATGAACGC CAGCCTGGGC AACAGCGCCG GCGTTCAATA ATCGGTTGCA GTGCGTTGCC      2040
GGGGGATATC CTTTCGCCCC CGGCTTTTTC GCCGCCGAAA GTTTTTTTAC GCCGCACAGA      2100
TTGTGGCTCT GCCCCGAGCA AAACGCGCTC ATCGGACTCA CCCTTTTGGG TAATCCTTCA      2160
GCATTTCCTC CTGTCTTTAA CGGCGATCAC AAAAATAACC GTTCAGATAT TCATCATTCA      2220
GCAACAAAGT TTTGGCGTTT TTTAACGGAG TTAAAAACCA GTAAGTTTGT GAGGTTCAGA      2280
CCAATGCGCT AAAAATGGCC GCTTAGCATA AATTTTCATG CTGGAACTGT TAACAAAAGG      2340
TTTTTTTTAT GTTTGTTTGC TGTTTCTCAC AGTCTGCGTA AATCCCCACT GGTTATATTG      2400
ACGACACCCC AAACAGTTGG CAACTTGATA GCCTCAGGGG TAAGAGCGAG AGTTGTTTGA      2460
GTGAATTCCA CGCGCTCAGA CGTCCCCGCC GCGATGCGTT CCATCCGGCA TTCTCTTCTC      2520
GTACGCCTTC TGCCTTCGGG CGCCGATCGC ACAGGCCACG CAATAAAAAA TACAGGTCTG      2580
GCGGCAATTA CACACATCAC ATCACACAAT GGAGCACTAA CGATGACACG TTCCTTGGGT      2640
AAATCGGGGA TTCTGAAATT CGGTATTGGG CTGATCGCGC TGACCGTGGC GGCCAGCGTA      2700
CAGGCCAAGA CGTTGGTTTA CTGTTCTGAA GGTTCCCCGG AAGGGTTCAA CCCGCAGCTG      2760
TTTACCTCCG GCACCACCTA TGACGCCAGC TCGGTACCAA TCTACAACCG GCTGGTCGAA      2820
TTCAAGACCG GCACCACCGA GCTGCAG                                          2847
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Figure 3.20 Nucleotide sequence of *Chi60* gene The fragment containing *Chi60* gene is 2847 bp in length. One open reading frame encoding for *Chi60* gene, was found from nucleotide 330 – 2021, encoding for a protein of 563 amino acid residues and predicted molecular weight of 60,900 kDa. The putative Shine-Dalgano (SD) sequence is double-underlined. The putative –10 and –35 sequences of the promoter of *Chi60* are underlined.

CLUSTAL X (1.64b) multiple sequence alignment

```
Burkholderia      MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNSLVKVKDAA
Serratia          MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNSLVKVKNAA
Enterobacter      MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNNLVKVKNAA
Pantoea           MRKFNKPLLALLIGSTLCSAAQAAAPGKPSIASGPTKFAIVEVDQQATAYNNLVKLKTAA
                  ******************************:** * ********** *****.***:* **

Burkholderia      DVSVSWNLWNGDTGTTAKVLLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGC
Serratia          DVSVSWNLWNGDTGTTAKVLLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGC
Enterobacter      DVSVSWNLWNGDAGTTAKILLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGC
Pantoea           DVSISWDLWSGDAGTTAKVLLDGKEVWSGASTGTSGTANFKVNKGGRYQMQVALCNADGC
                  ***:**:**.**:*****:**:***.***.***:*************************

Burkholderia      SASDATEIVVADTDGSHLAPLKEPLLEKNKPYKQNSGKVVGSYFVEWGVYGRNFTVDKIP
Serratia          SASDATEIVVADTDGSHLAPLKEPLLEKNKPYKQNSGKVVGSYFVEWGVYGRNFTVDKIP
Enterobacter      TASDATEIVVADTDGSHLAPLKEPLLEKNKPYKQNSGKVVGSYFVEWGVYGRNFTVDKIP
Pantoea           TASDVTQIVVADTDGSHLAPLKEPLLENNKPYKQDSGKVVGSYFVEWGVYGRNFTVDKIP
                  :***.*:********************:******:************************

Burkholderia      AQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDPFAALQKAQK
Serratia          AQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDPFAALQKAQK
Enterobacter      AQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDPFAALQKAQK
Pantoea           AQNLTHLLYGFIPVCGGDGINDSLKGVEGSFQALQRSCQGREDFKVSIHDPFAAVQKGQK
                  *************:***:******* :****************************:**.**

Burkholderia      GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF
Serratia          GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF
Enterobacter      GVTAWDDPYKGNFGQLMALKQARPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF
Pantoea           GVTAWDDPYKGNFGQLMALKQARPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF
                  **********************:************************************

Burkholderia      LQTWKFFDGVDIDWEFPGGKGANPNLGSPQDGETYVLLMKELRAMLDQLSAETGRKYELT
Serratia          LQTWKFFDGVDIDWEFPGGKGANPNLGSPQDGETYVLLMKELRAMLDQLSAETGRKYELT
Enterobacter      LQTWKFFDGVDIDWEFPGGKGANPNLGSPQDGETYVLLMKELRAMLDQLSAETGRKYELT
Pantoea           LQTWKFFDGVDIDWEFPGGGGANPKLGNAQDGATYVQLMKDLRAMLDQLSAETGRKYELT
                  ****************** ****:**.:*** *** ***:*******************

Burkholderia      SAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGAFDLKNLGHQTALNAPAWKPDTAYTT
Serratia          SAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGAFDLKNLGHQTALNAPAWKPDTAYTT
Enterobacter      SAISAGKDKIDKVAYNVAQNSMDHIFLMSYDFYGAFDLKNLGHQTALNAPAWKPDTAYTT
Pantoea           SAISAGKDKIDKVDYNTAQNSMDHIFLMSYDFYGAFDLKNLGHQTALKARPGNRHGLYHG
                  ************* **.******************************:* . : .  *

Burkholderia      VNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNGYQNNIPFTGTATGPVKGTWENGIVDYR
Serratia          VNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNGYQNNIPFTGTATGPVKGTWENGIVDYR
Enterobacter      VNGVNALLAQGVKPGKIVVGTAMYGRGWTGVNGYQNNIPFTGTATGPVKGTWENGIVDYR
Pantoea           ER-RQCVAGQGVKPGKIVVGAAKYGRGWTGVSGYQNNNPFTGTATGPVKGTWENGIVDYR
                    .  :.: .***********:* ********.***** **********************

Burkholderia      QIAGQFMSGEWQYTYDATAEAPYVFKPSTGDLITFDDARSVQAKGKYVLDKQLGGLFSWE
Serratia          QIAGQFMSGEWQYTYDATAEAPYVFKPSTGDLITFDDARSVQAKGKYVLDKQLGGLFSWE
Enterobacter      QIASQFMSGEWQYTYDATAEAPYVFKPSTGDLITFDDARSVQAKGKYVLDKQLGGLFSWE
Pantoea           QIANEFISDEWQYSYDATAEAPYVFKPSTGDLITFDDPRSVQAKGKYVLDKQLGGLFSWE
                  ***.:*:*.****:************************.**********************

Burkholderia      IDADNGDILNSMNASLGNSAGVQ
Serratia          IDADNGDILNSMNASLGNSAGVQ
Enterobacter      IDADNGDILNSMNASLGNSAGVQ
Pantoea           IDADNGDILNNMNTSLGNSAGAQ
                  **********.**:*******.*
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Figure 3.21 Alignment of the deduced amino acid sequence of *Burkholderia cepacia* Chi60 with several bacterial chitinases *Serratia marcescens*BJL200 ChiA, *Enterobacter sp*. ChiA and *Pantoea agglomerans* ChiA. The asterisks indicated identical residues, colon indicated that very similar residues and dot indicated similar residues.

DNA sequence analysis of the *Chi60* gene

The sequence of 2.8 kb of DNA fragment from pKKChi60 was determined from the subcloned fragment by the method of Sanger *et al.* (1997) (Figure 3.20). There was a 1,689 bp open reading frame encoding a 563 amino acid protein, with a calculated molecular weight of 60,900 Da. The amino acid sequence of Chi60 exhibited 99% homology to chitinase A of *Serratia marcescens* BJL200. As shown in Figure 3.21. Homology modeling of the structure of Chi60 was accomplished by Swiss-Model Protein Modeling (SWISS-MODEL version 36.0002) (Guex and Peitsch, 1997; Peitsch, 1995; and Peitsch, 1996) (Figure 3.22). Furthermore, the water accessible surface model of Chi60 generated by chime 2.6 program was shown in Figure 3.23.

Characterization of Chi60

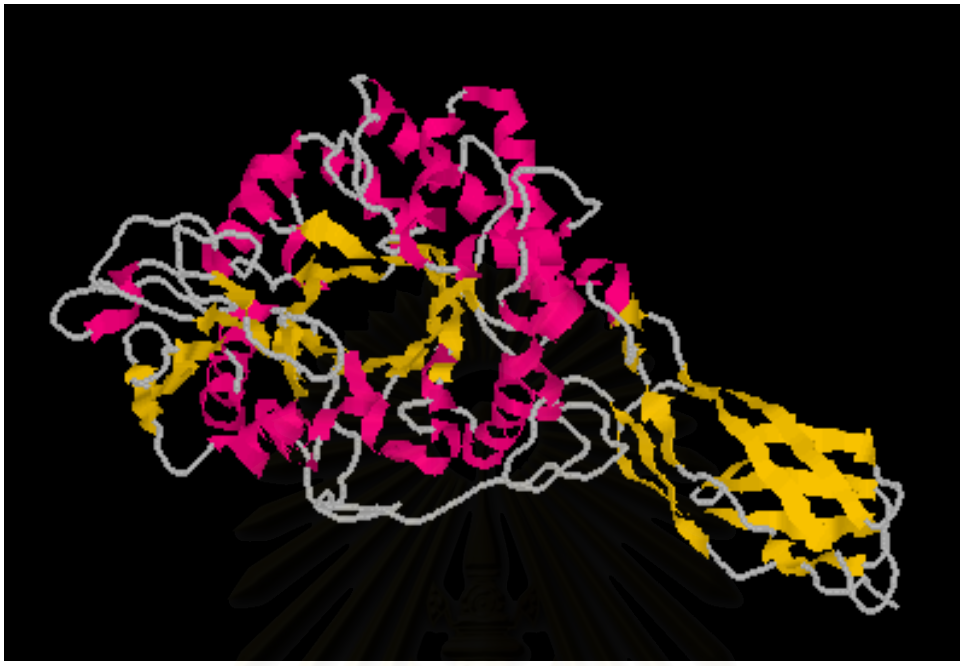
Chitinase production of Chi60

Chitinase activity was detected from *E. coli* XL-1Blue containing pKKchi60 when cultured in colloidal chitin minimum medium with 1% yeast extract, LB medium and LB medium with colloidal chitin. As shown in Figure 3.24. In three media, chitinase activity of Chi60 maximized in the second and third day of cultivation and remained constant until the seventh day. Chitinase activity detected in LB medium with colloidal chitin was higher than that detected in other medium, respectively.

Induction and repression of *Chi60* gene in *E. coli*

Transformant carrying chitinase gene was grown on medium plates including 0.02% colloidal chitin minimum medium plate, 0.2% colloidal chitin minimum medium plate containing ampicillin and 0.5% glucose, LB

A.



B.

Figure 3.22 Structure of Chi60 chitinase Structure of *Serratia marcescens* ChiA (A) displayed by Rasmol 2.6 and (B) Theoretical model of *Burkholderia cepacia* Chi60 accomplished by Swiss-Model Protein Modeling (SWISS-MODEL version 36.0002).

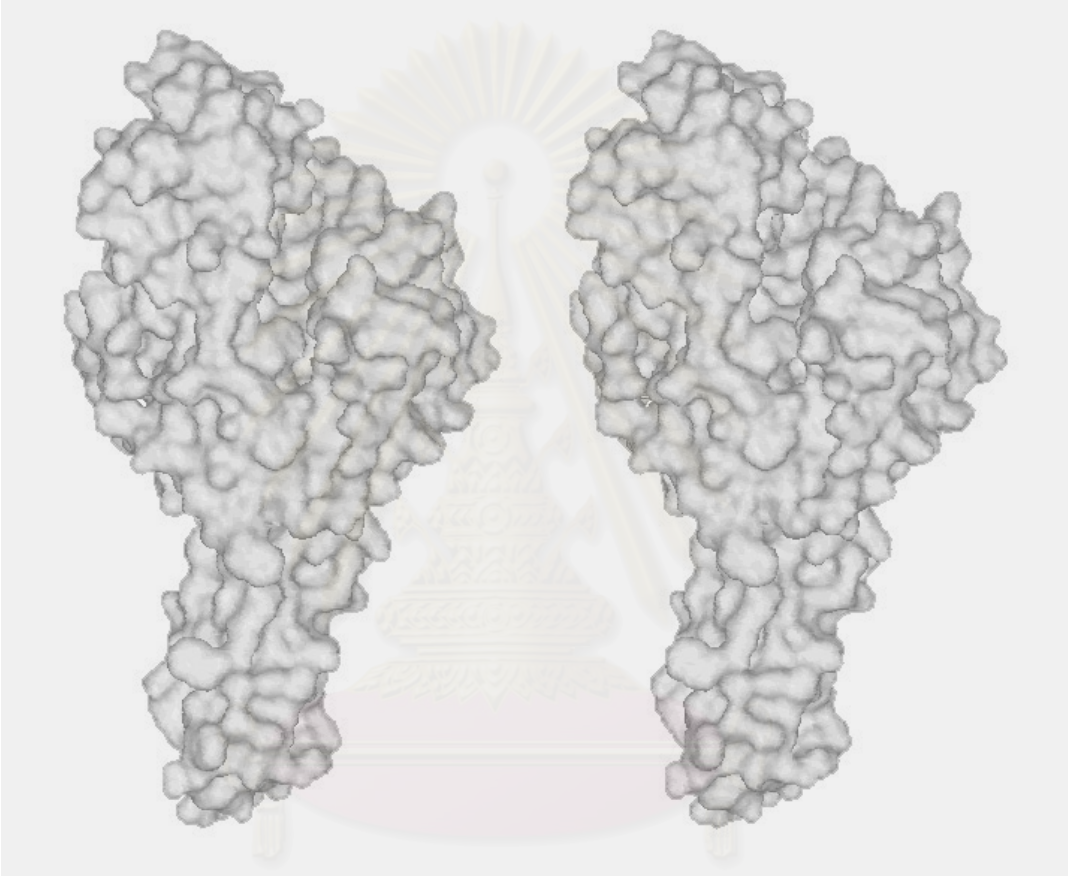


Figure 3.23 The water accessible surface model of *Burkholderia cepacia* Chi60 The water accessible surface model of Chi60 was generated by chime 2.6, MDL Information System, Inc.

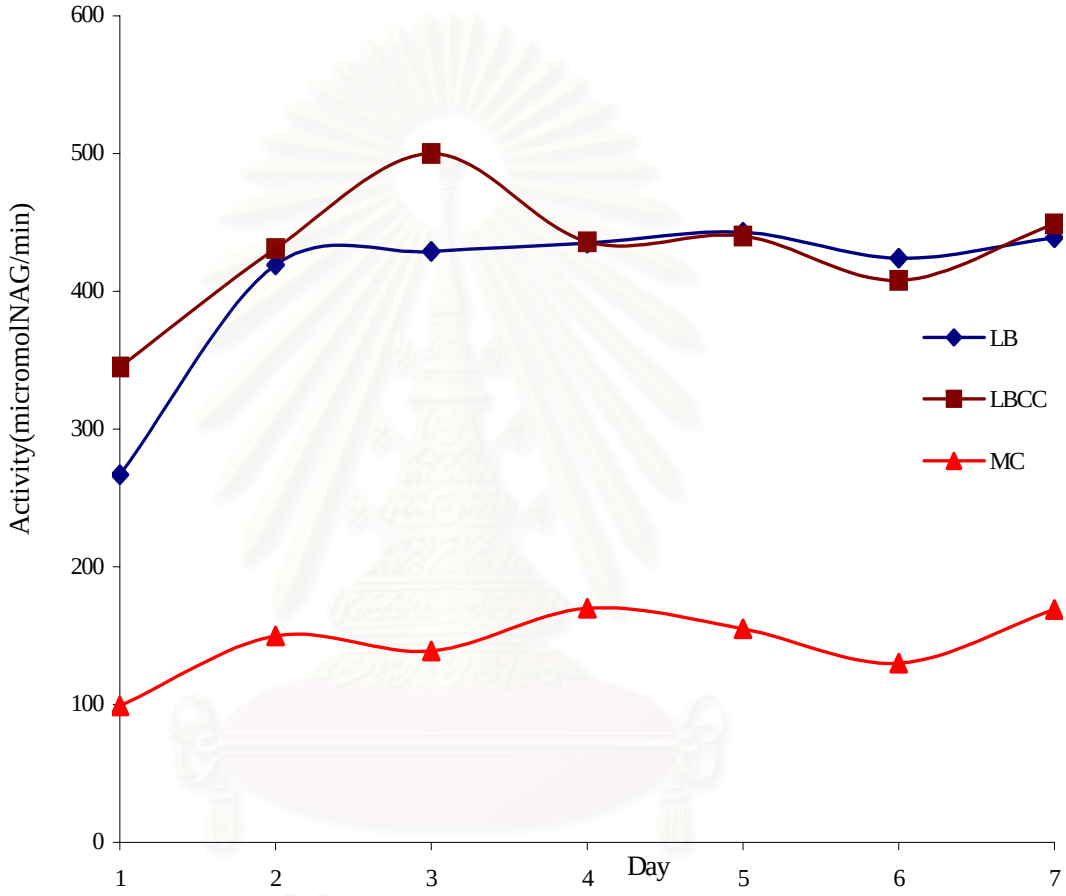


Figure 3.24 Profile of chitinase production in *E. coli* XL-1Blue profile of chitinase activity from *E. coli* XL-1Blue harboring Chi60 during culture for 7 days.

Abbreviations: LB, LBCC and MC show chitinase activity when *E. coli* XL-1Blue were grown in LB medium, LB medium with 0.2% colloidal chitin and minimum medium containing 0.2% colloidal chitin with 1% yeast extract, respectively.

A. CCMM plate with ampicillin

B. CCMM plate with ampicillin and 0.5% glucose

C. LB-colloidal chitin plate with ampicillin

D. LB-colloidal chitin plate with ampicillin and 0.5% glucose

Figure 3.25 Induction and repression of Chi60 gene in *E. coli* *E. coli* XL-1blue containing pKKChi60 was grown on CCMM, CCMM with 0.5% glucose, LB-colloidal chitin and LB-colloidal chitin with 0.5% glucose medium plates for 6 days

medium plate containing ampicillin and 0.2% colloidal chitin and LB medium plate containing ampicillin, 0.2% colloidal chitin and 0.5% glucose, then incubated at 37°C for 6 days. Clear zone was found around the colonies in all medium except the plate of LB medium with glucose and colloidal chitin, as shown in Figure 3.25.

Optimum pH

At the assay temperature of 37°C, Chi60 was active towards colloidal chitin in a broad pH range, with maximum activity at pH 4.0-5.0 (shown in Figure 3.26). The enzyme retained 60% of their activity in a broad pH range from pH 4.0-10.0.

Optimum temperature

Chi60 activity was measured at pH 5.0 at different temperature. Colloidal chitin was used as substrate. At pH 5.0, maximum activity was observed at 55°C. At least 60% of the maximum activity of Chi60 was obtained from 30-60 °C (shown in Figure 3.27).

Substrate specificity

Activity of Chi60 was analyzed on chitin and its derivative, at pH 5.0, 37°C. The results are shown in Figure 3.28. Chi60 exhibited the highest hydrolytic activity on 45% deacetylated chitosan, followed by colloidal chitin, power chitin, 80% deacetylated chitosan and flake chitin.

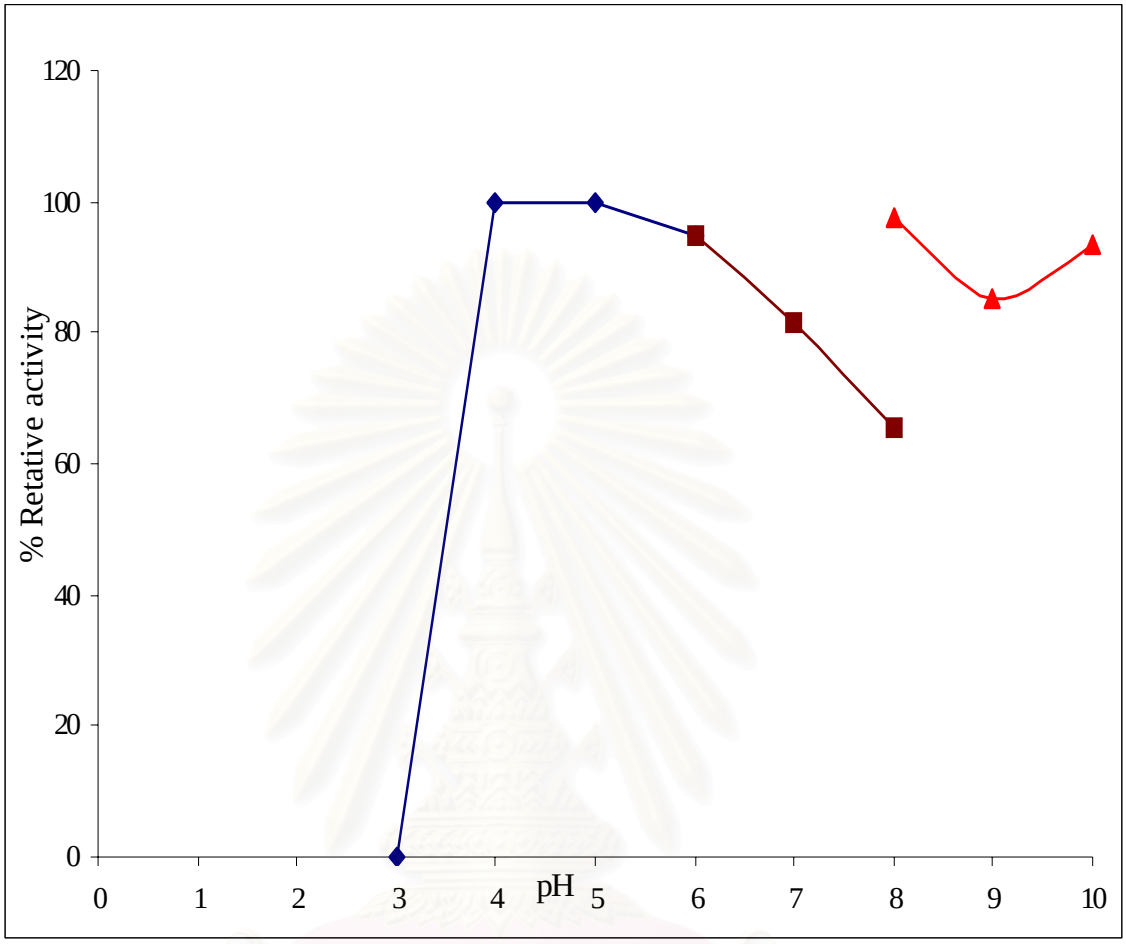


Figure 3.26 Optimum pH of Chi60 Effect of pH on Chi60 activity were measured at pH 3-10, colloidal chitin was used as substrate.
Buffer : pH 3-6, 0.1 M citrate buffer (♦)
: pH 6-8, 0.1 M phosphate buffer (■)
: pH 8-10, 0.1 M Tris-HCl buffer (▲)

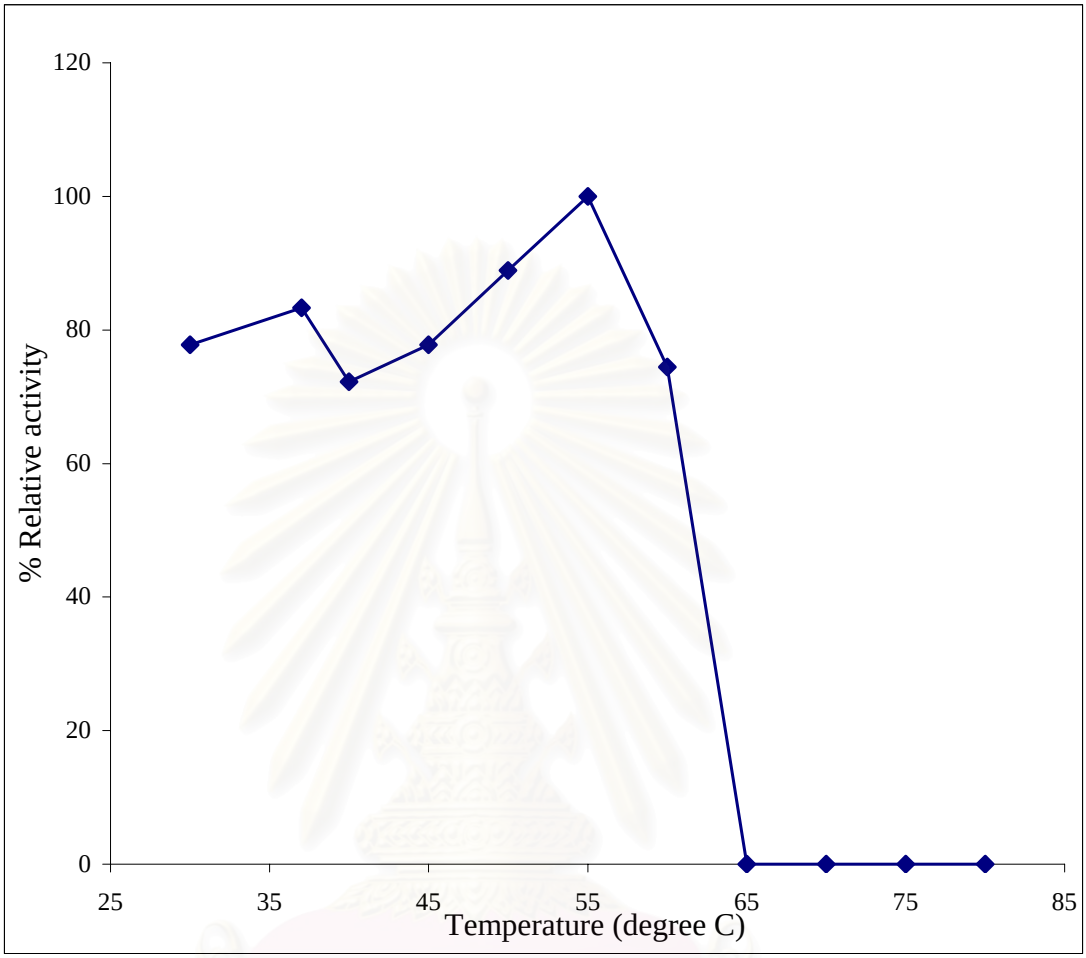


Figure 3.27 Optimum temperature of Chi60 Effect of temperature on Chi60 activity, were measured at 30- 80ºC. Colloidal chitin was used as substrate.

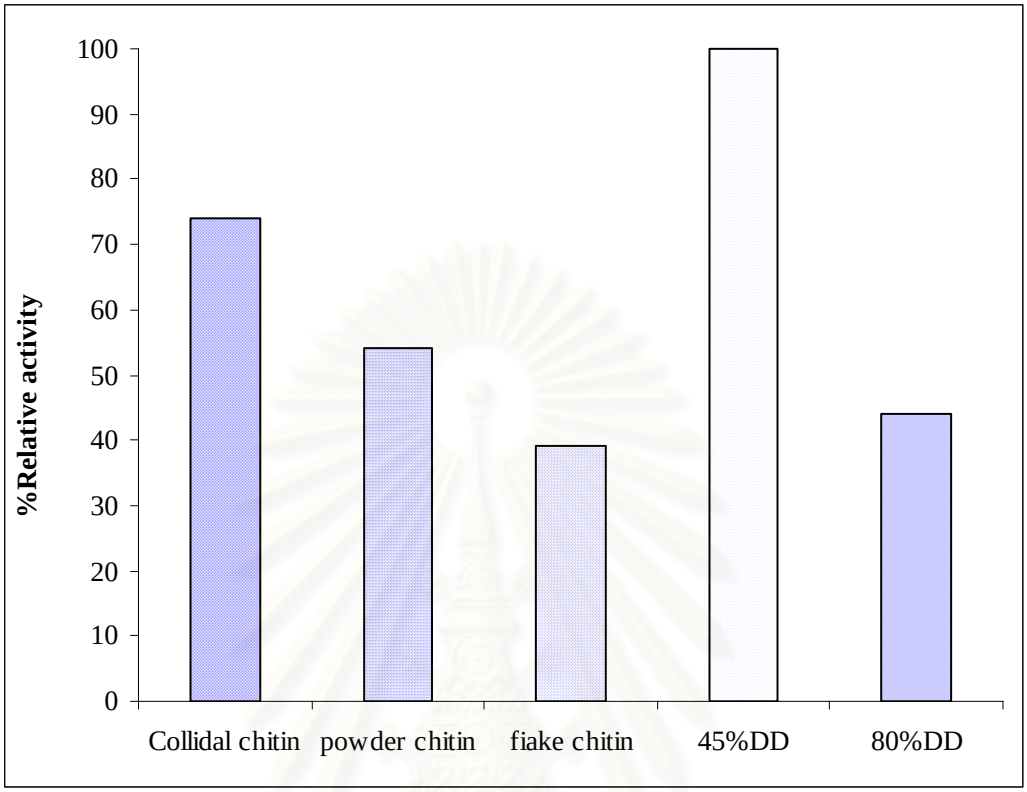


Figure 3.28 Substrate specificity of Chi60 Chitinase activity was measured at 37ºC in 0.1M citrate buffer pH 5.0 when colloidal chitin, powder chitin, flake chitin, 45% and 80% deacetylated chitin were used as substrate. The activity of the enzyme on each substrate was reported as percent relative activity.

Estimate molecular weight

Analysis of crude enzyme from a 48 hours culture and cell pellets of XL-1Blue harboring *Chi60* gene by 10% SDS-PAGE showed a single chitinase activity band (Figure 3.29) with an estimate molecular weight of 60 kDa.

Hydrolysis product of Chi60

The recombinant Chi60 hydrolyzed colloidal chitin to produce $(GlcANc)_2$ as the major product (Figure 3.30).

pH stability

Effect of pH on stability of chitinase from Chi60, the residual activity was determined using the standard assay method after incubating the enzyme at 4°C for different time intervals up to 72 hours. Activity of crude enzyme remained constant throughout 72 hours (Figure 3.31).

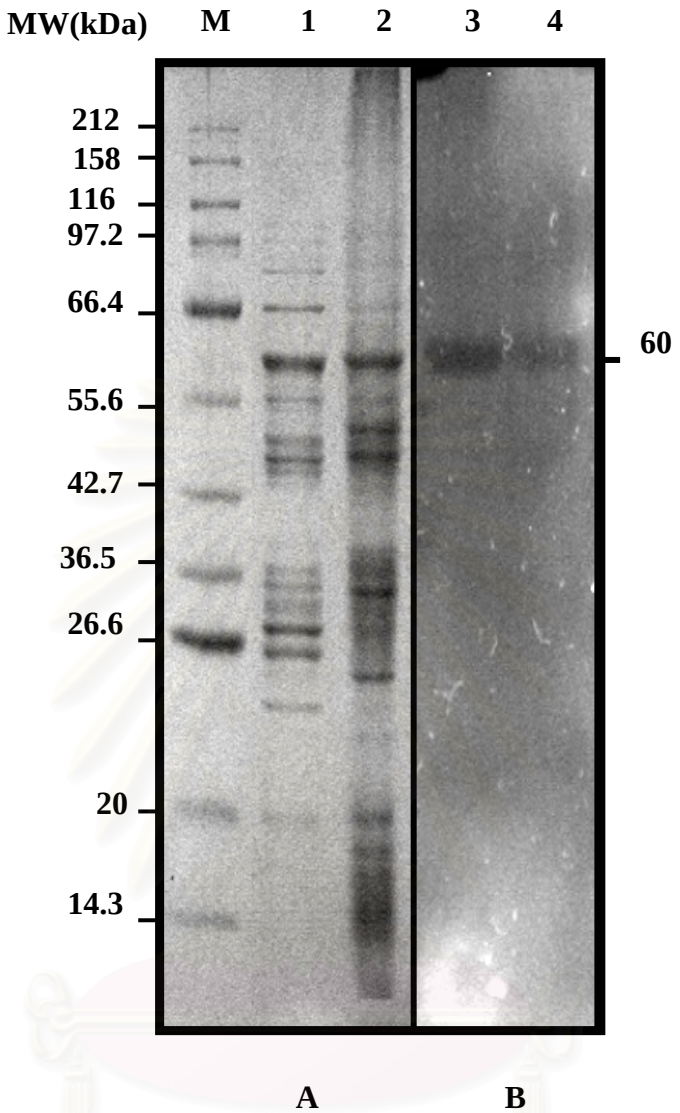


Figure 3.29 Molecular weight of Chi60 SDS-PAGE analysis of Chi60, *E.coli* XL-1Blue carrying plasmid pKKChi60 was grown in colloidal chitin medium. Proteins in the medium and cell lysate were analyzed by 10% SDS-PAGE. The gel was stained for activity and total protein.

Panel A: Protein staining by Coomassie Brilliant Blue R-250

Lane M protein marker

Lane 1 protein from culture medium

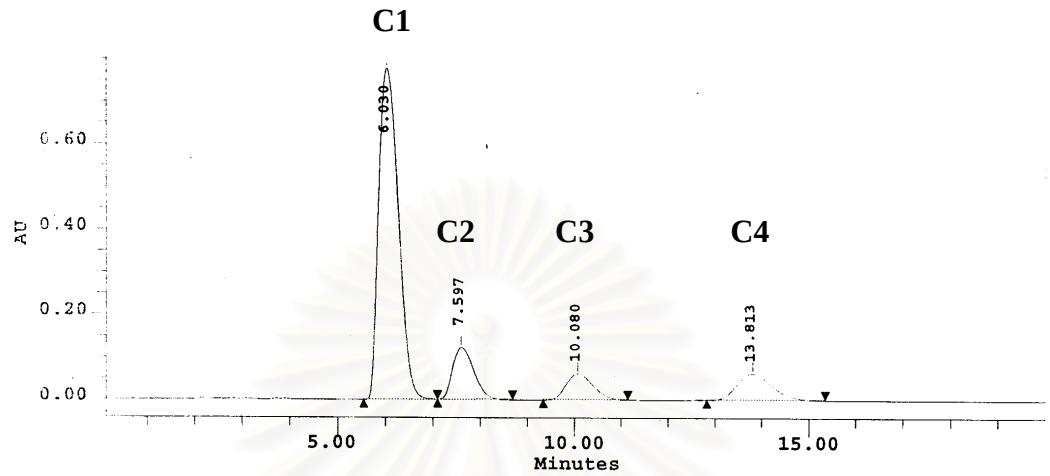
Lane 2 protein from cell lysate

Panel B: Chitinase activity staining

Lane 3 protein from culture medium

Lane 4 protein from cell lysate

A.



B.

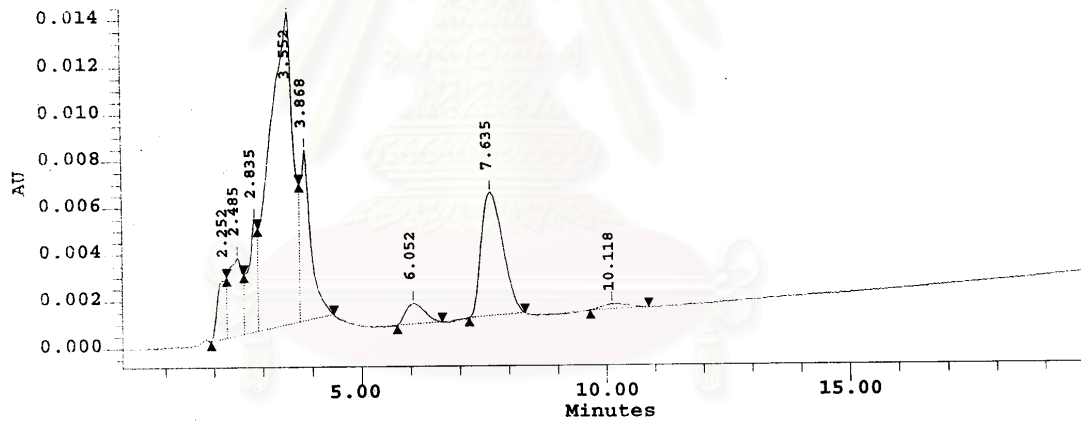


Figure 3.30 Determination of product size of Chi60 HPLC chromatogram of colloidal chitin hydrolyzed by the crude enzyme at 37ºC for 24 hours.

B. N-acetyl-chito-oligosaccharides standards C1 GlcNAc, C2 $(GlcNAc)_2$, C3 $(GlcNAc)_3$, and C4 $(GlcNAc)_4$.

C. Products of crude chitinase which colloidal chitin was used as substrate.

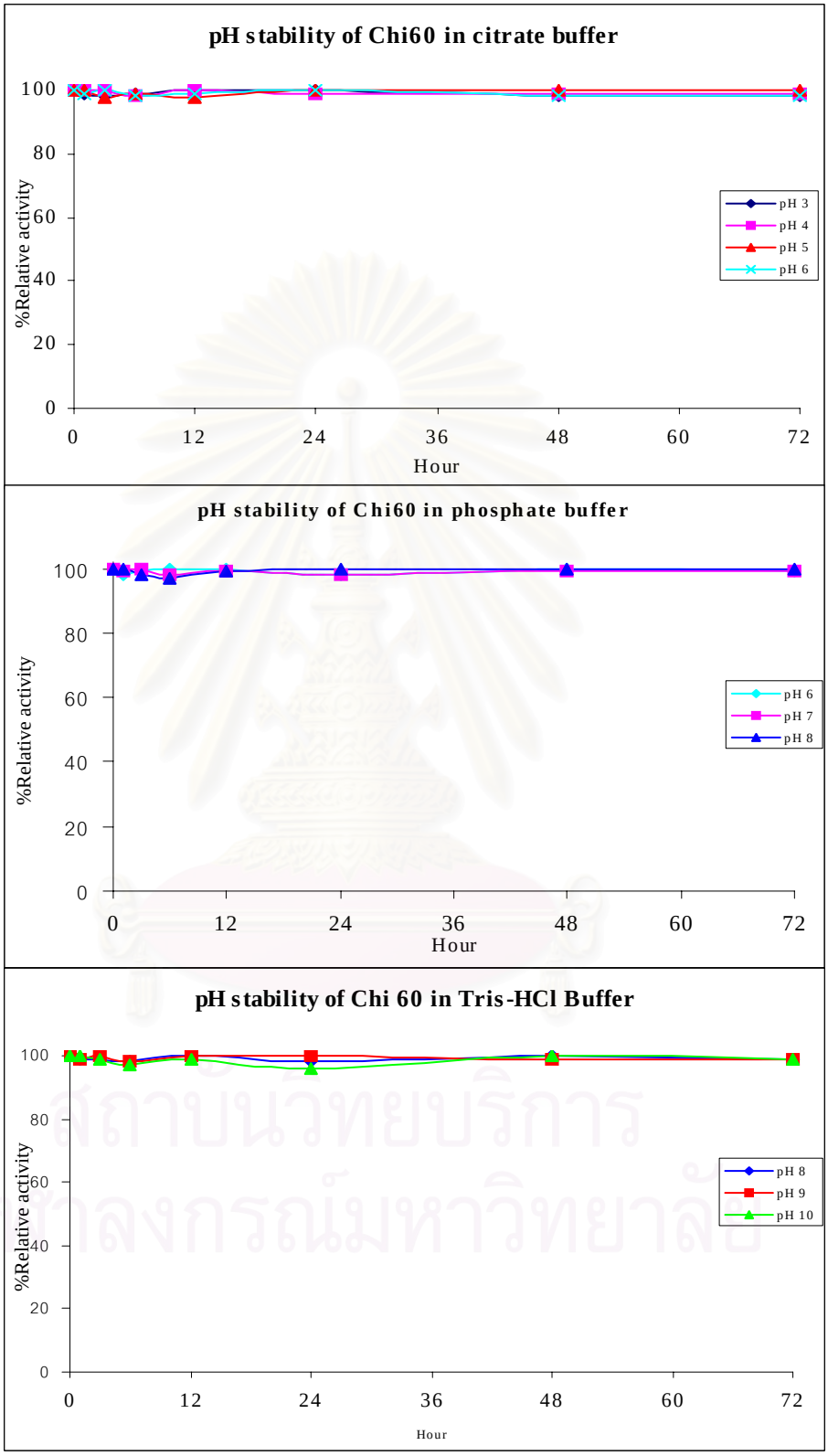


Figure 3.31 pH stability of *B. cepacia* Chi60

CHAPTER IV

DISCUSSION

Characterization of crude enzyme from *Burkholderia cepacia* TU09

Effect of pH and temperature on chitinase activity

After characterization of *B. cepacia* TU09 crude enzyme, we found that the *B. cepacia* TU09 can produce chitinase which works at wide pH and temperature, 4.0-10.0 and 30-60ºC, respectively. The enzyme had two optimum at pH 5.0 and 8.0, which was also a characteristic of other chitinases such as chitinase of *Bacillus licheniformis* B-6839 (Lesya *et al*., 1996) that had two optimum pH against colloidal chitin at 4.5-5.5 and 9.0-9.5 and possessed two pH optima of activity against glycol chitin, i.e., in acidic and alkaline solution. Furthermore chitinase from silk worm (Koga *et al*., 1997) and plant yam (Tsukamoto *et al*., 1984) also showed two optimum pH values 4 and 8-10 toward glycol chitin. The optimum temperature of *B. cepacia* crude enzyme were found to be 37ºC and 55ºC. However, the two optimum pH and temperature of the crude enzyme observed could be a result from multiple enzymes with different optimum pH and temperatures.

Substrate specificity

The crude enzyme had the highest hydrolytic activity on 45% deacetylated chitosan, followed by colloidal chitin, powder chitin, 80% deacetylated chitin and flake chitin. This indicated that the crude chitinase works on soluble substrate in homogeneous reaction better than heterogeneous reaction. Furthermore, crude enzyme had higher hydrolytic activity on amorphous than crystalline chitin. The relative hydrolytic activity on crystalline chitin were 62% and 82% of the activity

observed on soluble chitin and amorphous chitin, respectively. The crude chitinase was able to hydrolyze chitin and chitosan, but the activity drops when the percent deacetylation of the substrate increases, suggesting that the crude enzyme was able to hydrolyze only β-1,4 glycosidic bond between *N*-acetyl-D-glucosamine not glucosamine residues.

Estimation of molecular weight

The studies of chitinase activity in crude enzyme in the culture medium after 2 days, showed that at least 4 protein bands with chitinase activity with a molecular weight of 40, 50, 60 and 90 kDa on a 12% SDS-PAGE was observed.

Hydrolysis product of crude enzyme

We found that 90% of the product from colloidal chitin digestion were monomers (*N*-acetyl-glucosamine) and traces of dimer and trimer. Most of bacterial chitinase produced the mixture of monomers and dimers, but crude enzyme *B. cepacia* TU09 produces mostly GlcNAc. However, the cloned Chi60 gene produces exclusively $(GlcNAc)_2$. These results suggest that the crude enzyme of *B. cepacia* TU09 contains a high-activity hexosaminidase. Moreover, crude enzyme of *B. cepacia* TU09 has been successfully used for the production of GlcNAc from crystalline chitin (Pichyangkura *et al*., 2002).

Cloning of chitinase gene from *B. cepacia* TU09

One chitinase gene, *Chi60* had been isolated from the library of *B. cepacia* TU09 chromosomal DNA by shot gun cloning. When the colony containing plasmid *Chi60*, pKKChi60, was grown on CCMM plate, distinct clear zone formed around the colony where chitin was digested by chitinase. When *E. coli* containing pKKChi60 was grown on colloidal chitin plates with and without IPTG,

both shown clear zone around their colonies. This suggested that the Chi60 has its own promoter. *E. coli* containing pKKChi60 was grown on various medium plates such as colloidal chitin plate, colloidal chitin with glucose plate, LB with colloidal chitin plate and LB with colloidal chitin and glucose plate to investigate the expression of *Chi60* gene. We detected clear zone around the colonies in all medium, except the colonies grown in LB with colloidal chitin and glucose. This demonstrated that the endogenous promoter of *Chi60* can work well in *E. coli* and would only be turned off in rich medium with glucose.

Production of Chi60 in *E. coli*

Chitinase production in *E. coli*, XL-1Blue harboring pKKChi60 during cultivation in CCMM, CCMM with 1% yeast extract and LB medium with colloidal chitin (all containing ampicillin) was observed. In all three media, chitinase activity maximized in 2 days and remained constant throughout the cultivation period of 7 days. Maximum activity was observed in LB medium with colloidal chitin. These results suggest that Chi60 is stable in the medium, in contrast to *B. cepacia* TU09 culture where the Chi60 degrades and lost activity after 3 days as demonstrated by SDS-PAGE followed by activity staining (data not shown).

Characterization of Chi60

Analysis of chitinase gene

A positive clone was detected among 8,000 transformant colonies. The positive clone contains a plasmid (pKKChi60) with 2.8 kb inserted fragment. The DNA fragment was subcloned and nucleotide sequenced and the open reading frame of *Chi60* was determined. As shown in Figure 3.20, one open reading frame of 1,689 bp coding for 563 amino acids, which correspond to a protein of 60,900

Da was found. The data obtained from amino acid comparison revealed that *Chi60* is very similar to ChiA of *Serratia marcescens* BJL200 and their high level of sequence similarity suggests that these chitinases must have similar three-dimensional structure. Homology modeling of Chi60 by SWISS-MODEL version 36.0002 reinforces this hypothesis. However, crystallographic studies on Chi60 has to be perform to prove our hypothesis.

Optimal conditions for enzyme action

Chi60 was active in a broad pH range (4.0-10.0) with maximum activity at pH 5.0, similar to ChiA from *Serratia marcescens* (Brurberg *et al*., 1996), chitinaseA1 from *Bacillus circulans* WL-12 (Park *et al*., 1997), chitinase from *Pyrococcus kodakaraensis* KOD1 (Tanaka *et al*., 1999) and *Streptomyces erythraeus* (Okazaki *et al*., 1995).

The optimmum temperature at 55ºC in citrate buffer pH 5.0 is also similar to chitinaseA from *Serratia marcescens* (Brurberg *et al*., 1996). And at least 60% of the maximum activity of Chi60 was obtained at 30-60ºC.

Substrate specificity

The crude Chi60 had a highest hydrolytic activity on 45% deacetylated chitosan, followed by colloidal chitin, powder chitin, 80% deacetylated chitin and flake chitin, indicating that crude Chi60 works in homogeneous reaction better than heterogeneous reaction. Chi60 exhibited a high hydrolytic activity on both chitin and colloidal chitin substrates. The relative hydrolytic activity of Chi60 for powder chitin (α–chitin crab shells) were 50% and 70% of the activity observed on soluble chitin and amorphous chitin substrate, respectively. The crude Chi60 was able to hydrolyze chitin better than chitosan and hydrolyzed chitosan with lower degree of deacetylation better than high degree of deacetylation chitosan suggests

that the crude enzyme was able to hydrolyze β-1,4 glycosidic bond between *N*-acetylglucosamine, but not β-1,4 glycosidic bond between glucosamine.

Estimation of molecular weight

When the molecular weight of Chi60 was estimated by SDS-PAGE, only a single band was detected after activity staining of the extracellular and intracellular proteins with molecular weight of 60 kDa. Also, the chitinase activity band of Chi60 on SDS-PAGE corresponded to the 60 kDa chitinase activity band from crude enzyme of native strain. This suggests that we have successfully cloned the 60 kDa chitinase gene from *B. cepacia* TU09. Furthermore, the size of cloned enzyme from culture medium is slightly smaller than the size of cloned enzyme from cell lysate. This result agreed with the presence of an enzyme precursor with a signal peptide. The secreated chitinase lacks signal peptide that was probably cleaved off during protein transport into the periplasmic space.

Hydrolysis product of Chi60

The chitinase activity of Chi60 hydrolyzed colloidal chitin produced dimer $(GlcNAc)_2$ as the major product, with small amounts of GlcNAc and $(GlcNAc)_3$. This result also agrees with the homology modeling studies, results which also suggest that Chi60 produces $(GlcNAc)_2$ from chitin. Therefore, this enzyme can be applied in the production of $(GlcNAc)_2$.

pH stability of Chi60

Crude chitinase was stored at 4ºC for 72 hours without significant loss of activity similar to chitinaseA from *Serratia marcescens* (Brurberg *et al*., 1996). Purified chitinase could be stored at 4ºC in Tris-HCl buffer, pH 8.0 for several

months without loss of activity. This demonstrates that Chi60 is a very stable enzyme and has a good potential for industrial application.

CHAPTER V

CONCLUSION

Crude enzyme of *B. cepacia* TU09 can work in a wide range of pH and temperature, 4.0-10.0 and 30-60ºC, respectively. The optimum temperature and optimum pH was found at 37ºC and 55ºC and 5.0 and 8.0. Studies of chitinase activity in crude enzyme, after 2 days of cultivation, showed that at least 4 protein bands posses chitinase activity with a molecular weight of 40, 50, 60 and 90 kDa estimated by SDS-PAGE. Determination of hydrolysis product (of the crude enzyme), found that 90% of the product from colloidal chitin, α-chitin and β-chitin digestion were monomers (N-acetyl-D-glucosamine) and with small amounts of dimers and trimers.

One chitinase gene (*Chi60* gene) has been isolated from a library of *B. cepacia* TU09 chromosomal DNA by shot gun cloning. The transformant contains a plasmid, pKKChi60 with a 2.8 kb inserted fragment that harboring *Chi60* gene. pKKChi60 was nucleotide sequenced. One open reading frame of 1689 bp was found coding a 564 amino acid protein, which correspond to a protein of 60,900 Da. The data obtained from amino acid comparison revealed that the *Chi60* is the similar to ChiA of *Serratia marcescens* BJL200.

Chi60 was active in a broad pH range from 4.0-10.0 with maximum activity at pH 5.0. Optimum temperature was observed at 55ºC and at least 60% of the maximum activity of Chi60 was obtained at temperature 30-60ºC. When Chi60 was estimated by SDS-PAGE, only one band was detected after activity staining with a molecular weight of 60 kDa. Chi60 hydrolyzed colloidal chitin and produces $(GlcNAc)_2$ as the major product, with small amounts of GlcNAc and $(GlcNAc)_3$. Crude chitinase can be stored at 4ºC in buffer with pH 3-10, for 72 hours without significant loss of activity.

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APPENDICES

APPENDIX A

Subcloning of pKK243B

Krusong (1999) cloned a 7.8 kb *BamHI* digested DNA fragment from *B. cepacia* TU09 into a pBluescriptSK$^-$ (designated pKK243B) that was picked from chitinase gene screening by dot blot analysis with DNA probe specific for chitinase, CB1. After digested pKK243B with *PstI*, 1.8 kb band found in the Southern blot analysis of the genomic DNA was liberated. The results suggested that pKK243B should contain chitinase gene. To confirm the result, subcloning of 1.8 kb *PstI* fragment of pKK243B (named pKK1.8PP) was performed. After that, pKK1.8PP was sequenced by method of Sanger *et al*. (1997). When comparing the deduced amino acid sequences from the 1.8 kb DNA fragment with protein sequences in the Genbank database revealed a partial open reading frame of 206 amino acid which is similar to the amino acid sequence of putative sensor proteins, and an open reading frame of 244 amino acid sequence similar with the amino acid sequence of putative two component transcriptional regulator. No open reading frame which have similarity with chitinase gene was found in pKK1.8PP.

Recent work on regulation of chitinase gene from *Streptomyces thermoviolaceus* OPC-520 (Tsujibo *et al*., 1999) showed that *Streptomyces thermoviolaceus* OPC-520 produces both family18 chitinase (Chi40 and Chi30) and family19 (Chi35 and Chi25). Tsujibo et al. found that Chi40 is regulated by a two-component sensor-regulator system, *ChiS* and *ChiR*. The location of *ChiS* and *ChiR* is directly upstream flanking the *Chi40* gene (figure A.32). The gene construct of this two-component sensor-regulator system was similar to the sequence obtained from the 1.8 kb *PstI* fragment.

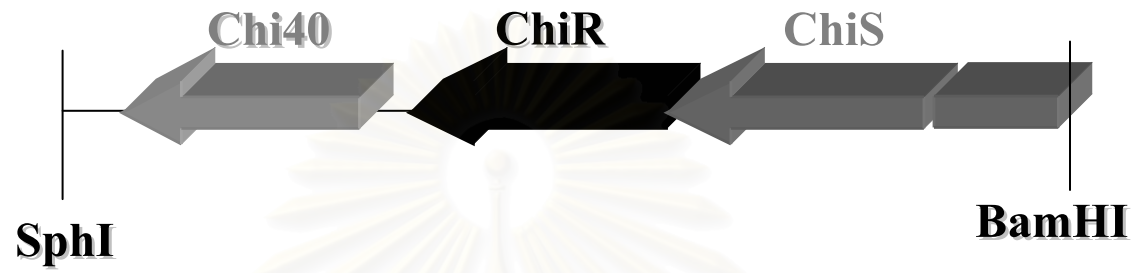
From recent finding by Tsujibo *et al*. (1999), we predict that pKK243B should contain a chitinase gene following the two-component regulator system. Therefore, the 1.0 kb *HindIII* - *PstI* fragment (designated pKK1.0HP), which is

adjacent to the 1.7 kb *PstI* fragment (shown in figure A.33) was subcloned and sequenced (the results shown in figure A.34) to determine whether or not there is a chitinase gene following the putative sensor gene and putative two-component transcriptional regulator gene similar to *Streptomyces thermoviolaceus* OPC-520.

The results showed that pKK1.0HP does not have chitinase gene after alignment of deduced amino acid sequences of pKK1.0HP with amino acid sequences of chitinase from Genbank. However, an the open reading frame of 81amino acids similar with atrophin, an open reading frame of 121amino acids of an unknown protein and a partial open reading frame of 101amino acids putative transporter protein, was found.

Furthermore, the 3.4 kb *EcoRI* fragment and 2.3 kb *HindIII* fragment (shown in figure A.33) were subcloned to determine chitinase activity. *E. coli* DH5α that containing pBluescriptSK$^-$ and pBluescriptSK$^-$ containing inserted fragments 3.4 kb *EcoRI* fragment (pKK3.4EE), 2.3 kB *HindIII* fragment (pKK2.3H), 1.0 kb *HindIII* - *PstI* fragment (pKK1.0HP) and 1.8 kb *PstI* fragment (pKK1.8PP) was grown on glycol chitin minimum medium plate for 7 days. As shown in table A.4. Clear zone of glycol chitin was formed around the colonies that contained the plasmid with inserted DNA fragment, although the inserted fragment does not caotain chitinase gene. This result suggests that phenotype screening for chitinase gene by glycol chitin plate was unsuitable.

A.



B.

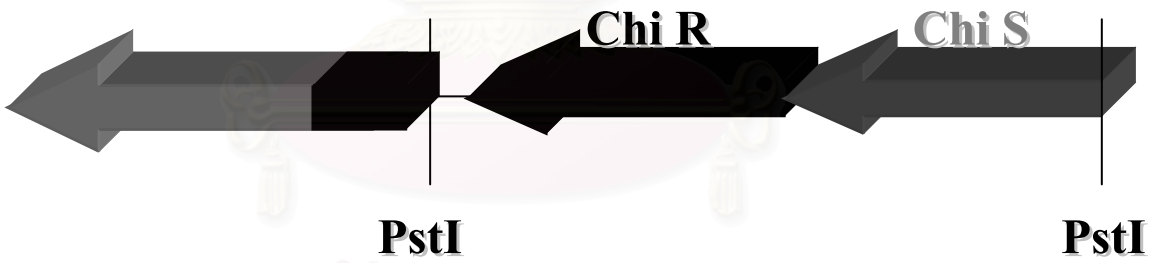


Figure A.32 Restriction map of *Streptomyces thermoviolaceus* Chi40 (A) Chi40 from *Streptomyces thermoviolaceus* OPC-520 and (B) proposed location of chitinase gene in 7.8 kb insert fragment of pKK243B.

Arrows indicated the ORF and the direction of transcription. S and R are representative of putative sensor protein and putative 2-component transcription regulator

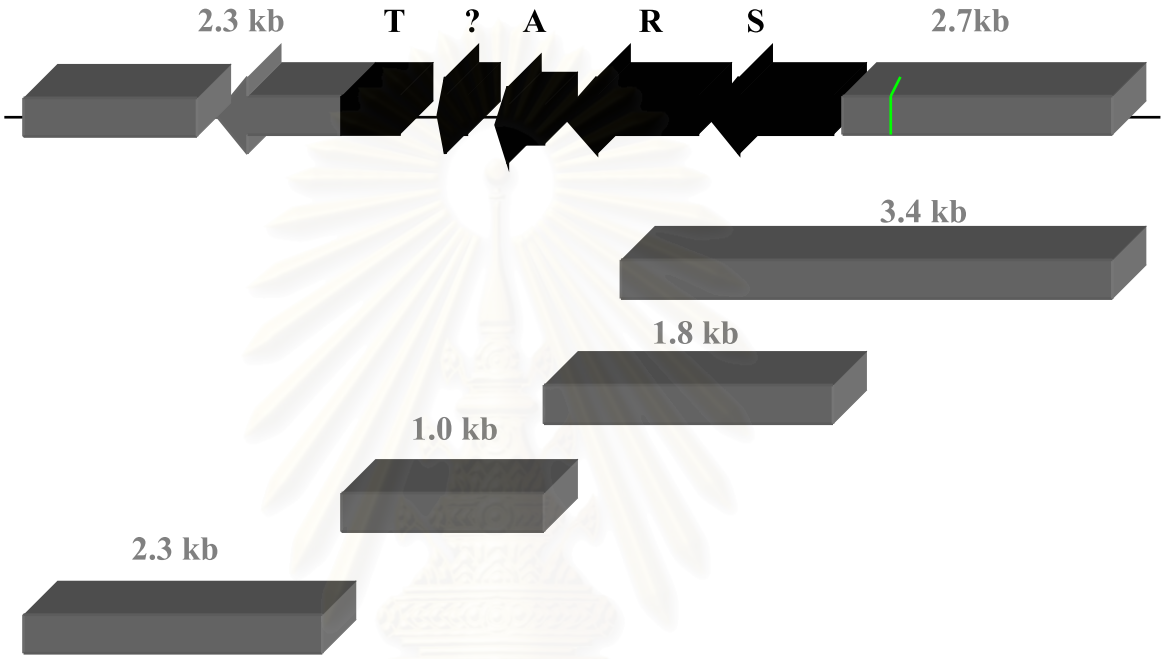


Figure A.33 Subcloned of pKK243B

Arrows indicated the ORF and the direction of transcription. S, R, A, ?, and T are representative of putative sensor protein, putative 2-component transcription regulator, putative atrophin, unknown protein and putative transporter protein, respectively.

```
   1    CTGCAGAGCA AGATTAACCC GCACTTCCTG TTCAACGCGC TGAACGCCAT CTCTTCCTCC   60
  61   ATCCGCCTGA ATCCGGACAC CGCGCGCCAG CTGATCATCA ACCTGTCGCG CTACCTGCGC   120
 121   TACAACCTGG AGCTGAACGA CGAACTGATC GATATCCGTA AGGAACTGCA TCAGATTCAG   180
 181   GACTATATCG CCATCGAACA GGCGCGCTTC GGCGCCAAGC TGACGGTGAT TTACGACATC   240
 241   GACGACGACG TCTCGGTGCG CATTCCGAGC CTGCTGATCC AGCCGCTGGT GGAGAACGCC   300
 301   ATCGTGCACG GCATCCAGCC CTGCAAGGGT AAAGGGGTGG TGGTGATCGC GGTGAAAGAT   360
 361   CAGGGCGATC GGGTGAAGAT TTCGGTGAAG GATACCGGCC ACGGCATCAA CCAGGAAACC   420
 421   ATCGACCGGG TGGCGCGCAA CGAGATGCCG GGCCACAATA TCGGCCTGCT CAACGTGCAT   480
 481   CACCGCGTGT CGCTGCTGTA CGGTGAAGGG TTGCATATCC GCCGCCTGGA GCCGGGCACC   540
 541   GAAATCGCGT TCTATATCAG CAAAAACGGC GGCAAGCTGC ATCAGGAACC GAGCGCGCCG   600
 601   CCGGTCGGGG AGGCCTCATG AAAGCTATCA TCGTGGAAGA CGAATTCCTC GCGCAGGAAG   660
 661   AACTCAGCTA CCTGATCAAG AAACACAGCA ATATCGATAT CATCGCTACC TTCGAGGACG   720
 721   GCCTCGACGT TCTGAAATAC CTGCAAACCC ACCAGGTCGA CGCCATTTTT CTCGACATCA   780
 781   ACATCCCGTC GCTGGACGGC GTGCTGCTGG CGCAAAACAT CAGCAAGTTC GCCCATCGGC   840
 841   CGTCTATCGT GTTCATCACC GCCTATAAAG AACACGCGGT GGAAGCCTTC GAGATCGAGG   900
 901   CGTTCGACTA TATCCTCAAG CCCTATCACG AAGCGCGCAT CGTCACCATG TTGCAAAAAC   960
 961  TGGAGGCGCT GCATCATCGC CCCGCCGGCG CGGCGGAACC GACCAGCGCG CCGAGCCGCG  1020
1021 GCAGCCACAG CATCAACCTG ATCAAAGACG AGCGGATCAT CGTTACCGAC ATCAACGACA  1080
1081 TCTATTACGC CGCCGCCGAT GAAAAGGTGA CGCGGGTCTA TACTCGCCGG GAAGAATTCG  1140
1141 TGATGCCGAT GAATCTCACC GAGTTTTACG GCCGGCTGCC GGAAGAGCAT TTCTTCCGCT  1200
1201 GCCACCGCTC TTACTGCGTT AACCTGGCCA AGATCCGCGA GATCGTGCCC TGGTTCAACA  1260
1261 ATACCTACAT TCTGCGGCTG AGCGATCTTG AGTTTGAAGT GCCGGTCAGC CGCAGCAAGG  1320
1321 TGAAAGAATT TCGCAAGCTG ATGCGCCTGT AAGCGCTTAC CAGCGCGGGC CCGGACGGTA  1380
1381 GTAATAGCCG GCGGCGGCGG TGGCGGCATG GCGGTAATGC GGCCGATCTC GCCAGTCGCG  1440
1441 CCGCGGCGGC CCGTAGTAAA TCACCCGTGG CGGCGGGCCG TAATAGTACC CGCGCGAACG  1500
1501 TTCATACCGG TGATGATCGG CCCACCAGCG CGGATCGCGC CAGCGATAGC CGTCCCAGTA  1560
1561 GTGGCCGCGA TGATCGCGAT CGCCGATATG CAACGACAGG CCCGGCACGT TCACGCCGAT  1620
1621 GGACACGTCG GCCTGGCTCG CCAGCGGCAG CGCCAGCAGC GCAGCCAGTA ACAACAGCGT  1680
1681 TTTTTTCATT TCATGGACTC CTGCAGAGCG GACCTCGTCC GATCGCAGAT AACATAGTCC  1740
1741 CGCGCTCGCC GCCGCTCTAT AGGCGCAATG CCGATTTACG CCTTGCAGAC GCATCCTTCA  1800
1801 CAATTTCACC ACAATCGTTC ACCGGCGATT AACAATAAAT CGTTGATATC TTTGATGTAG  1860
1861 ATCACGTGTT ACTCCCGGCA TTTCACGCGC TTTTAGGCGT AAAAACGGCA ACTCACGCCG  1920
1921 TATAAAATGC ATTTCATTCC TTCTGCCGTG CAATTCATTC CCGATCCGCC GTCCGCCGGG  1980
1981 GGGGCCGGCC ATATAATAAG CCCAGACCGC GGGAACACAG ATTCACCCCG CAAATTTCAG  2040
2041 CCAGCGGCTC ACGCCCGGCA ACGCACACAC AGGAAGGAGA CCGGCCATGA ACACCAAACC  2100
2101 GGCAAATCGC AACTTAATCG TTCTCGGCAC CATCATCTGT CAGATGGGTC TTGGGCACCA  2160
2161 TCTACACCTG GAGCCTGTTC AACCAAGCCG CTGGTCGACA AGGTTCCACT GGGGACTGGC  2220
2221 GGACGTCGCC ACCACCTTCT CCATCACCAG CTTCTTCCTG GCTTTCGGCC ACGCTGTTCG  2280
2281 CCGGCAAGCT GCAAGAACGC TTCGGCATCC GCAACCTGAC GCTTGTGTTC CGGCATCCTG  2340
2341 GTCGGCCTGG GCCTGATCGG CCAGCGCCCA TGTCAGCTCG CTCGACATGA TCTACCTGCT  2400
2401 GGCCGGCGTG GTGGTGGGCT TTGCGGTCGG TATCGCCTAC ATCTCCACCC TGTCCAACCT  2460
2461 GATTAAATGG TTCCCGGCCA ACAAAGGCCT GATTTCCGGC ATCTCCGTCG GCGCCTTCGG  2520
2521 CAGCGGCAGC CTGCTGTTCA AATACGTCAA CGCCGCCCTG ATCGCCGACG TCGGCGTTTC  2580
2581 AGGCGCCTTC TTCTACTGGG GTGCCATCGT GATGGGCCTG ATCGTCGTCG GCTCCCTGTT  2640
2641 GCTGAAAGAG CCGGTGCTGG CAACCAGCGC CGCACAGCCA GGCGCCAACG GTCTGGGCAA  2700
2701 CGACTTCAGC GTGCGCCAGA TGCTGGCCAC CAAAGAAGCT T    2741
```

Figure A.34 Nucleotide sequence of 1.0 kb and 1.8 kb insert fragment of pKK1.0HP and pKK1.8PP, respectively from pKK243B Arrows indicated the ORF and the direction of transcription of partial sequence of putative sensor protein (blue), complete sequence of putative two component regulatory (red), complete sequence of putative atrophin (pink), unknown protein (violet) and partial sequence of putative transporter protein (green).

Table A.4 Determination of the DNA fragment subcloned from pKK243B with chitinase activity on glycol chitin plate

Cell culture	Clear zone
B. cepacia	+++
E. coli containing pKK243B	++
E. coli containing pKK1.8PP	+
E. coli containing pKK1.0HP	+
E. coli containing pKK2.3H	+
E. coli containing pKK3.4EE	+
E. coli containing pBluescriptIISK -	0

APPENDIX B

Quantitative production of 2-Acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase

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Abstract

Fine powdered α- and β-chitin can be completely hydrolyzed with chitinase (EC 3.2.1.14) and β-*N*-acetylhexosaminidase (EC 3.2.1.52) for the production of 2-acetamido-2-deoxy-D-glucose (GlcNAc). Crude chitinase from *Burkholderia cepacia* TU09 and *Bacillus licheniformis* SK-1 were used to digest α- and β-chitin powder. Chitinase from *Burkholderia cepacia* TU09 produced GlcNAc over 85% yield from β-chitin and α-chitin within 1 day and 7 days, respectively. *Bacillus licheniformis* SK-1 chitinase completely hydrolyzed β-chitin within 6 days, giving the final GlcNAc yield of 75% along with 20% of chitobiose. However, only a 41% yield of GlcNAc was achieved from digesting α-chitin with *Bacillus licheniformis* SK-1 chitinase.

Key words: Chitin, Chitinase, *N*-Acetyl-D-glucosamine, 2-Acetamido-2-deoxy-D-glucose, *Bacillus licheniformis*, *Burkholderia cepacia*

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2-Acetamido-2-deoxy-D-glucose (*N*-Acetyl-D-glucosamine, GlcNAc) and 2-amino-2-deoxy-D-glucose (D-glucosamine, GlcN) have recently been promoted for treatment or as nutriceutical agents for patients with osteoarthritis and inflammatory bowel disease.[1,2] In contrast to GlcN hydrochloride or sulfate, both of which have a bitter taste, GlcNAc has sweet taste which can be conveniently used in daily consumption. However, GlcNAc has not been widely commercialized mainly due to the lack of an economical process for production of this compound that is acceptable for food and medicine. The current acid hydrolysis of chitin using concentrated HCl is inefficient, and poses environmental and technical concerns.[3] On the other hand, hydrolysis of chitin with enzymes can produce GlcNAc under mild and environmentally friendly conditions. An approach whereby commercially available crude enzymes were used to hydrolyze amorphous chitin substrate was carried out.[4] Unfortunately, this method added an additional substrate preparation step into the production of GlcNAc. The work on commercially available crude enzymes was also extended to a production of GlcNAc by direct hydrolysis of β-chitin powder.[5, 6] These reports have shown that enzymatic hydrolysis of chitin can produce GlcNAc in relatively higher yields than the acid hydrolysis. Nevertheless, the remaining major impediment of an enzymatic hydrolysis process is the extremely low hydrolytic susceptibility of the natural chitin substrate, due to its high crystallinity. We would thus like to show herein for the first time that crystalline chitin in both α- and β-forms could be cleanly hydrolyzed, producing GlcNAc in virtually quantitative yield.

Powdered α-chitin (14 μm in size) from crab shells and β-chitin (3 μm in size) from squid pens were used as substrates for digestion by crude bacterial chitinase from *Burkholderia cepacia* TU09 and *Bacillus licheniformis* SK-1. A typical reaction contains100 mU/mL (1 unit = the amount of enzyme that produces 1 μmole of GlcNAc per minute from colloidal chitin) of the enzyme and 10-40 mg/mL of the substrate, unless indicated otherwise. Digestion reactions were carried out in 3-5 mL of 0.1 M citrate-phosphate buffer, pH 6.0,

in 10 mL glass vials. The reactions were incubated in a shaking water bath, with moderate shaking, at 37°C and 50°C when the enzyme from *Burkholderia cepacia* TU09 and *Bacillus licheniformis* SK-1 was used, respectively. At each time point, a portion of the reaction mixture was withdrew, diluted with H_2O then mixed with CH_3CN (at the ratio 31/69), filtered, and analyzed by HPLC (column: Shodex Asahipak NH2P-50; flow rate: 1 mL/min; mobile phase: 31/69 H_2O-CH_3CN; detection: UV at 210 nm). The amount of GlcNAc in the reaction mixture was determined from a calibration curve of GlcNAc standard.

The percent yield of GlcNAc production increased with the reduction of substrate/enzyme ratio. Although, at the substrate/enzyme ratio of 100 mg/U, chitinase from *Bacillus licheniformis* SK-1 completely hydrolyzed β-chitin, it gave a mixture of GlcNAc and *N,N'*-diacetylchitobiose [$(GlcNAc)_2$] (**Table 1**). The gradual increase of the GlcNAc/$(GlcNAc)_2$ product ratio with incubation time implied the presence of low β-*N*-acetylhexosaminidase (EC 3.2.1.52) activity in the crude enzyme from *Bacillus licheniformis* SK-1 under the reaction conditions. On the other hand, hydrolysis of β-chitin with chitinase from *Burkholderia cepacia* TU09 gave mostly GlcNAc with a trace amount of chitotriose. At the substrate/enzyme ratio of 100 mg/U, a 90% yield of GlcNAc was obtained within one day, and a quantitative yield was realized upon prolonged incubation (**Table 2**).

The tightly packed chitin strands of α-chitin are known to have low susceptibility to enzymatic hydrolysis. We found that when chitinase from *Bacillus licheniformis* SK-1 was used, it was unable to completely hydrolyze α-chitin. Only 41% of α-chitin was hydrolyzed in 6 days, even when the concentration of enzyme used in the reaction was 10-fold of the amount that was used to completely hydrolyze β-chitin (**Table 3**). We speculate that the crystalline domains in α-chitin were completely resistant to digestion by chitinase from *Bacillus licheniformis* SK-1. The GlcNAc produced was probably liberated from amorphous regions of the substrate. Chitinase from

Burkholderia cepacia TU09 showed superior characteristic in hydrolyzing α-chitin as 85% yield of GlcNAc was achieved after 7 days of incubation (**Table 4**). It is worth noting that the hydrolysis of α-chitin with chitinase *from Burkholderia cepacia* TU09 consists of two steps. First, a rapid hydrolysis step in the first 24 hours, where we believe that the amorphous portion (~40%) of the chitin particle is hydrolyzed. The second step is a slower step, where the remaining tightly packed chitin is slowly hydrolyzed. Because of this slower degradation rate, 300 mU/mL of enzyme was used to ensure sufficient amount of active enzyme present throughout the hydrolysis. The isolation and characterization of the enzymes used here will be published elsewhere.

We have demonstrated here for the first time that chitinase from certain bacteria can completely hydrolyze both powdered α- and β-chitin to give GlcNAc in very high to quantitative yield. The cleanliness of the reaction, mild conditions, ease of substrate preparation, and high production yield undeniably render the approach of using enzyme more attractive than the current acid hydrolysis process for the production of GlcNAc. Despite all these beneficial factors in using bacterial chitinase, care must be taken in further development to ensure food safety and enhance cost efficiency for industrial production of GlcNAc.

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Table C.5 Production of GlcNAc from β-chitin by chitinase from *Bacillus licheniformis* SK-1

β-chitin/enzyme[a] (mg/U)	Digestion time (day)	% Yield[b]		
		GlcNAc	$(GlcNAc)_2$	Total
400	1	9	22	31
	3	18	27	45
	6	25	22	47
200	1	16	18	34
	3	34	38	72
	6	46	29	75
100	1	28	50	78
	3	53	40	93
	6	75	20	95

[a] [E] = 0.1 U/mL in 0.1 M citrate-phosphate buffer, pH 6.0.

[b] HPLC yield.

Table C.6 Production of GlcNAc from β-chitin by chitinase from *Burkholderia cepacia* TU09.

β-chitin/enzyme[a] (mg/U)	Digestion time (day)	% Yield[b] GlcNAc
400	1	31
	3	57
	6	65
200	1	62
	3	81
	6	84
100	1	90
	3	96
	6	100

[a] [E] = 0.1 U/mL in 0.1 M citrate-phosphate buffer, pH 6.0.

[b] HPLC yield.

Table C.7 Production of GlcNAc from α-chitin by chitinase from *Bacillus licheniformis* SK-1

α-Chitin/Enzyme (mg/U)	Digestion time (day)	% Yield GlcNAc[a]
	1	32
10	3	40
	6	41

[a] [E] = 1 U/mL in 0.1 M citrate-phosphate buffer, pH 6.0.

[b] HPLC yield.

Table C.8 Production of GlcNAc from α-chitin by chitinase from *Burkholderia cepacia* TU09.

α-Chitin/Enzyme[a] (mg/U)	Digestion time (day)	% Yield[b] GlcNAc
100	1	37
	3	54
	7	57
33	1	41
	3	57
	7	85

[a] [E] = 0.1 U/mL in 0.1 M citrate-phosphate buffer, pH 6.0.

[b] HPLC yield.

Quantitative production of 2-Acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase

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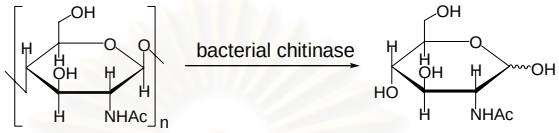
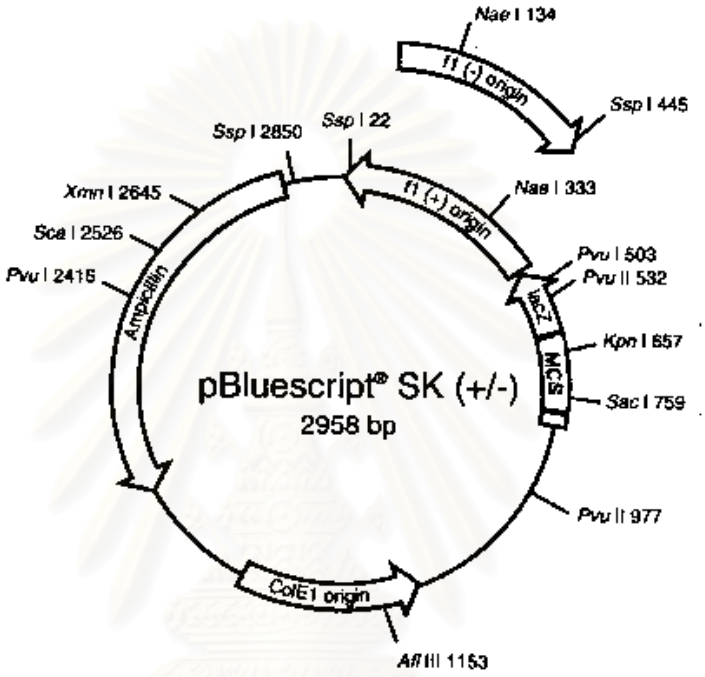


APPENDIX C

RESTRICTION MAP OF pBluescriptSK^{-}



BIOGRAPHY

Miss. Kamontip Kuttiyawong was born on April, 19th (1976) in Mahasarakham, Thailand. She graduated with a Bachelor Degree in Chemistry, Faculty of Science, Silapakorn University in 1997. She was enrolled in the M.Sc.Biochemistry Program since 1998.