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CONSTRUCTION AND CHARACTERIZATION OF CHIMERIC CHITINASE BY HOMOLOGOUS RECOMBINATION

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ในปัจจุบัน ไคทิเนสลูกนำไปใช้ในกระบวนการผลิตไคทินสายสั้นซึ่งเป็นวัตถุดิบในการนำไปประยุกต์ใช้ได้อย่างหลากหลาย ไคทิเนส 60 จาก *Serratia* sp. TU09 (Chi60) ไคทิเนส 66 จาก *Bacillus licheniformis* SK1 (Chi66) และ ไคทิเนสบีจาก *Serratia marcescens* (ChiB) ถูกจัดอยู่ในกลุ่มไกลโคซิลไฮโดรเลส แฟมิลี 18 ซึ่งได้มีการศึกษาอย่างละเอียดแล้ว งานวิจัยนี้เป็นการผลิตไคทิเนสลูกผสมโดยการรวม Chi60 และ Chi66 เข้ากับ ChiB ด้วยวิธีฮอมอโลกัสรีคอมบิเนชัน HRCD66B ซึ่งเป็นไคทิเนสลูกผสมระหว่าง CatDChi66 กับ ChiB ถูกคัดเลือกด้วยวิธีการคัดแยกบนอาหารเลี้ยงเชื้อแข็ง LB ที่มีแอมพิซิลลิน ร่วมกับการคัดแยกเชื้อในอาหารเลี้ยงเชื้อ CCMM ที่มีแอมพิซิลลิน จากวิธีดังกล่าวทำให้สามารถคัดเลือกเชื้อที่มียีนไคทิเนสลูกผสมได้ 6 แบบ ซึ่งเมื่อนำมาวิเคราะห์พบว่ายีนขนาด 1.67 1.68 1.34 และ 1.76 kb ที่เกิดการประสานกันแล้วยังคงสภาพเป็นยีนที่สามารถถอดรหัสเป็นโปรตีนได้ ส่วน HRCHI60B ซึ่งเป็นไคทิเนสลูกผสมระหว่าง Chi60 กับ ChiB ถูกคัดเลือกด้วยวิธีการเดียวกับการคัดเลือก HRCD66B ยีนลูกผสม 87 ยีนจากเชื้อที่คัดเลือกมาได้ถูกแบ่งออกเป็น 5 กลุ่มตามขนาดของยีน จากการวิเคราะห์พบว่ายีนขนาด 1.49 1.55 และ 2.0 kb ที่เกิดการประสานกันแล้วยังคงสภาพเป็นยีนที่สามารถถอดรหัสเป็นโปรตีนได้ ส่วนของยีนบริเวณที่เกิดการรวมกันระหว่าง 2 ยีนมากที่สุดคือบริเวณที่ถอดรหัสได้เป็นบริเวณเร่ง ซึ่งเป็นบริเวณที่สำคัญและพบได้ในไคทิเนส แฟมิลี 18 ทั่วไป ไคทิเนสลูกผสมที่คัดเลือกได้ ถูกนำไปแสดงออกและศึกษาการทำงานของไคทิเนส ผลคือตรวจไม่พบแอกทิวิตีของไคทิเนสที่มี ไคทินคอลลอยด์ PNAC และไคทินสายสั้นเป็นสารตั้งต้น

ภาควิชา.....ชีวเคมี..... ลายมือชื่อนิสิต.....ธนากร ธีรภานนท์.....

สาขาวิชา.....ชีวเคมี..... ลายมือชื่อ อ.ที่ปรึกษาหลัก.....

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THANAKORN THEERAPANON : CONSTRUCTION AND CHARACTERIZATION OF CHIMERIC CHITINASE BY HOMOLOGOUS RECOMBINATION. THESIS ADVISOR : ASSISTANT PROFESSOR RATH PICHYANGKURA, Ph. D., 140 pp.

Nowadays, chitinases play important roles in chitooligosaccharides production which are applied and used in many fields. Chitinase 60 from *Serratia* sp. TU09 (Chi60), Chitinases 66 from *Bacillus licheniformis* SK-1 (Chi66) and Chitinase B from *Serratia marcescens* (ChiB) which belong to family 18 of glycosylhydrolase have been intensively studied. In this work, chimeric chitinase that recombined Chi60 and Chi66 with ChiB have been constructed via *in vivo* homologous recombination. HRCD66B, chimeras that were constructed from recombination between CatDChi66 and ChiB, are screened by LB agar plate containing ampicillin and CCMM broth containing ampicillin. Six chimeras with chimeric chitinase genes were observed and only 4 of them with gene size of 1.67, 1.68, 1.34 and 1.76 kb have in frame recombination and could be translated to amino acid sequences. HRCHI60B, chimeras constructed from recombination between Chi60 and ChiB were also screened by the same method as HRCD66B screening. Eighty seven chimeras were observed and have been grouped into 5 groups based on chimeric gene size. Only 3 of them with gene size of 1.49, 1.55 and 2.0 kb have in frame recombination and could be translated to amino acid sequences. Homologous recombination is mostly found to occur at conserved region encoding for active site motif of family 18 chitinases. These chimeras were expressed and assayed for chitinolytic activity on colloidal chitin, PNAC and chitooligosaccharides but no activity was observed.

Department : Biochemistry Student's Signature Thanakorn Theerapanon
Field of Study : Biochemistry Advisor's Signature
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LIST OF ABBREVIATIONS

A	Absorbance
bp	Base pair
BSA	Bovine serum albumin
BLAST	Basic local alignment search tool
°C	Degree Celsius
CatD	Catalytic domain
ChBD	Chitin binding domain
Chi60	Chitinase 60 from *Serratia* sp. TU09
Chi66	Chitinases 66 from *Bacillus licheniformis* SK-1
ChiB	Chitinase B from *Serratia marcescens*
CCMM	Colloidal chitin minimum medium
DNA	Deoxyribonucleic acid
FnIIID	Fibronectin typeIII-like domain
g	Gram
GlcNAc, NAG	*N*-acetyl-D-glucosamine
hr	Hour
Ile, I	Isoleusine
kb	Kilo base
kDa	Kilo Dalton
L	Liter
Lys, K	Lysine
Leu, L	Leusine
M	Molar
ml	Milliliter

mg	Milligram
ng	Nanogram
μg	Microgram
μl	Microliter
min	Minute
pBS/SK$^-$	pBluescript SK$^-$
rpm	Revolution per minute
w/w	weight by weight
PNAC	Partially *N*-acetylated chitin

CHAPTER I

INTRODUCTION

Chitin

Since 1811 that chitin has been discovered, it was then widely studied due to the fact that, second to only cellulose, chitin appears to be the most abundant polysaccharides with high potential for various applications. Chitin is naturally found as one of the major structural components of many living organisms as shown in Table 1 (Knoor, 1984). It constitutes the exoskeleton of arthropods, such as the shell of shrimps, crabs and insects and also found in cell wall of fungi and yeast. Yearly, around 10 gigatons of chitin are produced on earth (Tharanathan and Kittur, 2003) made it to be one of a large carbohydrate resource available for researching.

Chemistry of chitin

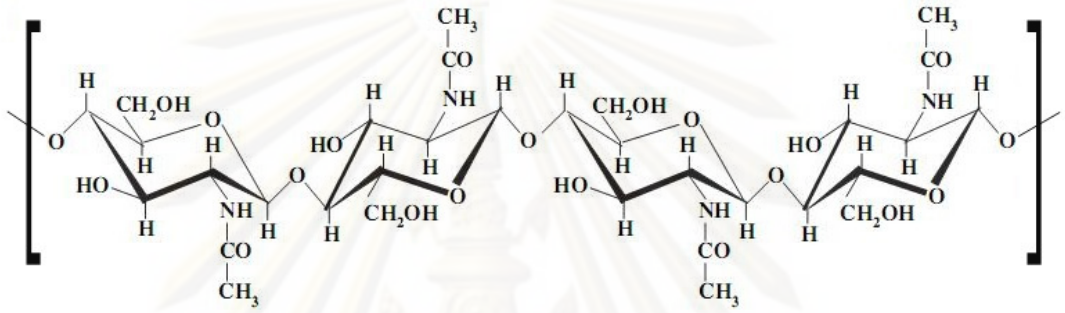
Chitin is a homopolymer of *N*-acetyl- glucosamine (GlcNAc) linked with β-(1,4) glycosidic bond. This β configuration makes GlcNAc unit 180^{o} twisted every one unit, thus, the repeating unit of chitin chain is dimer of GlcNAc as shown in Figure 1.1. GlcNAc unit is chiral and specifically linked from C-1 of one unit to C-4 of the contiguous one, the "left" and "right" direction, therefore, could be assigned to the chain with reducing and non-reducing terminal. Structure of chitin found in nature is roughly divided into 3 polymorphic forms depends on the direction of chitin chains that packed together. First one is so called α-chitin, the most commonly found in many organisms including crustaceans, insects and fungi. Chitin chains of α-chitin are stacked in antiparallel direction which is stabilized by hydrophobic interaction between the surfaces of sugar ring along the chain. Additionally, half of CH_2OH groups in chitin chain are bonded with CH_2OH groups of the adjacent one thus help stabilizing structure, while another half of CH_2OH groups bonded with carbonyl group within the same stack. Second form is β-chitin, a less common allomorph found in the spines of the polychaete Aphrodite, the pen of the squid Loligo, the tubes of

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

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

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

A



B

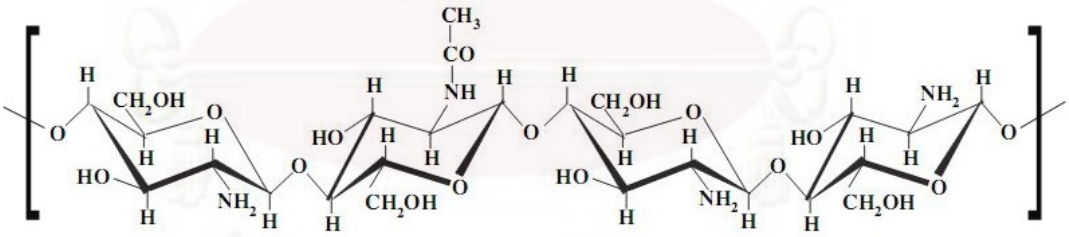


Figure 1.1 Chemical structures of chitin and chitosan

This figure shows chemical structure of chitin (A) and chitosan (B).

Pogonophora and the spines of certain marine diatoms. This form has parallel arrangement of chitin chains. Differ from α-chitin, chitin chains in the β-chitin are stacked together with only hydrophobic interactions, whereas all CH_2OH groups are bonded with carbonyl groups in the same chain. The last rarely existed form is γ-chitin. It has both parallel and antiparallel conformation with a 3 chain unit in which two parallel chains are followed by an opposite direction one. This form has been only found in stomach lining of Loligo and not yet to be analyzed in detailed, but X-ray diffraction patterns and NMR spectra of this allomorph shows to be distinguished from α- and β-chitin which confirms that this conformation is not the distortion of other forms. The graphical structure of these 3 forms of chitin is shown in Figure 1.2. (Blackwell, 1969)

Similar to cellulose, chitin is the aggregate of linear polysaccharides into crystalline structure which contributes to the strength in chitinous materials of the above organisms, and also to the water insoluble properties. Chitin cannot dissolve in common solvent unless it is degraded to oligosaccharides. It can only dissolve in some strong solvent, such as dimethylformamide with lithium chloride, hexafluoroisopropanol, hexafluoroacetone sesquihydrate, or 1,2 chloroethanol with sulfuric acid, made it hard to be further applied. In contrasts, chitosan, a deacetylated derivative of chitin, could dissolve in weak acid like acetic acid and lactic acid. Chitosan is a copolymer composed of GlcNAc and GlcN randomly distributed in *β*-(1,4)-linked linear chain at higher percentage of GlcN. Molecular weight of chitin and chitosan could be up to 10^6 Dalton depended on degree of polymerization and, in case of chitosan, degree of deacetylation. These two are important parameters to determine physical, chemical and biological properties which also dictate the use of chitin and chitosan for various applications.

Nowadays, chitin and chitosan serve as the biopolymer materials to many fields of industry with environmentally friendly characteristics which concern those who are aware of ecological safety. They are from natural resources, biodegradable, and almost non-toxic. Chitosan has potentials to be applied and used in many fields. In pharmaceutical uses, it shows possibility to be used as controlled drug releasing agents (Shu, Zhu and Song, 2001), and has been shown to facilitate wound healing (Lloyd et al., 1998). In food industry, addition of chitosan helps improve texture of

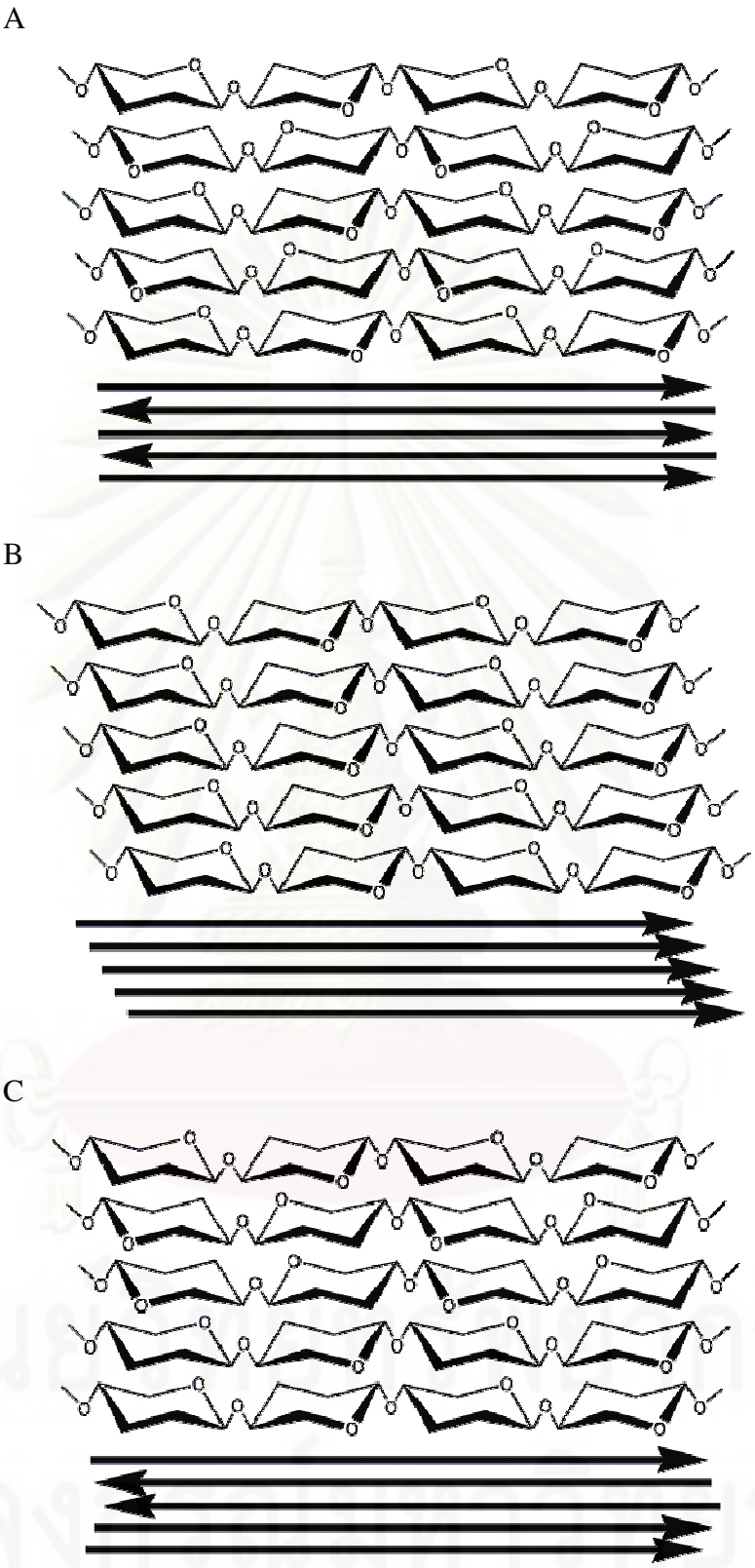


Figure 1.2 Simplified structure of α-chitin, β-chitin and γ-chitin

Structure of α-chitin (A), β-chitin (B) and γ-chitin (C) are displayed. Heads and tails of arrows represent reducing and non-reducing ends of chitin chain, respectively.

cold-set chicken salt-soluble protein gel (Kachanechai, Jantawat and Pichyangkura, 2008). It also has antimicrobial activity which could be used as non-chemical preservatives (Knoor, 1984). In ecology, chitosan powders could be used to purify heavy metals contaminated water (Muzzarelli et al., 1989). In agriculture, coating with chitosan could help enhance the quality of strawberry after harvested (Hernandez-Munoz et al., 2008), and it could induce plant resistance response system (Walker-Simmons, Hadwiger and Ryan, 1983).

To be applied more efficiently, chitin and chitosan was degraded into low molecular weight chitin/chitosan and chitooligosaccharides. Application potentials of chitooligosaccharides specifically change with different degree of polymerization. $(GlcNAc)_6$ and $(GlcN)_6$ have shown antitumor activity, immuno-enhancing effects, and enhancing protective effects against infection with pathogens in mice (Suzuki et al., 1986). Oligomers with six or more residues show strong physiological activities to elicit plant defense responses (Shibuya and Minami, 2001). They show the possibility to be used as prebiotics by stimulating beneficial gut bacterium. Importantly, studies showed that chitooligosaccharides could promote growth and enhance quality in some plants including orchid (Kananont et al., 2010), since Thailand is one of the world's largest orchid exporters.

Production of chitooligosaccharides could be achieved by various methods of degradation. Chemical method use strong acids (hydrochloric acid or nitrous acid) to randomly hydrolyze chitin/chitosan which give mostly monomers and other non-specific products. This method also causes the hazardous acid waste that brings about further treatment problems. Physical method undergoes irradiation with gamma ray which causes less environmental pollution (Choi et al., 2002). This method still gives non-specific products. Enzymatic method has become an important degradation process due to the fact that the reaction is done under mild condition, controllable and gives specific products which is up to the enzyme used and reaction conditions (Jung et al., 2007).

Chitinases

Chitinase (EC 3.2.1.14) is one of the chitinolytic enzymes the same as lysozyme (EC 3.1.2.17) and chitosanase (EC 3.2.1.132) which is found in many organisms with

their own specific biological purposes. In higher plants, chitinases are used as defensive response agents against pests and fungal pathogens. In insects and crustaceans, chitinases are used for cuticle degradation during the ecdysis. Microorganisms produce chitinases to hydrolyze chitinous substances and utilize as carbon source. Recently, chitinases are also found in fish and mammals. Chitinases have a wide range in size varied from 30 kDa in plants to as high as 120 kDa in some vertebrates (Koga et al., 1999).

Classification of chitinases

Chitinases are classified into family 18 and 19 of glycosylhydrolase families, based on amino acid sequences similarities which correlate with structure and mechanism of catalysis. Family 18 chitinases is the largest found in eukaryotes, prokaryotes and viruses which have several conserved amino acids regions. Figure 1.3A shows the conserve sequences found in the active site, including a glutamic acid (E) residue (residues in white letter) of the chitinases from *Bacillus circulans*, *Serratia marcescens*, *Pyrococcus kadakaraensis* and *Trichoderma harzianum*. These signature sequences played a crucial role in catalytic mechanism. Family 19 chitinases are mostly found in plants with the exception of *Streptomyces griseus*,. The conserved amino acids from these families are also found in the active site which two glutamic acid residues (residues in white letter) are important in the catalysis mechanism of family 19. Sequences represented by barley chitinase (*Hordeum valgare*), potato chitinase (*Solanum tuberosum*), pea chitinase (*Pisum sativum*) and *Arabidopsis thaliana* are shown in Figure 1.3B.

Structures of chitinases

X-ray crystallography and NMR spectroscopy are important procedures for obtaining 3-dimensional (3D) structure of enzyme which indeed helps for studying structures and mechanisms of catalysis of the enzymes. Most chitinases consist of at least 2 major domains, catalytic domain containing substrate binding cleft and active site and noncatalytic domain which are reported to facilitate chitin binding properties.

A

```
B.circulans          IISVGGWTYSNRFFDGVDIDWETPVS
P.kodakaraensis      LISVGGWTLSKYFFDGVDLDWEYPVS
S.marcescens         LPSIGGWTLSDPFFDGVDIDWEFPGG
T.harzanum           ILSIGGWTWSTNFFDGVDIDWEYPAD
                     : *:**** *  ******:**E * .
```

B

```
A.thaliana   KREIAAFLGQTSHETTGGWPTAPADGPYAWGYCFLREQNP-SDYCQASSEFPCASG
H.valgare    KREVAAFLAQTSHETTGGWATAPADGAFAWGYCFKQERGASSDYCTPSAQWPCAPG
P.sativum    KREVAAFFGQTSHETTGGWATAPADGPYSWGYCFKQEQNPASDYCEPSATWPCASG
S.tuberosum  KREIAAFFAQTSHETTGGWASAPADGPYAWGYCFLRERGNPGDYCPPSSQWPCAPG
             ***:***:.****E*****.:*****.::***** :E:.  .*** .*: :***.*
```

Figure 1.3 Amino acid sequences alignment in the active site of family 18 chitinases and family 19 chitinases

Alignment of amino acid sequences in the active site of family 18 chitinases (A) and family 19 chitinases (B) are displayed.

Structures of family 18 and 19 chitinases show significant differences especially in the catalytic domain.

1. Catalytic domain of chitinases

1.1 Catalytic domain of chitinases family 18

The catalytic domain structure of family 18 chitinases is $(\beta/\alpha)_8$ barrel composed of eight α-helices forming a ring toward the outside and eight strands of β-sheet bend into a barrel structure. In addition, many chitinases in this family have one or more noncatalytic domains found at both the N- or C- termini. Figure 1.4(A) shows the 3D structure of chitinase B from *S. marcescens*, a representative of family 18 chitinases. There is a small (β+α) domain stitches to the $(\beta/\alpha)_8$ barrel to form catalytic domain with chitin binding domain anchoring at C-terminus. The crystal structures of catalytic domain of other glycosylhydrolases family 18, such as chitinase A from *S. marcescens* (Perrakis et al., 1994), hevamine from *Hevea brasiliensis* (Terwisscha van Scheltinga et al., 1994), endo-β-*N*-acetyl-glucosaminidase F1 from *Flavobacterium meningosepticum* (Roey et al., 1994), and endo-β-*N*-acetyl-glucosaminidase H from *Streptomyces plicatus* (Rao, Guan and Roey, 1995), exhibit similar barrel structure.

1.2 Catalytic domain of chitinases family 19

In contrast to family 18, structure of catalytic domain of family 19 chitinases is essentially composed of α-helices with one three-stranded β-sheets. Most chitinases of family 19 also consist of additional binding domain, but only one at the N-termini, except for chitinase from *Urtica dioica* which has more than two modules containing a tandem repeat of two N-terminal chitin binding domains. Interestingly, the folding pattern of catalytic core of this family is found to be conserved among other glycosylhydrolases (family 22, 23, 24 and 46) which are not only chitinase but also chitosanase and lysozyme. A model structure family 19 is chitinase from barley *Hordeum vulgare* as shown in Figure 1.4(B).

2. Substrate-binding subsites of chitinases

According to subsite nomenclature for glycosylhydrolase proposed by Davies *et al*., 1997, chitinases have 6 binding subsites within catalytic cleft in which

A

B

Figure 1.4 Crystal structures of chitinase family 18 and 19.

Chitinase B from *S. marcescens*: 1E6N (A), a representative of chitinases family 18, and chitinase from barley seeds: 1CNS (B), a representative of chitinases family 19 are shown. The 3D models are visualized by PyMOL 0.99rc6.

have several residues involved in binding interactions. Substrate binding mechanism was proposed mainly by X-ray crystallography. In Family 18, the molecular dynamics simulations of $(GlcNAc)_6$ binding to *S. marcescens* chitinase A indicated that the binding cleft are represented by (-4)(-3)(-2)(-1)(+1)(+2). The -1 sugar was distorted to boat conformation and this was found to be critical in catalysis mechanism (Brameld and Goddard, 1998b). Family 19 was firstly reported to has (-4)(-3)(-2)(-1)(+1)(+2) subsites the same as those in hen egg white lysozyme (John Hart et al., 1995) but the study on barley chitinase revealed that the binding cleft seems to be longer, so that the binding subsites should be (-3)(-2)(-1)(+1)(+2)(+3) instead. In addition, the simulation study indicated that conformation of $(GlcNAc)_6$ substrate units that binds to barley chitinase are all in a chair conformation (Brameld and Goddard, 1998a). Hydrogen bonding interactions between catalytic residues and bound substrate are shown in figure1.5.

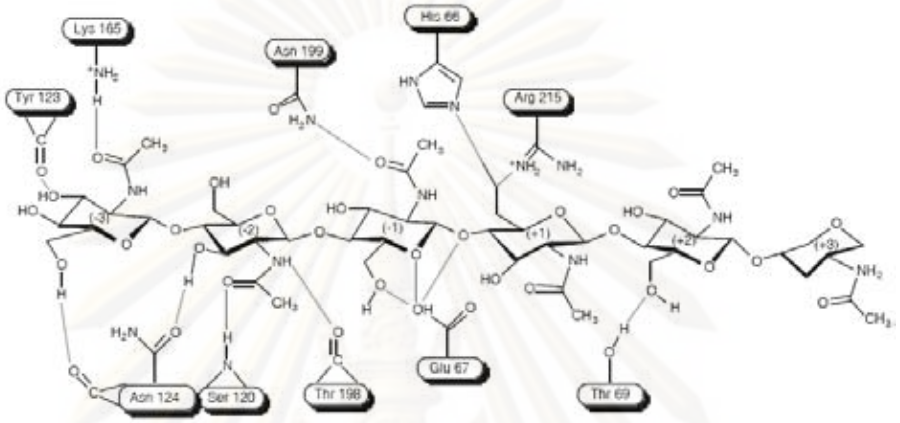
3. Noncatalytic domains of chitinases

Most chitinases of both family 18 and 19 have noncatalytic modules attached to the catalytic domains for some specific purposes. The important one is chitin binding domain (ChBD) composed of only β-sheets folding into globular structure with exposed aromatic residues. This domain could bind to chitin via hydrophobic interactions, thus it is required for chitinase to bind insoluble chitin specifically (Ikegami et al., 2000). The other module that also found in chitinase is Fibronectin typeIII-like domain (FnIIID). This domain shows significant sequence similarity with Fibronectin typeIII module and found as tandem repeats. The function of Fibronectin typeIII-like domain is still unclear but deletion of this reduces the activity of chitinase on insoluble substrates (Watanabe et al., 1994).

Catalytic mechanism of chitinases

Chitinases catalyze hydrolytic by cleaving at β-(1,4) glycosidic bonds between GlcNAc units which, in general, results in two anomeric configuration of products, retention or inversion. The most powerful information has been obtained from X-ray crystal structures of the enzymes complexed with their substrate analogues. These structures were solved by several investigators, and their catalytic mechanisms were

A



B

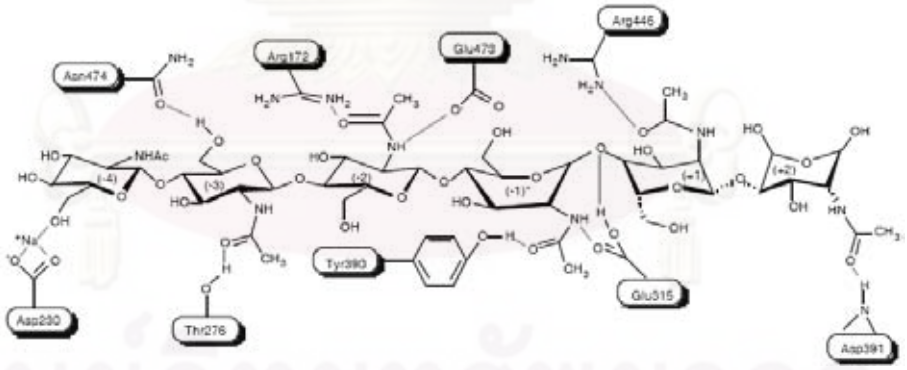


Figure 1.5 Hydrogen bonding interactions between $(GlcNAc)_6$ and catalytic residues within substrate binding subsites of chitinases

Hydrogen bonding interactions between $(GlcNAc)_6$ and catalytic residues within substrate binding subsites of barley chitinase (A) and *S. marcescens* chitinase A (B) are displayed.

discussed from the relative locations of catalytic residues to the bound substrate analogues. The catalytic mechanism of chitinases family 18 and 19 are significantly different which will be discussed further in details.

1. Catalytic mechanism of family 18 chitinases

The mechanism of family 18 chitinases has been proposed to retain anomeric configuration of the substrates. From the sequence comparison, the carboxylic amino acid residues involved in catalysis were found to be conserved in all chitinases of family 18. The study on chitinase A1 from *B. circulans* WL-12 showed that site-directed mutagenesis of Glu204 completely eliminated its activity while mutagenesis of Asp200 and Asp202 only decreased the enzymatic activity but did not completely eliminate the activity (Watanabe et al., 1994; Watanabe et al., 1993). From these results and the 3D structure analysis clearly showed that there is only one acidic residue at active site that is considered to be proton donor in the catalysis. Unlike other retaining glycosylhydrolases which undergo double replacement mechanism with two carboxylic catalytic residues, family 18 chitinases are proposed to involve a substrate-assisted mechanism due to lacking of enzymic nucleophile. This mechanism is supported by crystal structure and kinetic studies of the inhibitor allosamidin bound to hevamine, a family 18 chitinase from *Hevea brasiliensis*, which allosamidin is thought to be a transition state analog (Terwisscha van Scheltinga et al., 1995). Based on double displacement mechanism, the catalysis starts with proton donation from catalytic carboxylate to oxygen atom of β-(1,4) glycosidic linkage to form a oxazolinie ion intermediate stabilized by an anchimeric assistance of the sugar *N*-acetyl group instead of second carboxylic residue. Such stabilization might occur through a charge interaction between the C1 carbon and the carbonyl oxygen of the acetamido group (Figure 1.6 Scheme1). Quantum mechanical studies supported this substrate-assisted mechanism in family 18 chitinase (Brameld et al., 1998).

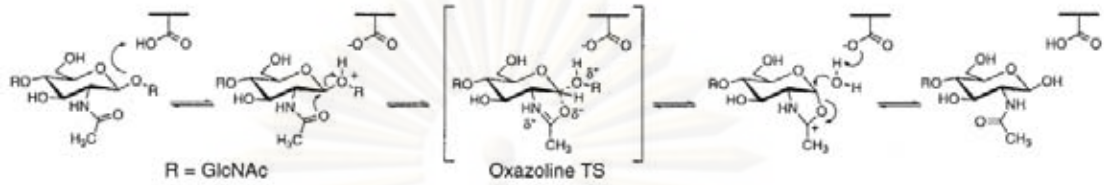
Recently, the latest catalytic mechanism in chitinase A from *S. marcescens* was proposed. Yannis and coworkers had studied on the active site and found that residues Asp313 and Tyr390 along with Glu315 were proton donor residues which play a central role in the catalysis as shown in Figure1.6 Scheme 2. After substrate binding, conformation of -1 sugar change from “chair” to the unstable “boat” form with acetamido group bend away from Tyr390 which provide spaces for water molecule

that H-bonded to both Tyr390 phenol hydroxyl group and the NH of acetamido group of the -1 sugar. Then Glu315 donates its proton to oxygen of the glycosidic C1(-1)-O4(+1) bond which subsequently break the bond and induce the acetamido group of -1 sugar to rotate around the C2-N2 bond toward Tyr390. So that the water molecule earlier bonded to Tyr390 was forced to translocate toward Glu315 residue. At this stage, water molecule donates proton to the carboxylic group of Glu315 while the remaining hydroxide anion is bonded to the positive charged C1 carbon of -1 sugar at the same configuration of the departed oxygen O4 of the +1 sugar. This completes the retaining mechanism without the formation of oxazoline ion intermediate (Papanikolau et al., 2001).

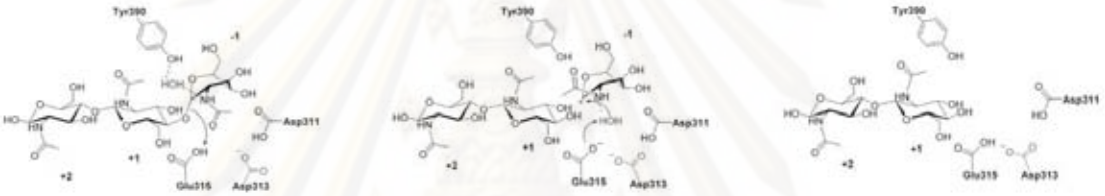
2. Catalytic mechanism of family 19 Chitinases

In contrast to family 18, family 19 chitinases were reported to yield α-anomer products which indicate inversion of hydrolytic mechanism. The major difference in structure especially in the active sites was thought to be the cause of this anomeric configuration difference. Two carboxylic amino acid residues were studied to be involved in catalysis mechanism. Site-directed mutagenesis study of barley chitinase showed that the mutation of Glu67 to Gln completely eliminated its activity, and that of Glu89 reduced the activity to 0.25 % compared with wild type. Thus, Glu67 and Glu89 are most likely to be a proton donor and a second catalytic residue respectively (Andersen et al., 1997). The distance between these two catalytic residues is also found to be closely related to the catalytic mechanism. In case of retaining enzymes, the average distance between the two catalytic residues is about 4-5 Å, while there is larger space to about 10-11 Å in inverting enzymes (Wang et al., 1994). With these characteristics, family 19 chitinases commonly undergo single displacement mechanism with two catalytic carboxylate residues, one as general acid and another one as general base. Figure 1.6 scheme 3 shows the single displacement mechanism. First, the general acid residue donates proton to oxygen atom of *β*-(1,4) glycosidic linkage whereas the basic residue activates nucleophilicity of the water molecule and stabilizes the oxocarbenium ion intermediate that formed. The direction of coming water molecule is α-side thus results in inversion of anomeric configuration (Brameld and Goddard, 1998a).

Scheme 1



Scheme 2



Scheme 3

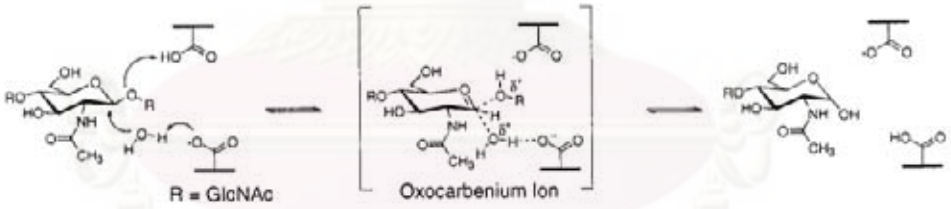


Figure 1.6 Hydrolysis mechanisms of chitinases

Scheme 1: Substrate-assisted mechanism of family 18

Scheme 2: Modified substrate-assisted mechanism proposed in 2001

Scheme 3: Single displacement mechanism of family 19

Bacterial chitinases of family 18 will be focused since they have been intensively studied in our laboratory and will be used in this work.

Chitinase 60 from *Serratia* sp. TU09

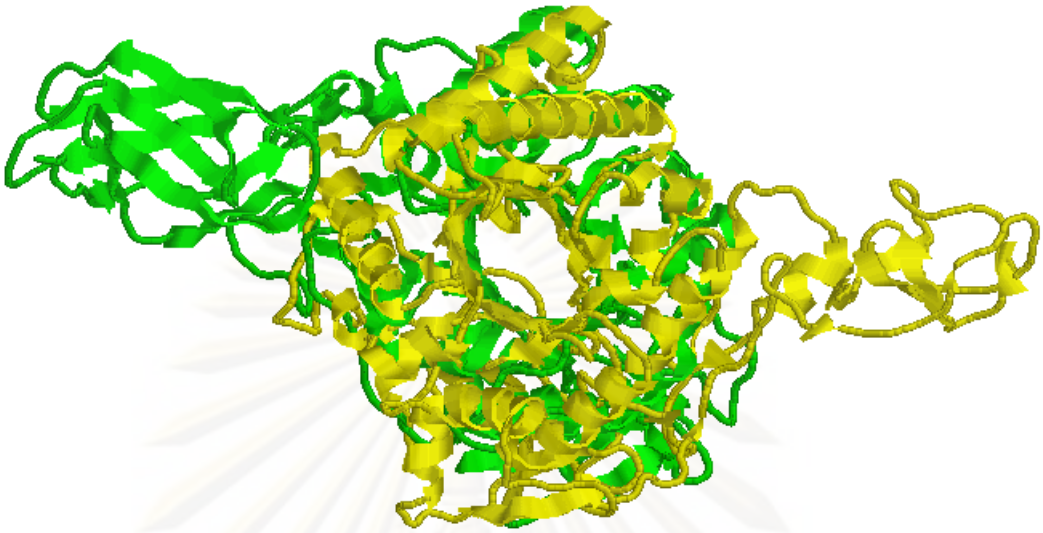
Chitinase 60 from *Serratia* sp. TU09 (Chi60) has almost the same structure as the very well studied chitinase A from *S. marcescens*. Structure of Chi60 consists of N-terminal FnIIID that acts as chitin binding module and catalytic domain. This chitinase hydrolyzes chitin with both endo- and exo-mode of catalysis depends on substrate morphology and ionic strength. Chi60 binds to chitin via hydrophobic interaction between sugar rings of GlcNAc units and aromatic residues on chitinase surface which guide chitin into catalytic cleft. Chi60 hydrolyzes chitin strands that are fed through FnIIID from the reducing end and yields major products of $(GlcNAc)_2$ (Kuttiyawong, Nakapong and Pichyangkura, 2008). Characterization of Chi60 was achieved by Kamontip Kuttiyawong (2001) which exhibits optimum pH at pH 5.0 and has the optimum temperature at 55ºC in citrate buffer pH 5.0.

Chitinase 66 from *Bacillus licheniformis* SK-1

Chitinase 66 from *B. licheniformis* SK-1 (Chi66) was characterized by Sanya Kudan and found to be similar to chitinase A1 from *B. circulans* WL-12 (Toratani et al., 2006). Chi66 consists of catalytic domain attached with FnIIID and ChBD at C-terminal; however, FnIID in Chi66 has a different folding structure from that in Chi60. FnIIID folding in Chi66 has a characteristic hydrophobic core of 3 conserved aromatic residues which is generally found between the substrate binding domain and the catalytic domain of various insoluble substrate-degrading enzymes. Interestingly, Chi66 also feeds substrate through N-terminal which is the nonreducing side of active site, although it has FnIIID and ChBD attached at C-terminal. Thus, Chi66 was suggested to hydrolyze chitin from reducing end. Chi66 showed two optimum pHs at pH 5.0 and pH 9.9 and has optimum temperature at 60-70ºC (Kudan, 2001).

Figure 1.7 Sequence alignment of the active site of Chi60, Chi66, and ChiB Sequences showed in the box are conserved motif in family 18.

A



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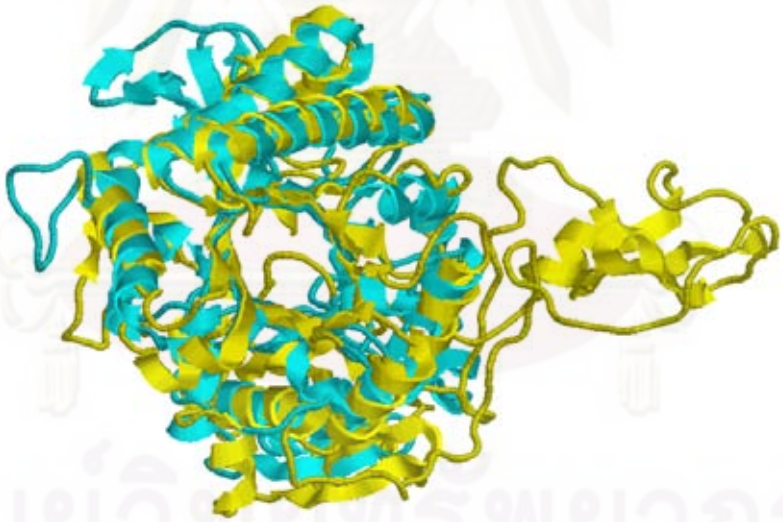


Figure 1.8 Superimposed structure of chitinases visualized by Rasmol 2.7.2.1

Superimposed structure of Chi60 (green) and ChiB (yellow) (A) and superimposed structure of CatDChi66 (blue) and ChiB (yellow) (B) are displayed.

Chitinase B from *S. marcescens*

Chitinase B from *S. marcescens* (ChiB) was intensively studied along with chitinase A from *S. marcescens*. Structure of ChiB consists of catalytic domain with ChBD located at the reducing side of active site and has porch loop that blocks chitin binding at nonreducing -4 subsite. This could imply that ChiB hydrolyzes chitin from nonreducing end and yields $(GlcNAc)_3$ as primary product before further degraded to monomer and dimer of GlcNAc. The optimum pH and temperature of ChiB are at pH 6.0 and 58ºC respectively (Brurberg, Nes and Eijsink, 1996; van Aalten et al., 2000).

From the studies above, direction of chitin hydrolysis of ChiB is different from Chi60 and Chi66 but amino acid sequences alignment of catalytic domains shows that there are many regions of conserved sequences especially in the active site which is a unique motif of family 18 chitinases (Figure 1.7). Moreover, the overall structure of catalytic domains of these 3 chitinases folded into the very similar TIM barrel structures. Figure 1.8 shows the superimposed structure of Chi60/ChiB and CatDChi66/ChiB. The notable difference in the structure is the blocked loop in ChiB which is absent in Chi60 and Chi66. The active cleft of Chi60 and Chi66 has extended substrate binding groove of both reducing and nonreducing side thus these two are proposed to have endo-mode of catalysis. ChiB, on the other hand, has a porch loop that limits the binding chitin from extending to nonreducing side, and also has a flexible loop that crosses over the active cleft. This tunnel-like model combined with the extended ChBD suggested that ChiB is a true exochitinase (van Aalten et al., 2000).

Enzyme manipulation of chitinases

From previous thesis work of Sanya Kudan (2004), deletion and shuffling of non-catalytic domains on Chi66 were constructed by genetic engineering. Results showed that relative activity on crystalline chitin substrates decreased as the ChBD was deleted and it decreased even more with both deletion of FnIIID and ChBD. On the contrary, the activity of the enzyme on colloidal chitin substrates increased when the non-catalytic domains were deleted, suggested that these domains contributes to

the exo-mode of hydrolysis. The mutant of Chi66 with N-terminal FnIIID of Chi60 was also constructed but had no significant difference in hydrolysis activity on both crystalline and colloidal chitin. Furthermore, mutant of only FnIIID of Chi60 attached to CatD of Chi66 showed a decrease in activity on crystalline chitin, the same as wild type without non-catalytic domains. This could be assumed that the non-catalytic domains of each chitinase might have their own specific functions.

Homologous recombination technique in genetic engineering

Chromosomal recombination in bacteria is a common phenomenon found in asexual reproduction and natural genetic exchange both within and cross bacterial strains which brings about diversity and adaptation to natural selection (Dykhuizen and Green, 1991). Homologous recombination occurs through various pathways with specific set of recombinase and each required different size of homologous nucleotide sequences. The RecA RecB recombination pathway, requires a large complete DNA sequence homology of around 70–200 bp while the RecE pathway can promote recombination with shorter sequences of as little as 6 bp of perfect homology (Keim and Lark, 1990). Recently, recombination in *E. coli* has been used in DNA cloning as the new alternative way that has advantages over traditional cloning by restriction enzyme. Strategy of using restriction enzyme imposes practical limitations that cloning and ligation of inserted DNA molecules can only be precisely combined between or near convenient restriction endonuclease sites (Zhang et al., 1998). The idea of homologous recombination cloning is shown in Figure 1.9. The cloning vector was first prepared with homology arms sequences at the cloning site. This linearized vector was then transformed into ET-competent *E. coli* hosts; *E. coli* with RecE/RecT activity, containing target gene in either plasmid, BAC or chromosomal DNA with the same homology arms as designed in cloning vector flanking at both side of target gene. The recombination occurred *in vivo* at homology arms resulting in fully insertion of target gene into the episome without any detectable mutational errors (Zhang et al., 2000).

In recent times, protein engineering by homologous recombination has become more and more fascinating strategy for studying the functional region within a conserved fold of proteins since the observation on identical folding proteins showed

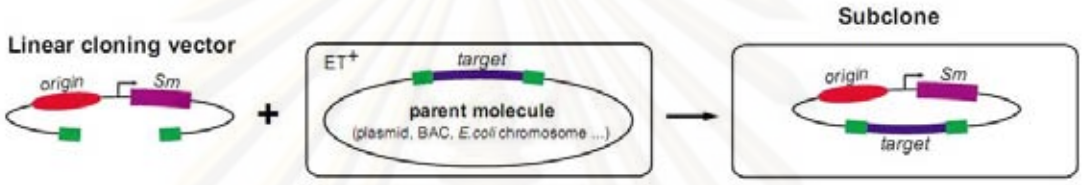


Figure 1.9 Schematic view of homologous recombination in DNA cloning (Zhang et al., 2000).

that not only sequences but also functions among these proteins were diverged. Thus, this approach has been further applied to construct chimeric proteins with fragments swapped between two parental sequences whether to pinpoint which sequence differences determine functional differences (Carbone and Arnold, 2007). In 1999, Hiromi and his team successfully constructed chimeric PQQ glucose dehydrogenase (PQQGDH) from *Acinetobacter calcoaceticus* and *E. coli*. PQQGDH is a β-propeller protein that has highly conserved primary structures among bacterial species but the EDTA tolerance property is different. That is, PQQGDH in *A. calcoaceticus* is very stable to EDTA treatment while in *E. coli* is not. They used homologous recombination to construct chimeric PQQGDH gene of these two strains at conserved region responsible for EDTA tolerance. Figure 1.10 shows schematic view of chimeric gene construction by homologous recombination. Plasmid containing two genes in tandem was firstly constructed before digested with restriction enzyme. Linearized plasmid was then transformed into *E. coli* JC8679, a RecE+ host, and a plasmid with chimeric gene was obtained. Chimeric PQQGDH showed complete EDTA tolerance as found in *A. calcoaceticus* which help defining a new region responsible for EDTA tolerance (Yoshida et al., 1999). Using homologous recombination in this way was also done in chimeric cyclodextrin glucanotransferases (CGTase) manipulation. In 2003, Anusak Keadsin constructed series of CGTase chimera between α-CGTase from *Paenibacillus macerans* and β-CGTase from *B. circulans* A11. Chimeric CGTase of different domains resulted in different ratios of α- β- γ- cyclodextrin production (Keadsin, 2003).

We are interested in constructing chimeric chitinase between reducing end-hydrolyzing chitinase and non-reducing end-hydrolyzing chitinase by homologous recombination based on hypothesis that chimera of these two types of chitinases should have recombination site within catalytic domain since it showed highest nucleotide sequences homology. Chimera with fused catalytic domain might have ability to bind substrate from both reducing and non-reducing end and this might affects mechanism of chitinase which causes product size to be changed. In this work, we have constructed series of chimeric chitinases gene combining of Chi60 and Chi66 with ChiB within the catalytic domain via homologous recombination. The chimeras' DNA sequences were analyzed to find recombining sites patterns and the expressions of chimeras were studied.

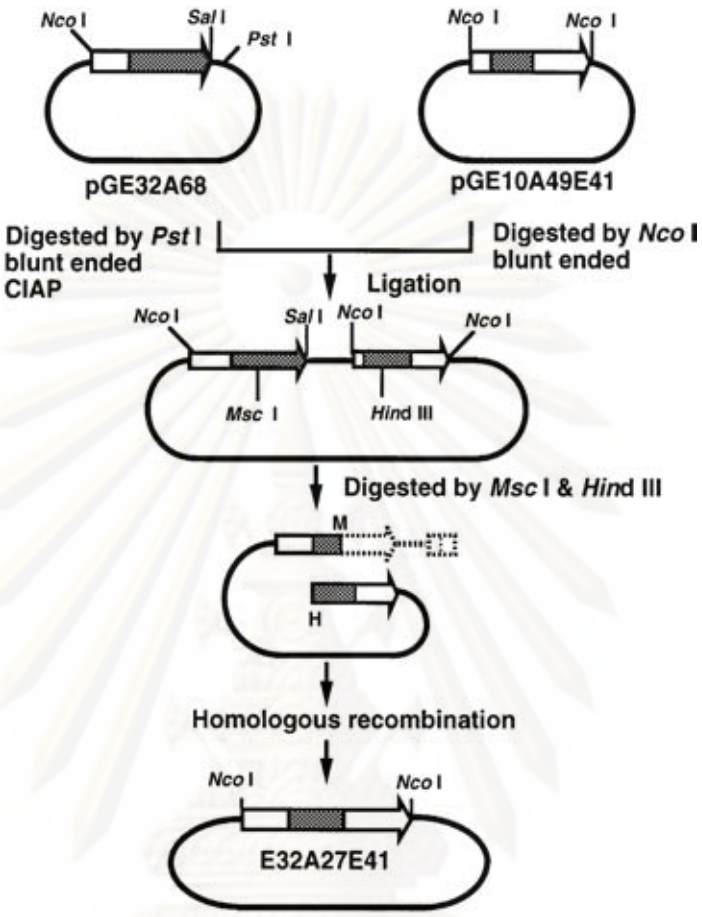


Figure 1.10 Schematic view of chimeric PQQGDH gene construction by homologous recombination (Yoshida et al., 1999).

CHAPTER II

MATERIALS AND METHODS

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
Gel mate 2000: TOYOBO, Japan
GS Gene linker UV chamber: Bio-Rad, USA.
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.
Mini-PROTEAN 3 Cell: Bio-Rad, 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.
Vortex: Model K-550-GE, Scientific Industries, Inc., U.S.A.
Water bath: Charles Hearson Co. Ltd., England

Chemicals

Acetone: Merck, Germany
Acrylamide: Merck, U.S.A.
Agarose: GIBCOBRL, U.S.A.
Ammonium persulphate: Sigma, U.S.A.
Ampicillin: Sigma, U.S.A.
Aniline: Merck, Germany

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.

Charcoal, activated: Sigma, U.S.A.

Chloroform: BDH, England

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

Diphenylamine: BDH, England

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

di-Sodium ethylenediaminetetra acetate: M&B, England

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

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

Ethidium bromide: Sigma, U.S.A.

Ethyl alcohol absolute: Carlo Erba Reagenti, Italy

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

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

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

Phenol: BHD, England

85%Phosphoric acid: Lab Scan, Ireland

Potassium acetate: Merck, Germany

Potassium ferricyanide: BDH, England

Potassium phosphate monobasic: Carlo Erba Reagenti, Italy

Qiaquick Gel Extraction Kit: Qiagen, Germany

Shrimp shell Chitin and squid pen chitin: Ta Ming Enterprises Co., Ltd, Samutsakon, Thailand

Silica gel plate (Kieselgel 60): Merck, Germany

Sodium carbonate anhydrous: Carlo Erba Reagenti, Italy

Sodium citrate: Carlo Erba Reagenti, Italy

Sodium chloride: Carlo Erba Reagenti, Italy

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

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: Scharlau, Spain

Yeast extract: Scharlau, Spain

Enzymes and Restriction Enzymes

Lysozyme: Sigma, U.S.A.

Proteinase K: Sigma, U.S.A.

Restriction Enzymes: New England BioLabs, Inc., U.S.A. and Fermentas, Canada.

RNase A: Sigma, U.S.A.

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

Taq DNA polymerase: Fermentas, Canada.

Bacterial Strains

E. coli Top 10 (Invitrogen) with genotype *F-, mcrA, Δ(mrr-hsdRMS-mcrBC), φ80lacZΔM15, ΔlacX74, nupG, recA1, araD139, Δ(ara-leu)7697, galE15, galK16, rpsL(Str^R), endA1, λ$^-$* was used as high efficiency cloning competent cell.

E. coli DH5α with genotype *F′, ∅80δ lacZΔM15, Δ(lacZYA-argV169), endA1, recA1, hsdR17 (r_K-m_{K+}), deoR, thi-1, supE44, λ$^-$gyrA96, relA1* was used as competent cell for chimeric chitinase expression.

E. coli Origami(DE3) with genotype *Δ, (ara–leu)7697, ΔlacX74, ΔphoA, PvuII, phoR, araD139, ahpC, galE, galK, rpsL, F'[lac+ lacIqpro], (DE3), gor522::Tn10, trxB, (KanR, StrR, TetR)* was used as competent cell for chimeric chitinase expression.

E. coli JC8679 with genotype *F′, thr-1, leu-6, thi1, lacY1, galK2, ara14, xy15, mtl1, proAZ, his4, argE3, str31, tsx33, supE44, λ⁻, recB21, recC22, sbcΔ23* was used for homologous recombination.

Chitinous substrate preparations

1. Partially *N*-acetylated chitin (PNAC)

Partially *N*-acetylated chitin (PNAC) was prepared from squid pen chitin by suspending 10 g of powdered squid pen chitin in 250 ml 40% (w/w) NaOH and vacuumed for 4 hours. 750 g crushed ice was then added and shook vigorously until the squid pen chitin was completely dissolved in NaOH solution. The mixture was stirred overnight at 4°C. Concentrated HCl (12M) was added to adjust the pH to 7, after that, 2 volumes of cold acetone was added to precipitate partially *N*-acetylated chitin (PNAC) which was collected by filtration. PNAC was dialyzed with water to remove salt before it was lyophilized and kept in dried fibrous form of PNAC. PNAC was further dissolved in 1% acetic acid to make 3 mg/ml PNAC solution and used in reaction as soluble chitin substrate at final concentration of 1 mg/ml.

2. Colloidal chitin

Colloidal chitin was prepared from flake shrimp shell chitin by the methods described by Jeuniaux, Elizabeth and Victor (1966) and Yamada and Imoto (1981) with some modification. Forty grams of shrimp shell chitin was hydrolyzed by adding 400 ml of concentrated HCl (12M) and stirred for 6 hours on ice with magnetic stirrer. After stirred at 37°C for a while, the chitin hydrolysate was filtrated into 4 L of chilled distilled water. The colloidal mixture was kept at 4 °C overnight. The colloidal chitin was collected by centrifugation at 8,000 g for 15 minutes and the pellet was washed by resuspending with distilled water until the pH was between 6 and 7. The colloidal chitin was finally resuspended in sterile distilled water and kept at 4°C. Wet weight and dry weight of chitin were determined, and used in reaction at final concentration of 1 mg/ml dry weight.

Media Preparation

1. Luria-Bertani (LB) medium

LB consisted of 1.0% tryptone, 0.5% yeast extract and 0.5% NaCl. Broth media pH was adjusted to 7.2 with 1N NaOH. For agar medium preparation, 1.5 - 2% agar was added. Medium was sterilized by autoclaving at 121°C for 15 minutes.

2. LB with colloidal chitin medium (LBCC)

LBCC was prepared by addition of 0.5% wet weight of colloidal chitin to LB with 1.5 - 2% agar before sterilization. LBCC agar plate was used for screening of chimera that could hydrolyze colloidal chitin and exhibit clear zone around colony.

3. Colloidal chitin minimum medium (CCMM)

CCMM consisted of 0.5% colloidal chitin (wet weight), 0.05% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.03% $MgSO_4.7H_2O$, 0.6% KH_2PO_4 and 1% K_2HPO_4 at pH 7.2. For agar medium preparation, 1.5 - 2% agar was added. Medium was sterilized as described above.

Standard commercial plasmids

Plasmid pBluescript SK^- (Stratagene) (pBS/SK^-) was used as a cloning vector to construct pKKCHI60, a plasmid carrying the wild-type *chi60* gene with its endogenous promoter from *Serratia* sp. TU09.

Plasmid pGEM®–T Easy (Promega) was used as cloning vector of *chiB* and chimeric chitinase genes.

Molecular cloning techniques

All basic molecular biology techniques including competent cell preparation, transformation by electroporation, plasmid preparation, DNA amplification by polymerase chain reaction (PCR), ligation, and agarose gel electrophoresis were carried out using standard protocols (Sambrook and Russell, 2001).

Plasmid construction

1. Construction of pKKCHI60

pKKCHI60 was previously constructed by Kamontip Kuttiyawong (2001). The PCR product of chi60 gene with promoter was cloned into pBS/SK$^-$ at *Pst*I restriction site and the *Bam*HI site of pBS/SK$^-$ was deleted. Thus, pKKCHI60 was also used to express *chi60* gene using its endogenous promoter.

2. Construction of pSKCatDCHI66

pSKCatDCHI66 was previously constructed by Sanya Kudan (2004). Catalytic domain of chi66 (*catDchi66*) gene was amplified by PCR and subcloned by replacing the *chi60* gene in pKKCHI60. Thus, pSKCatDCHI66 could express *catDchi66* using *chi60* promoter.

3. Construction of pCHIBXK and pCHIBHX

chiB gene from *S. marcescens* was amplified by PCR using *Taq* polymerase and ligated into pGEM-T Easy vector. Primers used for cloning of *chiB* were described in table 2.1. In pCHIBXK, *chiB* was amplified with FchiBXhoI and RchiBKpnI, whereas, FchiBHindIII and RchiBXhoI were used to amplified *chiB* in pCHIBHX. The PCR reaction conditions for *chiB* amplification are shown in table 2.2.

4. Construction of pCD66B

pCHIBXK was double digested with *Xho*I and *Kpn*I and digested plasmid fragments were separated by agarose gel electrophoresis. The resulted *chiB* fragment was extracted from agarose gel using Qiaquick Gel Extraction Kit. *chiB* was then ligated into pSKCatDCHI66 at *Xho*I and *Kpn*I sites which resulted in a plasmid consisted of *catDchi66* gene followed by *chiB* gene at the 3' side. Construction scheme of pCD66B is shown in figure 2.1A.

5. Construction of pCHI60B

pCHI60B consisted of chi60 gene at 5' side followed by chiB gene at 3' side. Construction of pCHI60 was achieved the same way as pCD66B, but using different restriction enzymes. pCHIBHX was double digested with *Hin*dIII and *Xho*I and the

Table 2.1 Primers used for cloning of *chiB*

Primer name	Nucleotide sequence
FchiBXhoI	*Xho*I 5’ CCG CTC GAG CCA TGT CCA CAC GCA AAG CCG 3’
FchiBHindIII	*Hin*dIII 5’ CCC AAG CTT ATG GCC ACA CGC AAA GCC 3’
RchiBKpnI	*Kpn*I 5’ CGG GGT ACC CTT TAC GCC AGG CGG CCC ACC 3’
RchiBXhoI	*Xho*I 5’ CCG CTC GAG TTT ACG CTA CGC GGC C 3’

Table 2.2 PCR condition for *chiB* amplification

Step	Temperature (^{o}C)	Time (min)	Cycle
Denaturation	95	5	1
Denaturation Annealing Polymerization	95 55 72	1 1 4	25
Final extention	72	10	1

After final extention step, PCR reaction was cooled down and hold at 10^{o}C.

resulted *chiB* gene was ligated to 3' side of *chi60* gene in pKKCHI60. Construction scheme of pCHI60B is shown in figure 2.1B.

Construction of chimeric chitinase

The diagrams for construction of chimeric chitinases are shown in figure 2.2

1. Chimeric chitinase of CatDChi66 and ChiB: HRCD66B

pCD66B was double digested with *Bgl*II and *Eco*RV which removes a small DNA fragment of *catDchi66* and *chiB* resulting in linearized plasmid with deletion of C-terminal of CatDChi66 and N-terminal of ChiB coding sequences. Linearized plasmid was purified using Qiaquick Gel Extraction Kit and transformed into *E. coli* JC8679 by electroporation. The chimeras were screened by the following methods.

1.1. Screening on LB agar plate with ampicillin

Transformants were cultured on LB agar plate containing 100 μg/ml ampicillin and incubate at 37°C for 12-16 hours. The forming colonies were picked up and cultured on LB broth containing 100 μg/ml ampicillin for plasmid preparation. Plasmids containing chimeric gene were linearized with *Kpn*I and analyzed on agarose gel electrophoresis.

1.2 Screening with CCMM broth with ampicillin

Aliquots of 50 μl from 1 ml transformants were cultured in 3 ml CCMM broth containing 100 μg/ml ampicillin and incubate at 37°C in incubator shaker at 250 rpm. The cultures were observed for 3 days and chimeras that could grow on CCMM were collected and plasmid extracted.

2. Chimeric chitinase of Chi60 and ChiB: HRCHI60B

pCHI60B was double digested with *Bst*Z17I and *Hin*dIII, which cut small 3' of *chi60* and 5' of *chiB*, resulting in linearized plasmid with two knockout genes. Linearized plasmid was purified using Qiaquick Gel Extraction Kit and transformed into *E. coli* JC8679 by electroporation and the chimeras were screened by the following methods. Screening of chimeric chitinase gene was done under the same

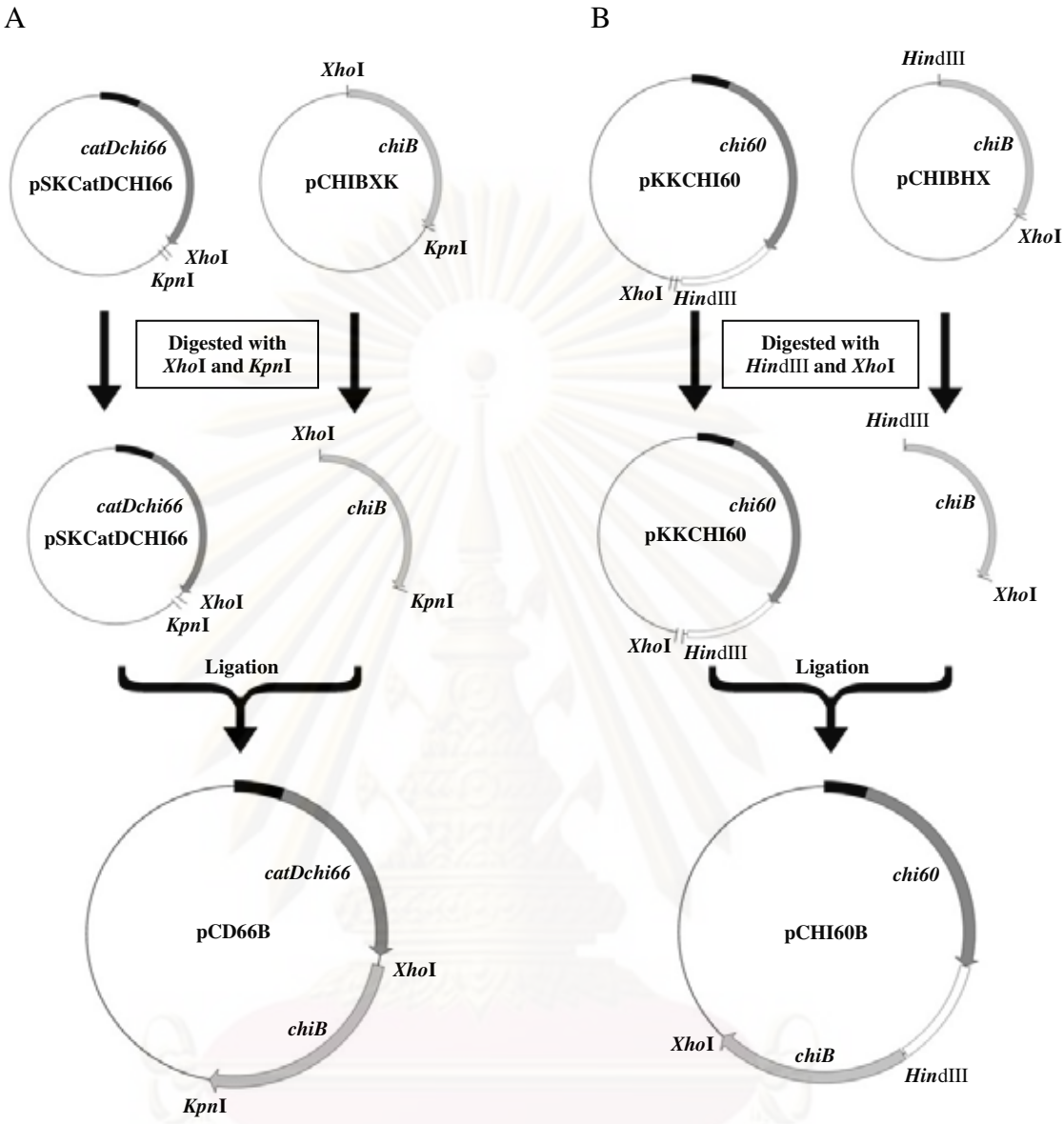


Figure 2.1 Plasmid construction schemes of pCD66B and pCHI60B

Plasmid construction schemes of pCD66B (A) and pCHI60B (B) are displayed**.** *chiB* genes were subcloned into 3'side of *catDchi66* gene and *chi60* gene in the pSKCatDCHI66 and pKKCHI60, respectively. Restriction sites used for ligation are also indicated. (■: promoter of *chi60*, □ : noncoding region left from *chi60* library)

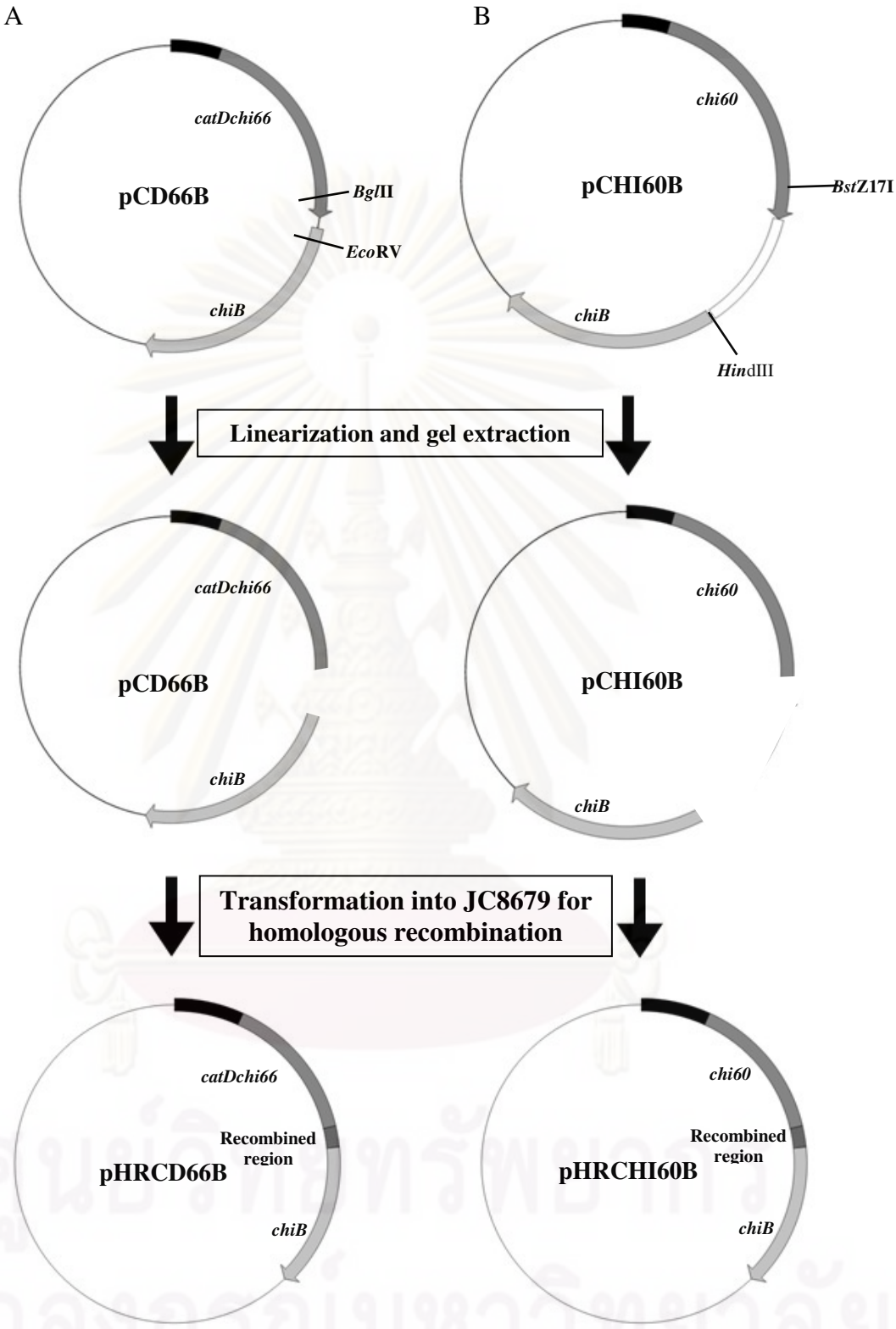


Figure 2.2 Chimeric plasmids construction diagrams of pHRCD66B and pHRCHI60B

Chimeric plasmids construction diagrams of pHRCD66B (A) and pHRCHI60B (B) are displayed.

methods as chimeric HRCD66B screening. Chimeric gene size of pHRCHI60B was checked by digesting with *Not*I and *Xho*I.

Sequence analysis of chimeric chitinase

Chimeric chitinase genes were sent to 1st BASE Pte. Ltd. for nucleotide sequencing. The gene sequences were then translated to amino acid sequences using EMBOSS Transeq program. The deduced amino acid sequence was sent to HHpred interactive server (Soding, Biegert and Lupas, 2005) for tertiary structure prediction by homology modeling.

Expression of chimeric chitinase

pHRCD66B and pHRCHI60B were transformed into *E. coli* DH5α and/or *E. coli* Origami(DE3) by electroporation. Transformant cultures were then spread on LB agar plate containing 100 μg/ml ampicillin and incubate at 37°C for 12-16 hours. Single colony of each chimera was picked and cultured in LB broth containing 100 μg/ml ampicillin and incubated at 37°C for 12-16 hours to be used as starter culture. Starter culture was diluted to 1:100 into 100 ml of LB containing 100 μg/ml ampicillin in 250 ml Erlenmeyer flask and the culture was incubated at 37°C in incubator shaker for chitinase production. The enzyme in supernatant was collected by centrifugation at 5,000 g for 10 minutes at 4°C. Crude enzyme of selected chimeras were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% of acrylamide gel (Weber and Osborn, 1969). 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. Electrophoresis was performed at a constant current of 15 mA per gel. After electrophoresis, proteins were stained with 0.25% Coomassie Brilliant Blue R-250 at room temperature for one hour and then destained with a mixture of 10% (v/v) acetic acid and 25% (v/v) methanol.

Chitinase activity assay

1. Determination of chitinase activity by measuring reducing sugar

Chitinase activity was assayed as described by Imoto and Yagishita (1971). Reducing sugar produced from a chitinolytic reaction was measured by colorimethod using ferricyanide solution as color reagent. Ferricyanide reagent was made by dissolving 0.5 g of potassium ferric cyanide in 1 liter of 1.5 M Na_2CO_3 and standard curve of 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35 μmol NAG was constructed. Seven hundred and fifty microliters of reaction mixture consisted of 1 mg/ml of colloidal chitin or PNAC and the desired amount of enzyme in 0.1 M of appropriated buffer was incubated at 37°C for 30 minutes. The reaction was stopped by adding 1 ml ferricyanide reagent and heat to 100°C for 15 minutes. Small particles were removed from the mixture by centrifugation at 5,000 g for 10 minutes. The absorbance of the sample (A1) at 420 nm was measured by a spectrophotometer using distilled water for baseline setting. Denatured enzyme was used instead of the enzyme in the reaction to obtain blank value (A0). The difference between A0 and A1 (ΔA) 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 that hydrolyzed chitin and produced 1 μmol of reducing sugar product per minute.

2. Determination of chitinase activity by thin layer chromatography (TLC)

The products from chitinolytic reaction with trimer (NAG_3) and tetramer (NAG_4) of chitoolgosaccharides as substrates were analyzed by silica-thin layer chromatography (TLC). The reaction mixture was applied to an activated charcoal column. The column was washed 3 times with water for salt elimination and the degradation product was eluted by 60% ethanol. The eluted mixture was dried at 60°C and the degradation products were dissolved in water. The desalted mixtures were chromatographed on silica gel plate with a solvent system consisted of isopropanol-ethanol-water (5: 2: 1[v/v/v]) and TLC plates were run twice. The product were detected by dipping the plate in aniline-diphenylamine reagent (4 ml of aniline, 4 g of diphenylamine, 200 ml of acetone and 20 ml of 85% phosphoric acid) and baking it at 130 °C for 3 min (Tanaka et al., 1999).

Mutation mediated by ultraviolet radiation of HRCD66B1

pHRCD66B1 was transformed into *E. coli* DH5α by electroporation and 100 μl aliquots were spread on 6 plates of CCMM agar plate containing 100 μg/μl ampicillin. Cultured plates were then radiated with UV for 0, 5, 10, 30, 50, and 80 seconds by using GS Gene linker UV chamber. Radiated plates were incubated at 37°C for 12-16 hours. Colonies formed were analyzed by clear zone observation and expressed for chitinolytic activity assay.

CHAPTER III

RESULTS

Plasmid construction

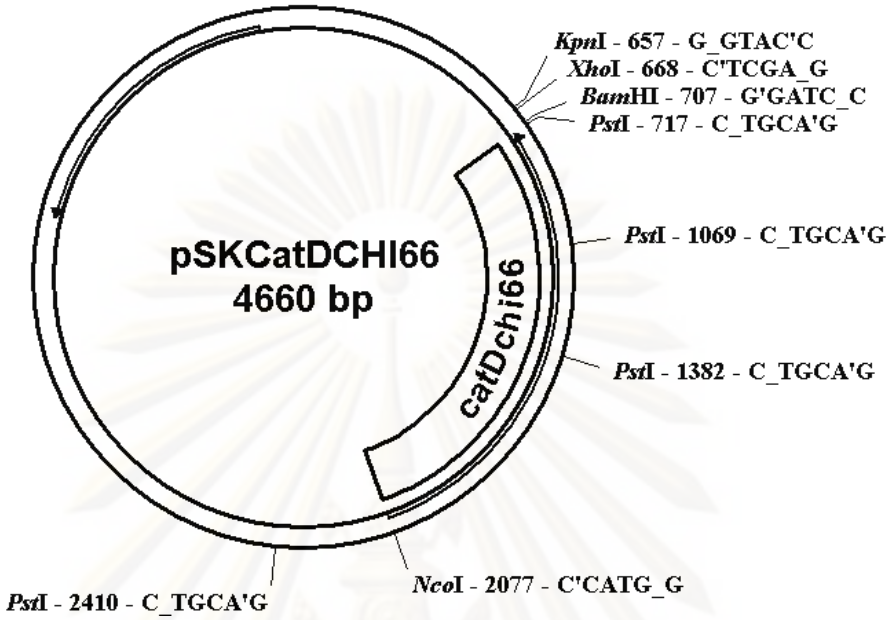
1. Construction of pCD66B

pCD66B was constructed as previously described, *chiB* gene was ligated into 3' side of *catDchi66* gene in pSKCatDCHI66. Firstly, pSKCatDCHI66 was extracted and digested with *Nco*I, *Bam*HI, *Nco*I+*Bam*HI, *Pst*I, *Xho*I and *Kpn*I. The digested fragments size were checked by agarose gel electrophoresis based on restriction map deduced from known sequences shown in figure 3.1A. pCHIBXK was also checked based on sequences of *chiB* from *S. marcescens* (figure 3.1B) by digestion with *Eco*RI, *Eco*RI+*Bam*HI, and *Xho*I+*Kpn*I. The digested product pattern separated by agarose gel electrophoresis of pSKCatDChi66 and pCHIBXK are shown in figure 3.2 and figure 3.3, respectively. Next, pCHIBHXK was double digested with *Xho*I and *Kpn*I and the resulted *chiB* was ligated into the *Xho*I/*Kpn*I-double digested pSKCatDChi66 using 3:1 (insert:vector) molar ratio. Finally, ligation reaction was transformed into *E. coli* Top10 and pCD66B was extracted. This plasmid was mapped by digestion with *Xho*I+ *Kpn*I, *Nco*I+ *Bam*HI, *Eco*RV, *Bgl*II and *Eco*RV+ *Bgl*II. The digestion pattern separated by agarose gel electrophoresis and the restriction map of pCD66B is shown in figure 3.4.

2. Construction of pCHI60B

Similarly to pCD66B, pKKCHI60 was extracted and digested with *Pst*I. The digested fragments size were analyzed by agarose gel electrophoresis based on restriction map deduced from known sequences shown in figure 3.5A. pCHIBHX was also checked based on sequences of *chiB* from *S. marcescens* (figure 3.5B) by digestion with *Hin*dIII+*Xho*I, *Eco*RV+ *Sal*I, *Pst*I and *Eco*RI+*Bam*HI. The digestion pattern separated by agarose gel electrophoresis of pKKCHI60 and pCHIBHX are shown in figure 3.6 and figure 3.7, respectively. pCHIBHX was then double digested with *Hin*dIII+ *Xho*I and *chiB* fragment was ligated into the *Hin*dIII/*Xho*I-double

A



B

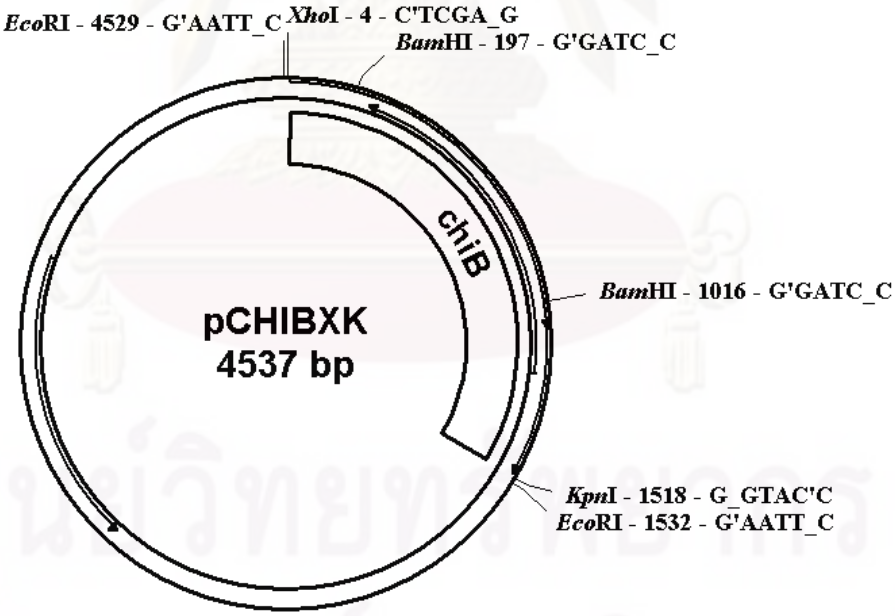


Figure 3.1 Restriction maps of pSKCatDCHI66 and pCHIBXK

Some of the restriction sites found in pSKCatDCHI66 (A) and pCHIBXK (B) are shown. This restriction map was generated by pDRAW32 version 1.1.106 program.

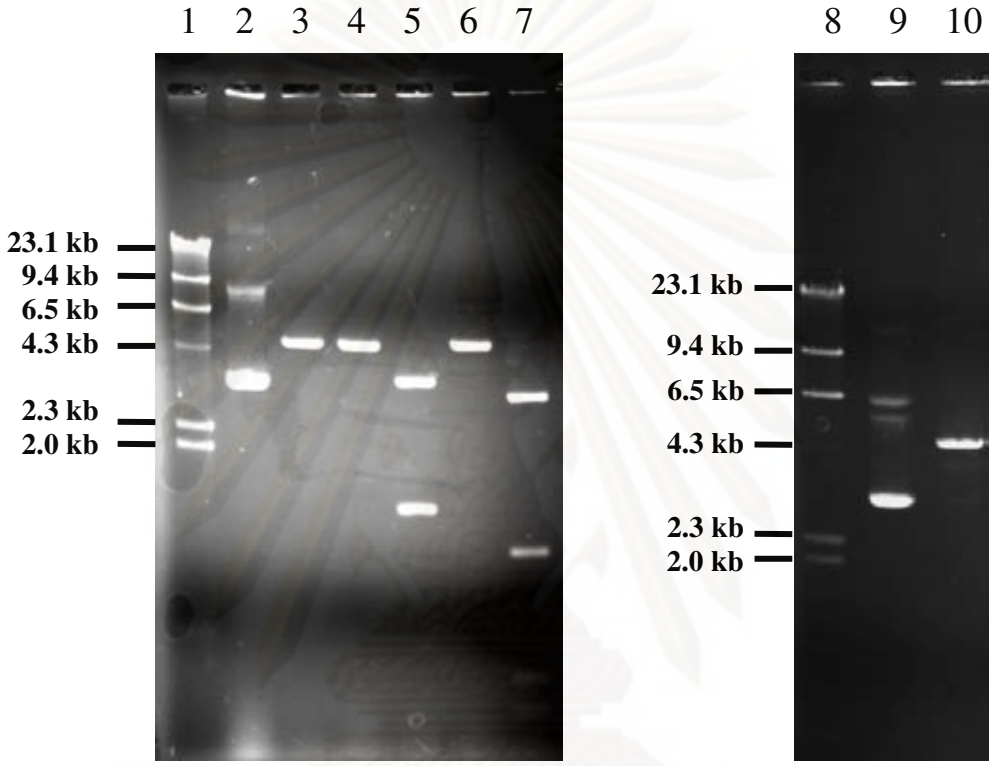


Figure 3.2 Restriction map of pSKCatDCHI66

Agarose gel electrophoresis analysis of single and double digested pSKCatDCHI66 with various restriction enzymes

Lane 1 λ/*Hin*dIII marker
Lane 2 pSKCatDCHI66
Lane 3 pSKCatDCHI66I/ *Bam*HI
Lane 4 pSKCatDCHI66/ *Nco*I
Lane 5 pSKCatDCHI66/ *Nco*I+ *Bam*HI
Lane 6 pSKCatDCHI66/ *Kpn*I
Lane 7 pSKCatDCHI66/ *Pst*I
Lane 8 λ/*Hin*dIII marker
Lane 7 pSKCatDCHI66
Lane 10 pSKCatDCHI66/ *Xho*I

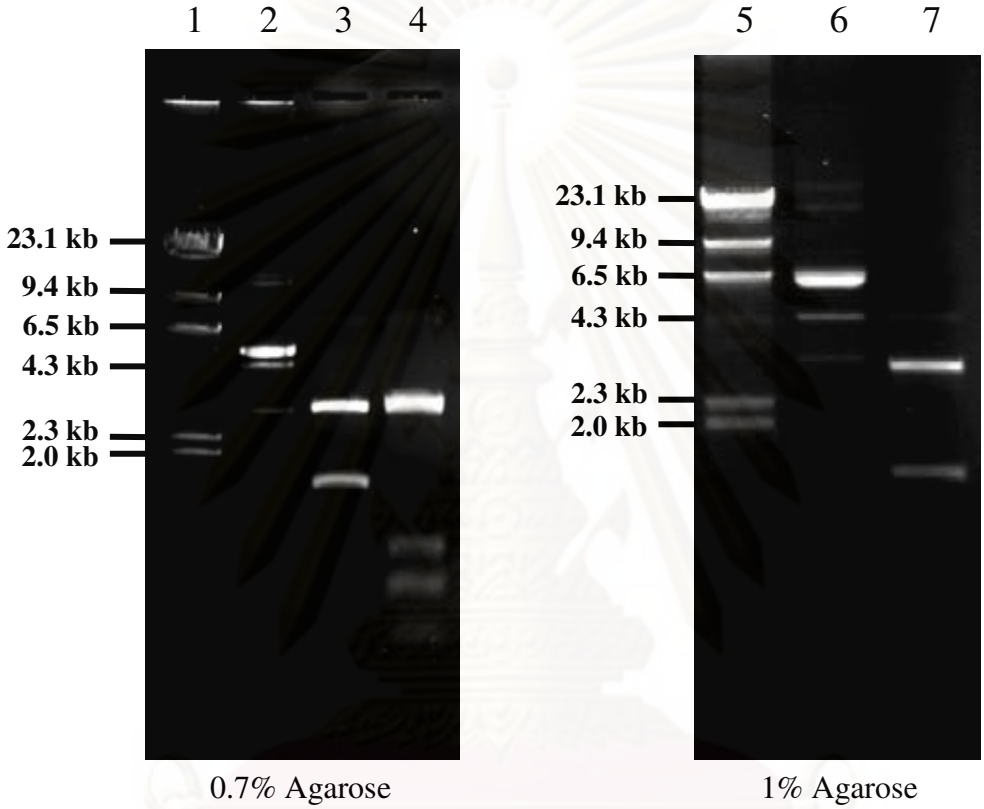


Figure 3.3 Restriction map of pCHIBXK

Agarose gel electrophoresis analysis of single and double digested pCHIBXK with various restriction enzymes

Lane 1 λ/*Hin*dIII marker
Lane 2 pCHIBXK
Lane 3 pCHIBXK/ *Eco*RI
Lane 4 pCHIBXK/ *Eco*RI+*Bam*HI
Lane 5 λ/*Hin*dIII marker
Lane 6 pCHIBXK
Lane 2 pCHIBXK/ *Xho*I+*Kpn*I

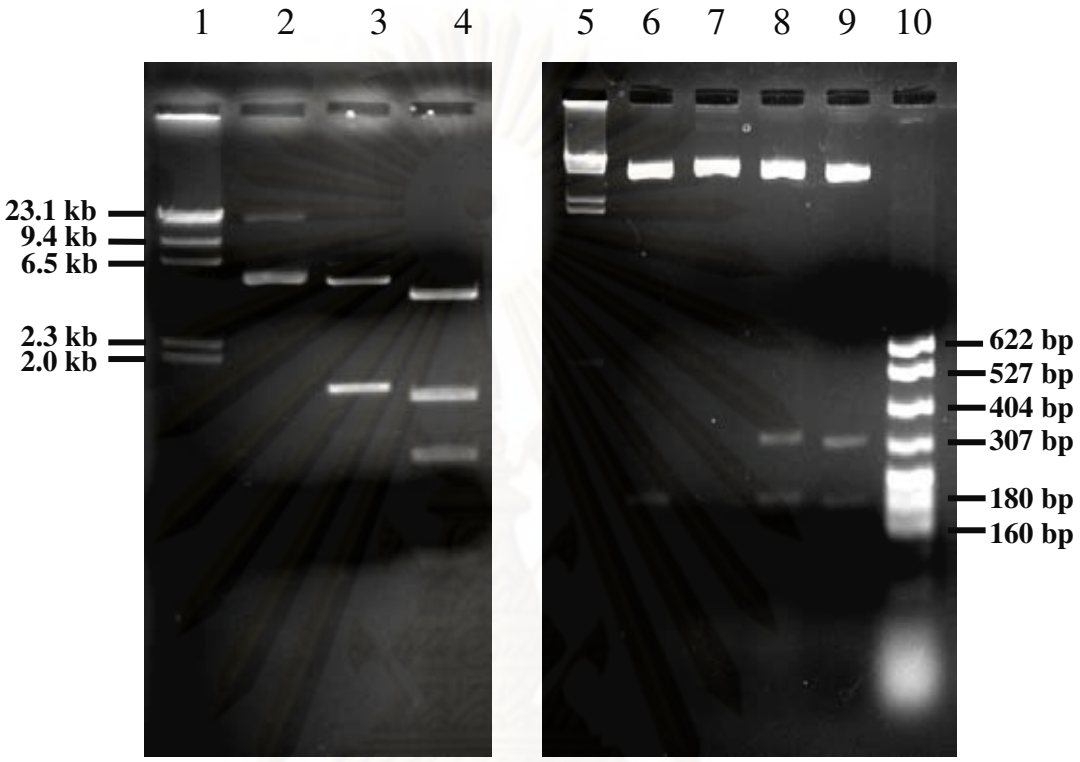


Figure 3.4 Restriction map of pCD66B

Agarose gel electrophoresis analysis of single and double digested pCD66B with various restriction enzymes

Lane 1 λ/*Hin*dIII marker
Lane 2 pCD66B
Lane 3 pCD66B/ *Xho*I+ *Kpn*I
Lane 4 pCD66B/ *Nco*I+ *Bam*HI
Lane 5 λ/*Hin*dIII marker
Lane 6 pCD66B/ *Eco*RV
Lane 7 pCD66B/ *Bgl*II
Lane 8 pCD66B/ *Eco*RV+ *Bgl*II
Lane 9 pCD66B/ *Eco*RV+ *Bgl*II
Lane 10 pBR322/ *Msp*I marker

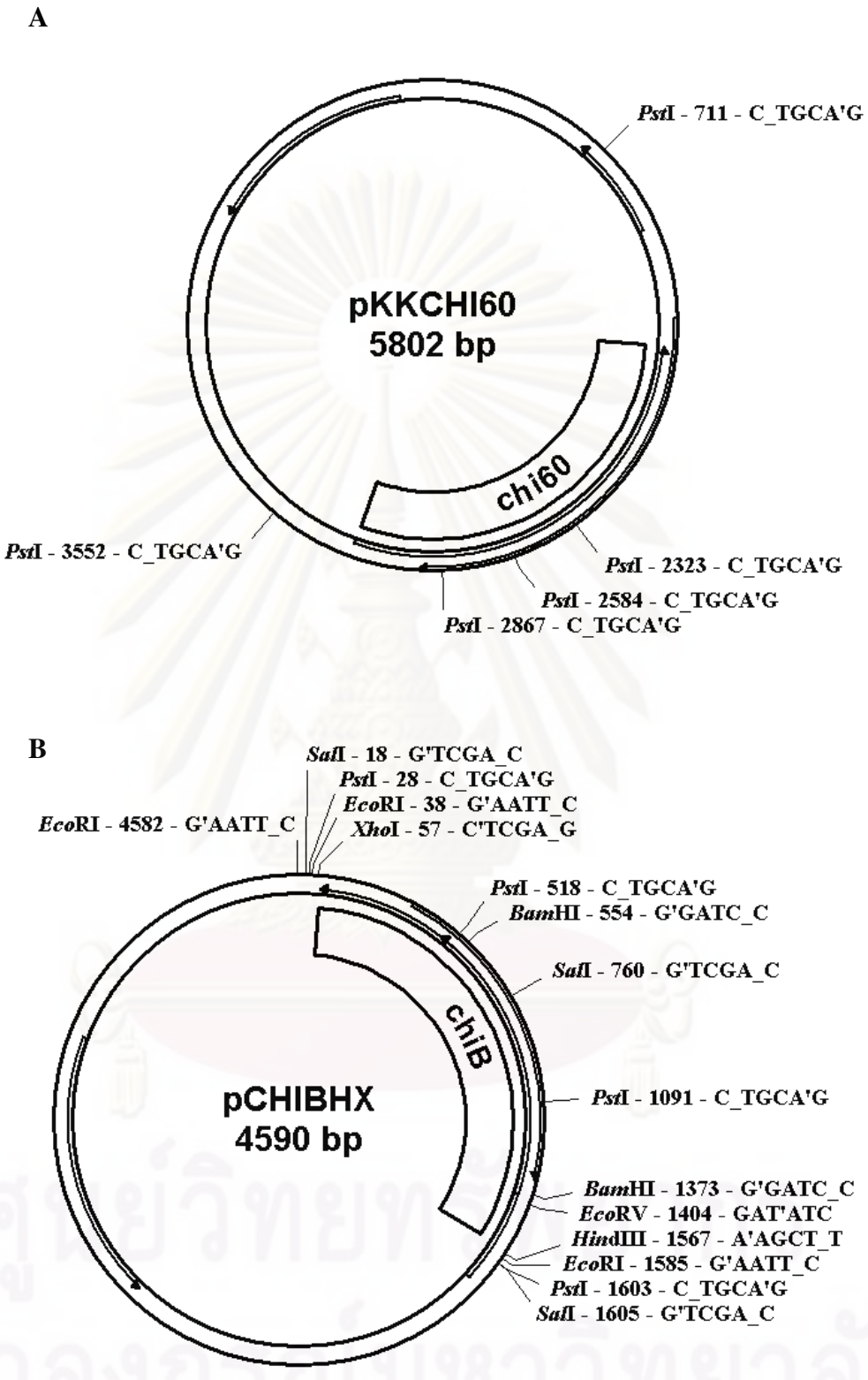


Figure 3.5 Restriction maps of pKKCHI66 and pCHIBHX

Some of the restriction sites found in pSKCatDCHI66 (A) and pCHIBXK (B) are shown. This restriction map was generated by pDRAW32 version 1.1.106 program.

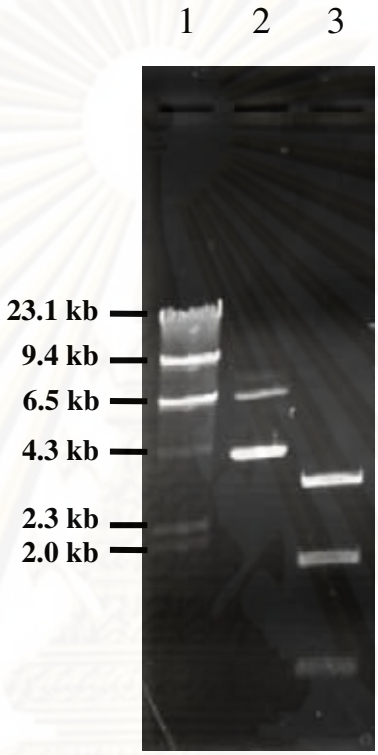


Figure 3.6 Restriction map of pKKCHI60

Agarose gel electrophoresis analysis of single and double digested pKKCHI60 with various restriction enzymes

Lane 1 λ/*Hin*dIII marker

Lane 2 pKKCHI60

Lane 3 pKKCHI60/ *Pst*I

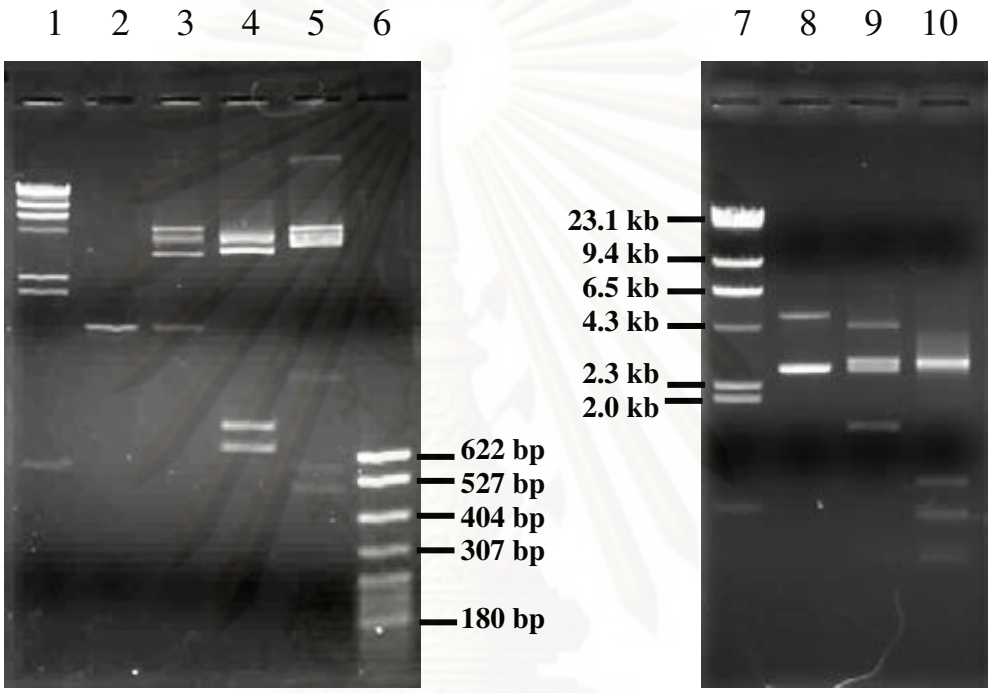


Figure 3.7 Restriction map of pCHIBHX

Agarose gel electrophoresis analysis of single and double digested pCHIBHX with various restriction enzymes

Lane 1 λ/*Hin*dIII marker
Lane 2 PCR product of *chiB*
Lane 3 pCHIBHX/ *Hin*dIII+*Xho*I
Lane 4 pCHIBHX/ *Eco*RV+ *Sal*I
Lane 5 pCHIBHX/ *Pst*I
Lane 6 pBR322/ *Msp*I marker
Lane 7 λ/*Hin*dIII marker
Lane 8 pCHIBHX
Lane 9 pCHIBHX/ *Hin*dIII+*Xho*I
Lane 10 pCHIBHX/ *Eco*RI+*Bam*HI

digested pKKCHI60 using 3:1 (insert:vector) molar ratio. Ligation reaction was finally transformed into *E. coli* Top10. The resulted pCHI60B was extracted. This plasmid was mapped by digestion with *Hin*dIII+*Xho*I and *Not*I+*Xho*I. The digestion pattern separated by agarose gel electrophoresis and the restriction map of pCHI60B is shown in figure 3.8.

Construction and expression of HRCD66B

1. Construction of HRCD66B

pCD66B was double digested with *Bgl*II and *Eco*RV and linearized plasmid was purified using Qiaquick Gel Extraction Kit. The digested pCD66B was transformed into *E. coli* JC8679 by electroporation and screened by the following methods.

1.1 HRCD66B screening from LB agar plate with ampicillin

One milliliter of transformants culture was spread on LB agar plate containing 100 μg/ml ampicillin and incubated at 37°C for 12-16 hours. The result showed that there were 39 colonies formed on agar plate. Each colony was picked and plasmid was extracted. Figure 3.9 shows the agarose gel electrophoresis of extracted plasmids that were digested with *Kpn*I and loaded along with the undigested ones. From the result, there were 12 colonies that contained plasmid of 4.7-6.3 kb in size. These plasmids which were from colony 1, 5, 10, 17, 20, 24, 26, 31, 32, 33, 35, and 38, were designated as pHRCD66B1-12.

This screening was repeated and 86 forming colonies were observed. Plasmids were extracted and linearized with *Kpn*I as shown in Figure 3.10. From these results, we observed only 8 more colonies from colony number 7, 23, 25, 40, 43, 58, 67 and 77 with plasmid size between 4.9-6.3 kb which designated as pHRCD66B13-20.

1.2 HRCD66B screening from CCMM broth with ampicillin

E. coli Top10 with pCD66B and pBS/SK$^-$ were used as positive and negative control, respectively. Aliquots of 100 μl from 1 ml transformants were cultured into 12 tubes of 3 ml CCMM broth containing 100 μg/ml ampicillin and incubate at 37°C in incubator shaker at 250 rpm. The cultures were observed for 3

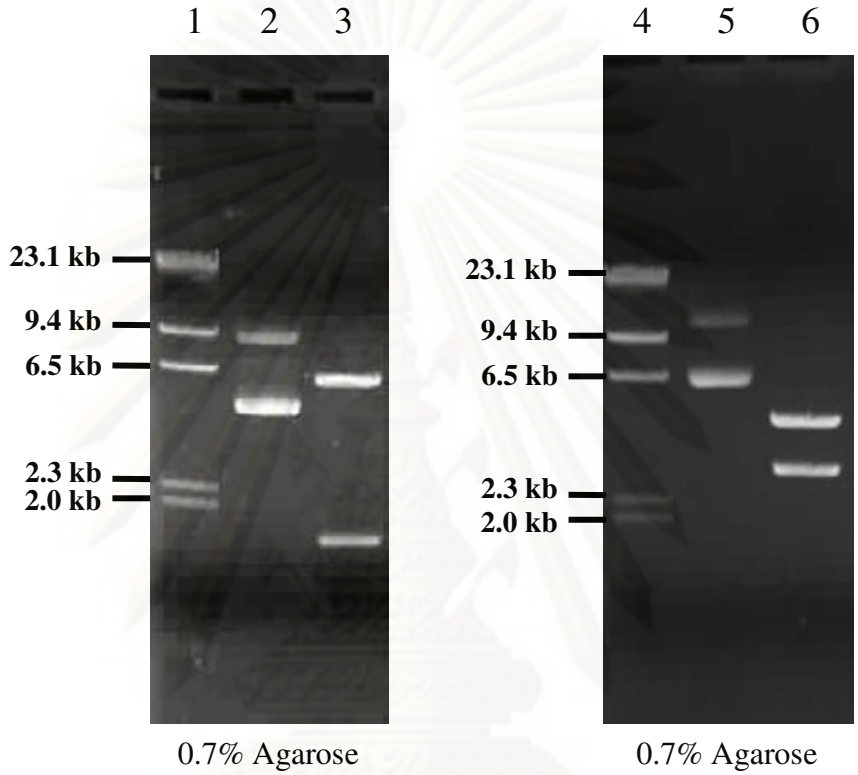


Figure 3.8 Restriction map of pCHI60B

Agarose gel electrophoresis analysis of single and double digested pKKCHI60 with various restriction enzymes

Lane 1 λ/*Hin*dIII marker

Lane 2 pCHI60B

Lane 3 pCHI60B/ *Hin*dIII+*Xho*I

Lane 4 λ/*Hin*dIII marker

Lane 5 pCHI60B

Lane 6 pCHI60B/ *Not*I+*Xho*I

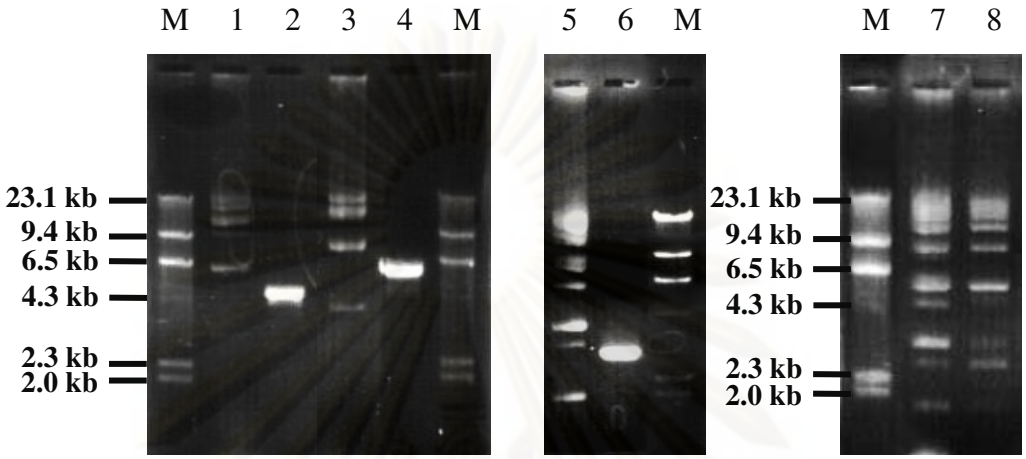


Figure 3.9 Agarose gel electrophoresis analysis of plasmids extracted from colony 1, 18, 22 and 12 of transformants of pCD66B that were first screened by LB agar plate with ampicillin

Plasmids extracted from colony 1, 18, 22 and 12 are representatives of normal plasmid, too large plasmid, too small plasmid and unidentified plasmid, respectively.

Lanes M contain λ/*Hin*dIII marker.

Lane 1 Plasmid from colony1

Lane 2 Plasmid from colony1/ *Kpn*I

Lane 3 Plasmid from colony8

Lane 4 Plasmid from colony8/ *Kpn*I

Lane 5 Plasmid from colony22

Lane 6 Plasmid from colony22/ *Kpn*I

Lane 7 Plasmid from colony12

Lane 8 Plasmid from colony12/ *Kpn*I

days and found that there were 2 chimeras that could grow on CCMM. Plasmids of these two were extracted and digested with *Nco*I and *Kpn*I. Figure 3.10 shows agarose gel electrophoresis of these two plasmids which indicated that one plasmid has appropriated size and designated as pHRCD66BM1.

2. Sequences analysis of pHRCD66B

pHRCD66B61-29 and pHRCD66BM1 were double digested with *Nco*I and *Kpn*I which cleaved at start codon of *catDchi66* and stop codon of *chiB*, respectively. Chimeric chitinase gene size was observed by agarose gel electrophoresis and found that pHRCD66B4, 7, 11, 12, and 18 were pCD66B which were further confirmed by *Eco*RV digestion as shown in as shown in figure 3.11. Other chimeras of pHRCD66B were sequenced. Sequences of each chimera were aligned using ClustalW2 program and found that there were 6 individuals with chimeric gene size of 1.34, 1.57, 1.65, 1.67, 1.68 and 1.76 kb as shown in table 3.1. Figure 3.12 shows nucleotide sequences alignment of chimeric chitinases gene from pHRCD66B. Nucleotide sequences of pHRCD66B1, 2, 5, 6, 15 and M1 were analyzed to find recombining site by using basic local alignment search tool (BLAST). Figure 3.13 shows the graphical view of recombination site between *catDchi66* and *chiB* of each chimera. Gene sequences were then translated to amino acid sequences using EMBOSS Transeq program which showed that pHRCD66B2 and pHRCD66B5 had single base frameshift and could not translate through the entire gene. HRCD66B1, 6, 15 and HRCD66BM1 were chosen to be representatives of chimeric chitinases encoding from 1.67, 1.68, 1.34 and 1.76 kb coding sequences, respectively. Amino acid sequences were also sent to BLAST to find recombining regions. Recombined regions obtained from nucleotide blast and protein blast of HRCD66B1, 6, 15 and HRCD66BM1 are shown in figure 3.14, 3.15, 3.16 and 3.17.

3. Three dimensional structure prediction of HRCD66B1, HRCD66B6, HRCD66B15 and HRCD66BM1

Amino acid sequences of HRCD66B1, HRCD66B6, HRCD66B15 and HRCD66BM1 were sent to HHpred interactive server for structure prediction. Predicted 3D structure of HRCD66B1, HRCD66B6, HRCD66B15 and HRCD66BM1 and visualized by Rasmol version 2.7.4.2 as shown in figure 3.18, 3.19, 3.20 and 3.21

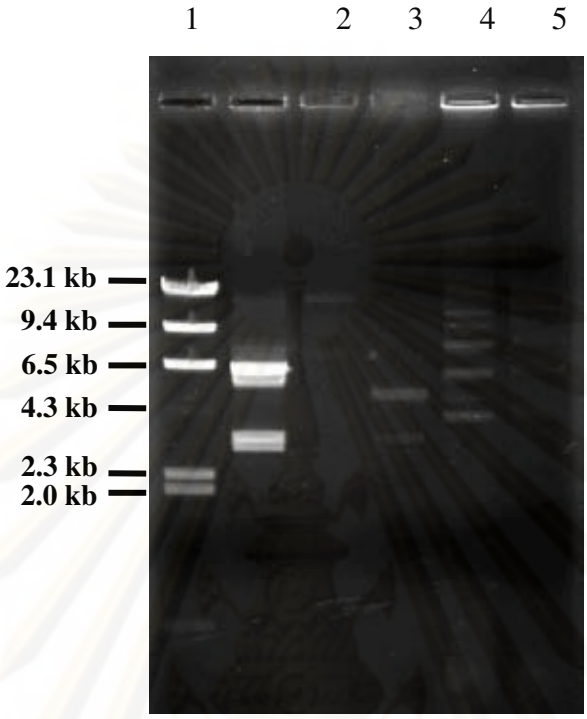


Figure 3.10 Agarose gel electrophoresis analysis of plasmids extracted from pCD66B transformants that were screened on CCMM broth with ampicillin

Lane 1 λ/*Hin*dIII marker

Lane 2 Plasmid from culture tube 1

Lane 3 Plasmid from culture tube 1/ *Nco*I+ *Kpn*I

Lane 4 Plasmid from culture tube 2

Lane 5 Plasmid from culture tube 2/ *Nco*I+ *Kpn*I

*Plasmid from tube 1 was designated as pHRCD66BM1.

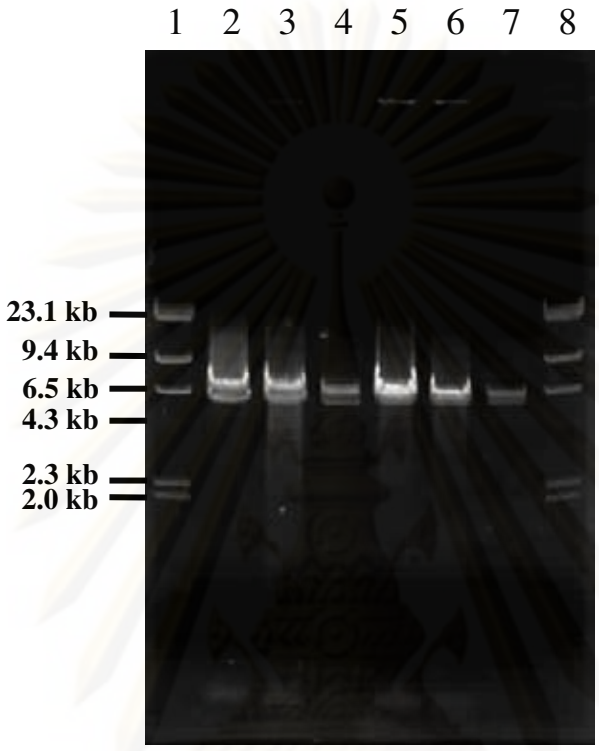


Figure 3.11 Agarose gel electrophoresis analysis of pHRCD66B4, 7, 11, 12, and 18 digested with *Eco*RV

Lane 1 λ/*Hin*dIII marker

Lane 2 pCD66B/ *Eco*RV

Lane 3 pHRCD66B4/ *Eco*RV

Lane 4 pHRCD66B7/ *Eco*RV

Lane 5 pHRCD66B11/ *Eco*RV

Lane 6 pHRCD66B12/ *Eco*RV

Lane 7 pHRCD66B18/ *Eco*RV

Lane 8 λ/*Hin*dIII marker

Table 3.1 Groups of pHRCD66B derivatives divided by chimeric gene size

Chimeric gene size	pHRCD66B
1.34 kb	pHRCD66B 15,16
1.57 kb	pHRCD66B 5
1.65 kb	pHRCD66B 2
1.67 kb	pHRCD66B 1, 3, 9, 10, 13, 14, 17, 19, 20
1.68 kb	pHRCD66B 6, 8
1.76 kb	pHRCD66BM1

```
hrcd66B10       ATGGAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B20       ATGGAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B9        ATGGAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B1        ATGGAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B3        ATGGAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B15       ATGGAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B19       ATGGAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B13       ATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B14       ATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B17       ATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B16       ATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B5        ATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B6        ATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B8        ATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66Bm1       ATGGAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
hrcd66B2        ATGAAAATCGTGTTGATCAACAAAAGCAAAAAGTTTTTCGTTTTTTCTTTCATTTTTGTT 60
                *** ************************************************************

hrcd66B10       ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B20       ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B9        ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B1        ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B3        ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B15       ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B19       ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B13       ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B14       ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B17       ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B16       ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B5        ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B6        ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B8        ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66Bm1       ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
hrcd66B2        ATGATGCTGAGCCTCTCATTTGTGAATGGGGAAGTTGCAAAAGCCGATTCCGGAAAAAAC 120
                ************************************************************

hrcd66B10       TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B20       TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B9        TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B1        TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B3        TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B15       TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B19       TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B13       TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B14       TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B17       TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B16       TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B5        TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B6        TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B8        TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66Bm1       TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
hrcd66B2        TATAAAATCATCGGCTACTATCCATCATGGGGTGCTTATGGAAGGGATTTTCAAGTTTGG 180
                ************************************************************
```

Figure 3.12 Nucleotide sequence alignment of chimeric chitinase genes of pHRCD66B: hrcd66B

```
hrcd66B10       GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B20       GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B9        GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B1        GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B3        GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B15       GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B19       GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B13       GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B14       GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B17       GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B16       GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B5        GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B6        GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B8        GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66Bm1       GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
hrcd66B2        GATATGGACGTTTCGAAAGTCAGCCACATTAATTATGCCTTTGCTGATATTTGCTGGGAG 240
                ************************************************************

hrcd66B10       GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B20       GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B9        GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B1        GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B3        GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B15       GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B19       GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B13       GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B14       GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B17       GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B16       GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B5        GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B6        GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B8        GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66Bm1       GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
hrcd66B2        GGAAGGCATGGAAACCCTGATCCGACAGGCCCCAATCCTCAAACGTGGTCATGCCAGGAT 300
                ************************************************************

hrcd66B10       GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B20       GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B9        GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B1        GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B3        GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B15       GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B19       GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B13       GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B14       GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B17       GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B16       GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B5        GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B6        GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B8        GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66Bm1       GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
hrcd66B2        GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC 360
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```

Figure 3.12 Nucleotide sequence alignment of chimeric chitinase genes of pHRCD66B: hrcd66B (continued)

```
hrcd66B10       GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B20       GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B9        GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B1        GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B3        GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B15       GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B19       GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B13       GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B14       GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B17       GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B16       GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGC------------ 408
hrcd66B5        GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B6        GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B8        GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66Bm1       GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
hrcd66B2        GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGCAACTTTAAACAA 420
                ************************************************

hrcd66B10       TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B20       TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B9        TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B1        TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B3        TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B15       TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B19       TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B13       TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B14       TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B17       TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B16       ------------------------------------------------------------
hrcd66B5        TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B6        TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B8        TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66Bm1       TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480
hrcd66B2        TTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGAAAACGTTCATATCGGTCGGGGGGTGG 480

hrcd66B10       ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B20       ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B9        ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B1        ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B3        ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B15       ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B19       ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B13       ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B14       ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B17       ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B16       ------------------------------------------------------------
hrcd66B5        ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B6        ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B8        ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66Bm1       ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
hrcd66B2        ACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAGATCCTGCGGCAAGGGAGAATTTCGCC 540
```

Figure 3.12 Nucleotide sequence alignment of chimeric chitinase genes of pHRCD66B: hrcd66B (continued)

```
hrcd66B10       GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAG 600
hrcd66B20       GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAG 600
hrcd66B9        GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAG 600
hrcd66B1        GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAG 600
hrcd66B3        GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAA 600
hrcd66B15       GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAA 600
hrcd66B19       GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAA 600
hrcd66B13       GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAG 600
hrcd66B14       GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAG 600
hrcd66B17       GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGCGTGGACATCGACTGGGAG 600
hrcd66B16       ------------------------------------------------------------
hrcd66B5        GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAA 600
hrcd66B6        GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAA 600
hrcd66B8        GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAA 600
hrcd66Bm1       GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAA 600
hrcd66B2        GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAA 600


hrcd66B10       TATCCGCAGGCGGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCT-- 658
hrcd66B20       TATCCGCAGGCGGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCT-- 658
hrcd66B9        TATCCGCAGGCGGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCT-- 658
hrcd66B1        TATCCGCAGGCGGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCT-- 658
hrcd66B3        TATCCGCAGGCGGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCT-- 658
hrcd66B15       TATCCGCAGGCGGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCT-- 658
hrcd66B19       TATCCGCAGGCGGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCT-- 658
hrcd66B13       TATCCGCAGGCGGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCT-- 658
hrcd66B14       TATCCGCAGGCGGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCT-- 658
hrcd66B17       TATCCGCAGGCGGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCT-- 658
hrcd66B16       ------------------------------------------------------------
hrcd66B5        TATCCG------------------------------------------------------ 606
hrcd66B6        TATCCGGTCAGCGGAGGATTGCCGGGGAACAGCACACGTCCGGAAGATAAAAGAAACTAC 660
hrcd66B8        TATCCGGTCAGCGGAGGATTGCCGGGGAACAGCACACGTCCGGAAGATAAAAGAAACTAC 660
hrcd66Bm1       TATCCGGTCAGCGGAGGATTGCCGGGGAACAGCACACGTCCGGAAGATAAAAGAAACTAC 660
hrcd66B2        TATCCGGTCAGCGGAGGATTGCCGGGGAACAGCACACGTCCGGAAGATAAAAGAAACTAC 660


hrcd66B10       -TGCTGAACCAGCAAACCAT-CGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCA- 715
hrcd66B20       -TGCTGAACCAGCAAACCAT-CGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCA- 715
hrcd66B9        -TGCTGAACCAGCAAACCAT-CGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCA- 715
hrcd66B1        -TGCTGAACCAGCAAACCAT-CGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCA- 715
hrcd66B3        -TGCTGAACCAGCAAACCAT-CGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCA- 715
hrcd66B15       -TGCTGAACCAGCAAACCAT-CGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCA- 715
hrcd66B19       -TGCTGAACCAGCAAACCAT-CGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCA- 715
hrcd66B13       -TGCTGAACCAGCAAACCAT-CGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCA- 715
hrcd66B14       -TGCTGAACCAGCAAACCAT-CGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCA- 715
hrcd66B17       -TGCTGAACCAGCAAACCAT-CGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCA- 715
hrcd66B16       ------------------------------------------------------------
hrcd66B5        ------------------------------------------------GTCAGCGGAGGA- 618
hrcd66B6        ACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTGACGCTGCAGAAGCAAAAGACGGCAAG 720
hrcd66B8        ACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTGACGCTGCAGAAGCAAAAGACGGCAAG 720
hrcd66Bm1       ACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTGACGCTGCAGAAGCAAAAGACGGCAAG 720
hrcd66B2        ACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTGACGCTGCAGAAGCAAAAGACGGCAAG 720
```

Figure 3.12 Nucleotide sequence alignment of chimeric chitinase genes of pHRCD66B: hrcd66B (continued)

```
hrcd66B10       ---TCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 765
hrcd66B20       ---TCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 765
hrcd66B9        ---TCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 765
hrcd66B1        ---TCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 765
hrcd66B3        ---TCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 765
hrcd66B15       ---TCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 765
hrcd66B19       ---TCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 765
hrcd66B13       ---TCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 765
hrcd66B14       ---TCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 765
hrcd66B17       ---TCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 765
hrcd66B16       --------------------GCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 441
hrcd66B5        ---TTGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATT-------ACAGCAAGCTG 668
hrcd66B6        GAATACTTGCTGACGATCGCATCCGGCGCAAGTCCCGATTATGTAAGCAACACTGAGCTC 780
hrcd66B8        GAATACTTGCTGACGATCGCATCCGGCGCAAGTCCCGATTATGTAAGCAACACTGAGCTC 780
hrcd66Bm1       GAATACTTGCTGACGATCGCATCCGGCGCAAGTCCCGATTATGTAAGCAACACTGAGCTC 780
hrcd66B2        GAATACTTGCTGACGATCGCATCCGGCGCAAATC--------------------------- 754
                                    *    *   **

hrcd66B10       GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 825
hrcd66B20       GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 825
hrcd66B9        GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 825
hrcd66B1        GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 825
hrcd66B3        GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 825
hrcd66B15       GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 825
hrcd66B19       GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 825
hrcd66B13       GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 825
hrcd66B14       GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 825
hrcd66B17       GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 825
hrcd66B16       GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 501
hrcd66B5        GCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 728
hrcd66B6        GATAAAATCGCTCAAACCGTGGATTGGATTAACATTATGACCTACGATCTGGCCGGCCCC 840
hrcd66B8        GATAAAATCGCTCAAACCGTGGATTGGATTAACATTATGACCTACGATCTGGCCGGCCCC 840
hrcd66Bm1       GATAAAATCGCTCAAACCGTGGATTGGATTAACATTATGACCTATGACTTTAATGGCGGA 840
hrcd66B2        ------GTCGCGCCACTCG---ATTACATCAACCTGATGACCTACGATCTGGCCGGCCCC 805
                      ***           ***  ** *** * ******** **  *    ***

hrcd66B10       TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 870
hrcd66B20       TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 870
hrcd66B9        TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 870
hrcd66B1        TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 870
hrcd66B3        TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 870
hrcd66B15       TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 870
hrcd66B19       TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 870
hrcd66B13       TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 870
hrcd66B14       TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 870
hrcd66B17       TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 870
hrcd66B16       TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 546
hrcd66B5        TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 773
hrcd66B6        TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 885
hrcd66B8        TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 885
hrcd66Bm1       TGGCAAAGCATAAGCGCCCATAATGCACCGCTGTTTTATGATCCAAAAGCGAAAGAAGCA 900
hrcd66B2        TGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGAC---------------GCG 850
                *** * *  ** * *  ***  * **  *******    **               **
```

Figure 3.12 Nucleotide sequence alignment of chimeric chitinase genes of pHRCD66B: hrcd66B (continued)

```
hrcd66B10       GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 906
hrcd66B20       GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 906
hrcd66B9        GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 906
hrcd66B1        GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 906
hrcd66B3        GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 906
hrcd66B15       GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 906
hrcd66B19       GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 906
hrcd66B13       GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 906
hrcd66B14       GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 906
hrcd66B17       GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 906
hrcd66B16       GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 582
hrcd66B5        GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 809
hrcd66B6        GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 921
hrcd66B8        GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 921
hrcd66Bm1       GGCGTTCCAAACGCTGAGACCTACAATATTGAAAACACTGTGAAACGCTACAAGGAAGCC 960
hrcd66B2        GCCGGGCCGACCTT------CTACAACG-------CACTGCGC-----------GAAGCC 886
                * **  ** * *       ******         ***** *           ******

hrcd66B10       AATCT-------------------------------------------GGGCTGGAGCTGG 924
hrcd66B20       AATCT-------------------------------------------GGGCTGGAGCTGG 924
hrcd66B9        AATCT-------------------------------------------GGGCTGGAGCTGG 924
hrcd66B1        AATCT-------------------------------------------GGGCTGGAGCTGG 924
hrcd66B3        AATCT-------------------------------------------GGGCTGGAGCTGG 924
hrcd66B15       AATCT-------------------------------------------GGGCTGGAGCTGG 924
hrcd66B19       AATCT-------------------------------------------GGGCTGGAGCTGG 924
hrcd66B13       AATCT-------------------------------------------GGGCTGGAGCTGG 924
hrcd66B14       AATCT-------------------------------------------GGGCTGGAGCTGG 924
hrcd66B17       AATCT-------------------------------------------GGGCTGGAGCTGG 924
hrcd66B16       AATCT-------------------------------------------GGGCTGGAGCTGG 600
hrcd66B5        AATCT-------------------------------------------GGGCTGGAGCTGG 827
hrcd66B6        AATCT-------------------------------------------GGGCTGGAGCTGG 939
hrcd66B8        AATCT-------------------------------------------GGGCTGGAGCTGG 939
hrcd66Bm1       GGTGTCAAGGGTGACAAATTAGTGCTTGGAACACCGTTCTACGGAAGGGGCTGGAGCTGG 1020
hrcd66B2        AATCT-------------------------------------------GGGCTGGAGCTGG 904
                 * *                                             ************

hrcd66B10       GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 984
hrcd66B20       GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 984
hrcd66B9        GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 984
hrcd66B1        GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 984
hrcd66B3        GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 984
hrcd66B15       GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 984
hrcd66B19       GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 984
hrcd66B13       GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 984
hrcd66B14       GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 984
hrcd66B17       GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 984
hrcd66B16       GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 660
hrcd66B5        GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 887
hrcd66B6        GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 999
hrcd66B8        GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 999
hrcd66Bm1       GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 1080
hrcd66B2        GAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAG 964
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Figure 3.12 Nucleotide sequence alignment of chimeric chitinase genes of pHRCD66B: hrcd66B (continued)

```
hrcd66B10       CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1044
hrcd66B20       CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1044
hrcd66B9        CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1044
hrcd66B1        CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1044
hrcd66B3        CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1044
hrcd66B15       CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1044
hrcd66B19       CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1044
hrcd66B13       CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1044
hrcd66B14       CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1044
hrcd66B17       CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1044
hrcd66B16       CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 720
hrcd66B5        CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 947
hrcd66B6        CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1059
hrcd66B8        CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1059
hrcd66Bm1       CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1140
hrcd66B2        CAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTAC 1024
                ************************************************************

hrcd66B10       GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1104
hrcd66B20       GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1104
hrcd66B9        GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1104
hrcd66B1        GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1104
hrcd66B3        GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1104
hrcd66B15       GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1104
hrcd66B19       GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1104
hrcd66B13       GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1104
hrcd66B14       GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1104
hrcd66B17       GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1104
hrcd66B16       GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 780
hrcd66B5        GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1007
hrcd66B6        GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1119
hrcd66B8        GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1119
hrcd66Bm1       GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1200
hrcd66B2        GGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACG 1084
                ************************************************************

hrcd66B10       CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1164
hrcd66B20       CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1164
hrcd66B9        CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1164
hrcd66B1        CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1164
hrcd66B3        CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1164
hrcd66B15       CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1164
hrcd66B19       CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1164
hrcd66B13       CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1164
hrcd66B14       CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1164
hrcd66B17       CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1164
hrcd66B16       CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 840
hrcd66B5        CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1067
hrcd66B6        CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1179
hrcd66B8        CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1179
hrcd66Bm1       CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1260
hrcd66B2        CCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTG 1144
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Figure 3.12 Nucleotide sequence alignment of chimeric chitinase genes of pHRCD66B: hrcd66B (continued)

```
hrcd66B10       CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1224
hrcd66B20       CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1224
hrcd66B9        CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1224
hrcd66B1        CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1224
hrcd66B3        CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1224
hrcd66B15       CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1224
hrcd66B19       CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1224
hrcd66B13       CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1224
hrcd66B14       CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1224
hrcd66B17       CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1224
hrcd66B16       CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 900
hrcd66B5        CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1127
hrcd66B6        CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1239
hrcd66B8        CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1239
hrcd66Bm1       CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1320
hrcd66B2        CGCGACAAGGATCCGCGCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAAC 1204
                ************************************************************

hrcd66B10       TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1284
hrcd66B20       TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1284
hrcd66B9        TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1284
hrcd66B1        TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1284
hrcd66B3        TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1284
hrcd66B15       TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1284
hrcd66B19       TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1284
hrcd66B13       TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1284
hrcd66B14       TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1284
hrcd66B17       TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1284
hrcd66B16       TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 960
hrcd66B5        TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1187
hrcd66B6        TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1299
hrcd66B8        TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1299
hrcd66Bm1       TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1380
hrcd66B2        TACGGCTATCAGCGGTTGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCGCAG 1264
                ************************************************************

hrcd66B10       AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1344
hrcd66B20       AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1344
hrcd66B9        AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1344
hrcd66B1        AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1344
hrcd66B3        AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1344
hrcd66B15       AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1344
hrcd66B19       AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1344
hrcd66B13       AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1344
hrcd66B14       AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1344
hrcd66B17       AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1344
hrcd66B16       AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1020
hrcd66B5        AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1247
hrcd66B6        AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1359
hrcd66B8        AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1359
hrcd66Bm1       AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1440
hrcd66B2        AACGGGCTGTTTGTCACCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATC 1324
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Figure 3.12 Nucleotide sequence alignment of chimeric chitinase genes of pHRCD66B: hrcd66B (continued)

```
hrcd66B10       AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1404
hrcd66B20       AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1404
hrcd66B9        AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1404
hrcd66B1        AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1404
hrcd66B3        AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1404
hrcd66B15       AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1404
hrcd66B19       AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1404
hrcd66B13       AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1404
hrcd66B14       AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1404
hrcd66B17       AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1404
hrcd66B16       AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1080
hrcd66B5        AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1307
hrcd66B6        AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1419
hrcd66B8        AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1419
hrcd66Bm1       AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1500
hrcd66B2        AAGCAGCAGCAGCTGGGCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGC 1384
                ************************************************************

hrcd66B10       GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1464
hrcd66B20       GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1464
hrcd66B9        GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1464
hrcd66B1        GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1464
hrcd66B3        GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1464
hrcd66B15       GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1464
hrcd66B19       GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1464
hrcd66B13       GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1464
hrcd66B14       GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1464
hrcd66B17       GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1464
hrcd66B16       GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1140
hrcd66B5        GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1367
hrcd66B6        GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1479
hrcd66B8        GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1479
hrcd66Bm1       GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1560
hrcd66B2        GATCTGCTGGCCGCGCTGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTG 1444
                ************************************************************

hrcd66B10       GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAACCTGCCGATCATGACC 1524
hrcd66B20       GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAACCTGCCGATCATGACC 1524
hrcd66B9        GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAACCTGCCGATCATGACC 1524
hrcd66B1        GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAACCTGCCGATCATGACC 1524
hrcd66B3        GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAACCTGCCGATCATGACC 1524
hrcd66B15       GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAACCTGCCGATCATGACC 1524
hrcd66B19       GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAACCTGCCGATCATGACC 1524
hrcd66B13       GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACC 1524
hrcd66B14       GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACC 1524
hrcd66B17       GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACC 1524
hrcd66B16       GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACC 1200
hrcd66B5        GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACC 1427
hrcd66B6        GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACC 1539
hrcd66B8        GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACC 1539
hrcd66Bm1       GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAACCTGCCGATCATGACC 1620
hrcd66B2        GATATGGGCACCGGCCTGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACC 1504
                ****************************************** ***************
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Figure 3.12 Nucleotide sequence alignment of chimeric chitinase genes of pHRCD66B: hrcd66B (continued)

```
hrcd66B10       GCGCCGGCCTATGTGCCGGGCACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1584
hrcd66B20       GCGCCGGCCTATGTGCCGGGCACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1584
hrcd66B9        GCGCCGGCCTATGTGCCGGGCACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1584
hrcd66B1        GCGCCGGCCTATGTGCCGGGCACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1584
hrcd66B3        GCGCCGGCCTATGTGCCGGGCACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1584
hrcd66B15       GCGCCGGCCTATGTGCCGGGCACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1584
hrcd66B19       GCGCCGGCCTATGTGCCGGGCACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1584
hrcd66B13       GCGCCGGCCTATGTGCCGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1584
hrcd66B14       GCGCCGGCCTATGTGCCGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1584
hrcd66B17       GCGCCGGCCTATGTGCCGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1584
hrcd66B16       GCGCCGGCCTATGTGCCGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1260
hrcd66B5        GCGCCGGCCTATGTGCCGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1487
hrcd66B6        GCGCCGGCCTATGTGCCGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1599
hrcd66B8        GCGCCGGCCTATGTGCCGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1599
hrcd66Bm1       GCGCCGGCCTATGTGCCGGGCACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1680
hrcd66B2        GCGCCGGCCTATGTGCCGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGC 1564
                ******************** ****************************************

hrcd66B10       TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1644
hrcd66B20       TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1644
hrcd66B9        TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1644
hrcd66B1        TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1644
hrcd66B3        TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1644
hrcd66B15       TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1644
hrcd66B19       TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1644
hrcd66B13       TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1644
hrcd66B14       TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1644
hrcd66B17       TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1644
hrcd66B16       TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1320
hrcd66B5        TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1547
hrcd66B6        TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1659
hrcd66B8        TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1659
hrcd66Bm1       TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1740
hrcd66B2        TACGTCTGGCAGACCAAGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGG 1624
                ************************************************************

hrcd66B10       CTGAAGGTGGGCCGCCTGGCGTAA 1668
hrcd66B20       CTGAAGGTGGGCCGCCTGGCGTAA 1668
hrcd66B9        CTGAAGGTGGGCCGCCTGGCGTAA 1668
hrcd66B1        CTGAAGGTGGGCCGCCTGGCGTAA 1668
hrcd66B3        CTGAAGGTGGGCCGCCTGGCGTAA 1668
hrcd66B15       CTGAAGGTGGGCCGCCTGGCGTAA 1668
hrcd66B19       CTGAAGGTGGGCCGCCTGGCGTAA 1668
hrcd66B13       CTGAAGGTGGGCCGCCTGGCGTAA 1668
hrcd66B14       CTGAAGGTGGGCCGCCTGGCGTAA 1668
hrcd66B17       CTGAAGGTGGGCCGCCTGGCGTAA 1668
hrcd66B16       CTGAAGGTGGGCCGCCTGGCGTAA 1344
hrcd66B5        CTGAAGGTGGGCCGCCTGGCGTAA 1571
hrcd66B6        CTGAAGGTGGGCCGCCTGGCGTAA 1683
hrcd66B8        CTGAAGGTGGGCCGCCTGGCGTAA 1683
hrcd66Bm1       CTGAAGGTGGGCCGCCTGGCGTAA 1764
hrcd66B2        CTGAAGGTGGGCCGCCTGGCGTAA 1648
                ************************
```

Figure 3.12 Nucleotide sequence alignment of chimeric chitinase genes of pHRCD66B: hrcd66B (continued)

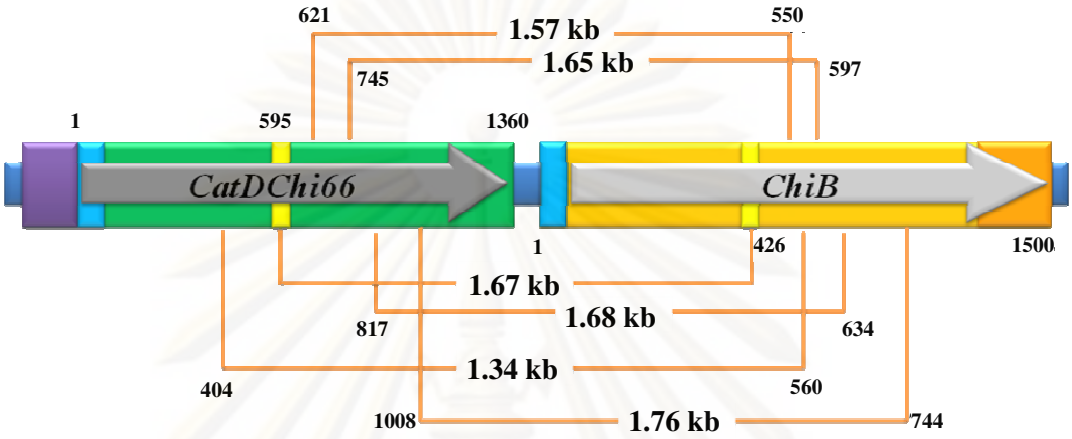

Figure 3.13 Graphical view of recombination site between *catDchi66* and *chiB* of each chimeric gene size

represents promoter of *chi60*

represents coding sequence of signal peptide

represents coding sequence of active site

Query: hrcd66B1 Molecule type: nucleic acid Query Length: 1668

Bacillus licheniformis strain SK-1 chitinase precursor (chiB) and putative chitinase precursor (chiA) genes, complete cds Length=4272

```
Query  541   GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  674   GCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGTTTGACGGGGTCGATCTTGACTGGGAA  733

Query  601   TATCCG  606
             ||||||
Sbjct  734   TATCCG  739
```

Serratia marcescens chiB gene for chitinase B (EC 3.2.1.14) Length=1700

```
Query  561   AAGGAAATACGGGTTTGACGGGGTCGATC-TTGACTGGGAGTATCCGCAGGCGGCGGAAG  619
             |||| | ||||| || ||||| || || | | ||||||||||||||||||||||||||||
Sbjct  511   AAGG-ATTACGGCTTCGACGGCGTGGA-CATCGACTGGGAGTATCCGCAGGCGGCGGAAG  568

Query  620   TGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCTTGCTGAACCAGCAAACCATCG  679
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  569   TGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCTTGCTGAACCAGCAAACCATCG  628
```

Query: HRCD66B1 Molecule type: amino acid Query Length: 555

Chitinase [Bacillus licheniformis]Length=598

```
Query  121   AQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENFA  180
             AQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENFA
Sbjct  121   AQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENFA  180

Query  181   ASAVEFLRKYGFDGVDLDWEYPQAAEVDG----------FIAALQEIRTLLNQQTIADGR  230
             ASAVEFLRKYGFDGVDLDWEYP +  + G          +   LQE+R  L+     DG+
Sbjct  181   ASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGK  240
```

Chitinase B [Serratia marcescens]Length=499

```
Query  131   DEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSN-------RFSDVAADPAARENFAASA  183
             D   R    +L  LK  +P L+   S+GGW +SN        + +    PAAR  FA S
Sbjct  68    DAKARDVVNRLTALKAHNPSLRIMFSIGGWYYSNDLGVSHANYVNAVKTPAARTKFAQSC  127

Query  184   VEFLRKYGFDGVDLDWEYPQAAEVDGFIAALQEIRTLLNQQTIADGRQALPYQLTIAGAG  243
             V  ++ YGFDGVD+DWEYPQAAEVDGFIAALQEIRTLLNQQTIADGRQALPYQLTIAGAG
Sbjct  128   VRIMKDYGFDGVDIDWEYPQAAEVDGFIAALQEIRTLLNQQTIADGRQALPYQLTIAGAG  187
```

>HRCD66B1

MEIVLINKSKKFFVFSFIFVMMLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVWDMDVSKVSHINYAFAD
ICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLK
TFISVGGWTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWEYPQAAEVDGFIAALQEIRTLLNQQTIAD
GRQALPYQLTIAGAGGAFFLSRYYSKLAQIVAPLDYINLMTYDLAGPWEKITNHQAALFGDAAGPTFYNALREANL
GWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFYGRAFKGVSGGNGGQYSSHSTPGEDPYPNADYW
LVGCDECVRDKDPRIASYRQLEQMLQGNYGYQRLWNDKTKTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQQLGGV
MFWHLGQDNRNGDLLAALDRYFNAADYDDSQLDMGTGLRYTGVGPGNLPIMTAPAYVPGTTYAQGALVSYQGYVWQ
TKWGYITSAPGSDSAWLKVGRLA

Figure 3.14 Recombination site of HRCD66B1

Fasta format of deduced amino acid sequence is displayed below. The underlined sequence shows recombination site between CatDChi66 and light grey highlighted ChiB. The dark grey highlighted sequence with white alphabets represents ChBD of ChiB.

Query: hrcd66B6 Molecule type: nucleic acid Query Length: 1683

Bacillus licheniformis strain SK-1 chitinase precursor (chiB) and putative chitinase precursor (chiA) genes, complete cds Length=4272

```
Query  721     GAATACTTGCTGACGATCGCATCCGGCGCAAGTCCCGATTATGTAAGCAACACTGAGCTC  780
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  854     GAATACTTGCTGACGATCGCATCCGGCGCAAGTCCCGATTATGTAAGCAACACTGAGCTC  913

Query  781     GATAAAATCGCTCAAACCGTGGATTGGATTAACATTATGACCTA  824
               |||||||||||||||||||||||||||||||||||||||||||
Sbjct  914     GATAAAATCGCTCAAACCGTGGATTGGATTAACATTATGACCTA  957
```

Serratia marcescens chiB gene for chitinase B (EC 3.2.1.14) Length=1700

```
Query  817     ATGACCTACGATCTGGCCGGCCCCTGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTC  876
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  634     ATGACCTACGATCTGGCCGGCCCCTGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTC  693

Query  877     GGCGACGCGGCCGGGCCGACCTTCTACAACGCACTGCGCGAAGCCAATCTGGGCTGGAGC  936
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  694     GGCGACGCGGCCGGGCCGACCTTCTACAACGCACTGCGCGAAGCCAATCTGGGCTGGAGC  753
```

Query: HRCD66B6 Molecule type: amino acid Query Length: 560

Chitinase [Bacillus licheniformis]Length=598

```
Query  181  ASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGK  240
            ASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGK
Sbjct  181  ASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAKDGK  240

Query  241  EYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYDLAGPWEKITNHQAALFGDAAGPTF  300
            EYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYD  G W+ I+ H A LF D
Sbjct  241  EYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYDFNGGWQSISAHNAPLFYDPKA---  297
```

Chitinase B [Serratia marcescens]Length=499

```
Query  242  -YLLTIASGASPDYVSN--TELDKIAQTVDWINIMTYDLAGPWEKITNHQAALFGDAAGP  298
             Y LTIA      ++S   ++L +I   +D+IN+MTYDLAGPWEKITNHQAALFGDAAGP
Sbjct  178  PYQLTIAGAGGAFFLSRYYSKLAQIVAPLDYINLMTYDLAGPWEKITNHQAALFGDAAGP  237

Query  299  TFYNALREANLGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFYGRAFK  358
            TFYNALREANLGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFYGRAFK
Sbjct  238  TFYNALREANLGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFYGRAFK  297
```

>HRCD66B6

MKIVLINKSKKFFVFSFIFVMMLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVWDMDVSKVSHINYAFAD
ICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLK
TFISVGGWTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVR
KKLDAAEAKDGKEYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYDLAGPWEKITNHQAALFGDAAGPTFYNAL
REANLGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFYGRAFKGVSGGNGGQYSSHSTPGEDPYP
NADYWLVGCDECVRDKDPRIASYRQLEQMLQGNYGYQRLWNDKTKTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQ
QLGGVMFWHLGQDNRNGDLLAALDRYFNAADYDDSQLDMGTGLRYTGVGPGNLPIMTAPAYVPGTTYAQGALVSYQ
GYVWQTKWGYITSAPGSDSAWLKVGRLA

Figure 3.15 Recombination site of HRCD66B6

Fasta format of deduced amino acid sequence is displayed below. The underlined sequence shows recombination site between CatDChi66 and light grey highlighted ChiB. The dark grey highlighted sequence with white alphabets represents ChBD of ChiB.

Query: hrcd66B15 Molecule type: nucleic acid Query Length: 1344

Bacillus licheniformis strain SK-1 chitinase precursor (chiB) and putative chitinase precursor (chiA) genes, complete cds Length=4272

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Query  301   GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  434   GAAAACGGAGTGATCGACGCGCCAAATGGAACAATCGTGATGGGCGATCCCTGGATTGAC  493

Query  361   GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGC  408
             |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  494   GCACAAAAGTCAAATCCCGGGGATGTCTGGGATGAACCGATCCGCGGC  541
```

Serratia marcescens chiB gene for chitinase B (EC 3.2.1.14)

Length=1700

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Query  404   GCGGCGCCTTCTTCCTGTCGCGCTATTACAGCAAGCTGGCGCAAATCGTCGCGCCACTCG  463
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  560   GCGGCGCCTTCTTCCTGTCGCGCTATTACAGCAAGCTGGCGCAAATCGTCGCGCCACTCG  619

Query  464   ATTACATCAACCTGATGACCTACGATCTGGCCGGCCCCTGGGAGAAGATCACCAACCACC  523
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  620   ATTACATCAACCTGATGACCTACGATCTGGCCGGCCCCTGGGAGAAGATCACCAACCACC  679
```

Query: HRCD66B15 Molecule type: amino acid Query Length: 447

Chitinase [Bacillus licheniformis]Length=598

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Query  61    DMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWID  120
             DMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWID
Sbjct  61    DMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWID  120

Query  121   AQKSNPGDVWDEPIRGAF  138
             AQKSNPGDVWDEPIRG F
Sbjct  121   AQKSNPGDVWDEPIRGNF  138
```

Chitinase B [Serratia marcescens]Length=499

```
Query  136   GAFFLSRYYSKLAQIVAPLDYINLMTYDLAGPWEKITNHQAALFGDAAGPTFYNALREAN  195
             GAFFLSRYYSKLAQIVAPLDYINLMTYDLAGPWEKITNHQAALFGDAAGPTFYNALREAN
Sbjct  188   GAFFLSRYYSKLAQIVAPLDYINLMTYDLAGPWEKITNHQAALFGDAAGPTFYNALREAN  247

Query  196   LGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFYGRAFKGVSGGNGGQY  255
             LGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFYGRAFKGVSGGNGGQY
Sbjct  248   LGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFYGRAFKGVSGGNGGQY  307
```

>HRCD66B15

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ICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKSNPGDVWDEPIRGAFFLSRYYSKLAQIVA
PLDYINLMTYDLAGPWEKITNHQAALFGDAAGPTFYNALREANLGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGV
PSAKIVMGVPFYGRAFKGVSGGNGGQYSSHSTPGEDPYPNADYWLVGCDECVRDKDPRIASYRQLEQMLQGNYGYQ
RLWNDKTKTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQQLGGVMFWHLGQDNRNGDLLAALDRYFNAADYDDSQL
DMGTGLRYTGVGPGNLPIMTAPAYVPGTTYAQGALVSYQGYVWQTKWGYITSAPGSDSAWLKVGRLA

Figure 3.16 Recombination site of HRCD66B15

Fasta format of deduced amino acid sequence is displayed below. The underlined sequence shows recombination site between CatDChi66 and light grey highlighted ChiB. The dark grey highlighted sequence with white alphabets represents ChBD of ChiB.

Query: hrcd66Bm1 Molecule type: nucleic acid Query Length: 1764

Bacillus licheniformis strain SK-1 chitinase precursor (chiB) and putative chitinase precursor (chiA) genes, complete cds Length=4272

```
Query  901   GGCGTTCCAAACGCTGAGACCTACAATATTGAAAACACTGTGAAACGCTACAAGGAAGCC  960
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1034  GGCGTTCCAAACGCTGAGACCTACAATATTGAAAACACTGTGAAACGCTACAAGGAAGCC  1093

Query  961   GGTGTCAAGGGTGACAAATTAGTGCTTGGAACACCGTTCTACGGAAGGGGCTGGAGC  1017
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1094  GGTGTCAAGGGTGACAAATTAGTGCTTGGAACACCGTTCTACGGAAGGGGCTGGAGC  1150
```

Serratia marcescens chiB gene for chitinase B (EC 3.2.1.14) Length=1700

```
Query  1008  GGGCTGGAGCTGGGAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGA  1067
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  744   GGGCTGGAGCTGGGAAGAGCTGACCCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGA  803

Query  1068  CGCCGCCGTGCAGCAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGG  1127
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  804   CGCCGCCGTGCAGCAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGG  863
```

Query: HRCD66BM1 Molecule type: amino acid Query Length: 587

Chitinase [Bacillus licheniformis]Length=598

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Query  241  EYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYDFNGGWQSISAHNAPLFYDPKAKEA  300
            EYLLTIASGASP+YVSNTELDKIAQTVDWINIMTYDFNGGWQSISAHNAPLFYDPKAKEA
Sbjct  241  EYLLTIASGASPEYVSNTELDKIAQTVDWINIMTYDFNGGWQSISAHNAPLFYDPKAKEA  300

Query  301  GVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSWEELTRAFPSPFSLTVDAAVQ  360
            GVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWS
Sbjct  301  GVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWS----------------------  339
```

Chitinase B [Serratia marcescens]Length=499

```
Query  299  EAGVPNAETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSWEELTRAFPSPFSLTVDAA  358
             A  P    YN        +EA +              GWSWEELTRAFPSPFSLTVDAA
Sbjct  234  -AAGPT--FYNA------LREANL--------------GWSWEELTRAFPSPFSLTVDAA  270

Query  359  VQQHLMMEGVPSAKIVMGVPFYGRAFKGVSGGNGGQYSSHSTPGEDPYPNADYWLVGCDE  418
            VQQHLMMEGVPSAKIVMGVPFYGRAFKGVSGGNGGQYSSHSTPGEDPYP+ DYWLVGC+E
Sbjct  271  VQQHLMMEGVPSAKIVMGVPFYGRAFKGVSGGNGGQYSSHSTPGEDPYPSTDYWLVGCEE  330
```

>HRCD66BM1

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ICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLK
TFISVGGWTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVR
KKLDAAEAKDGKEYLLTIASGASPDYVSNTELDKIAQTVDWINIMTYDFNGGWQSISAHNAPLFYDPKAKEAGVPN
AETYNIENTVKRYKEAGVKGDKLVLGTPFYGRGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFY
GRAFKGVSGGNGGQYSSHSTPGEDPYPNADYWLVGCDECVRDKDPRIASYRQLEQMLQGNYGYQRLWNDKTKTPYL
YHAQNGLFVTYDDAESFKYKAKYIKQQQLGGVMFWHLGQDNRNGDLLAALDRYFNAADYDDSQLDMGTGLRYTGVG
PGNLPIMTAPAYVPGTTYAQGALVSYQGYVWQTKWGYITSAPGSDSAWLKVGRLA

Figure 3.17 Recombination site of HRCD66BM1

Fasta format of deduced amino acid sequence is displayed below. The underlined sequence shows recombination site between CatDChi66 and light grey highlighted ChiB. The dark grey highlighted sequence with white alphabets represents ChBD of ChiB.

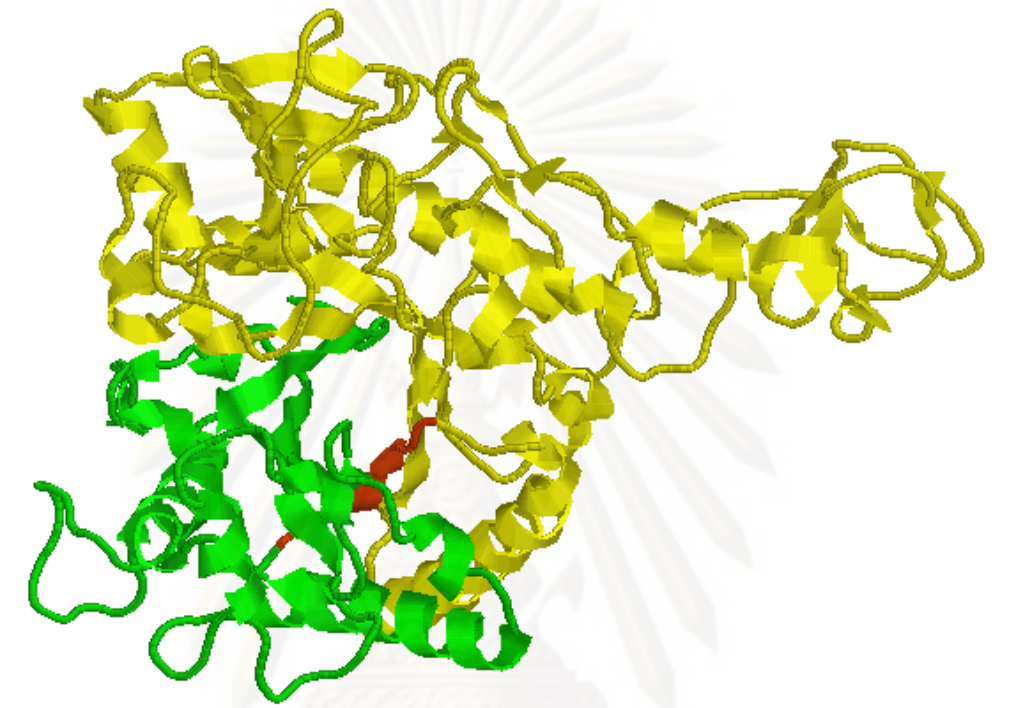


Figure 3.18 Homology modeling structure of HRCD66B1

Structure was visualized by Rasmol version 2.7.4.2. Green color indicates structure deduced from CatDChi66 sequences and yellow color indicates structure deduced from ChiB sequences. Conserved motif in family 18 active site (FDGVDLDWE) is displayed in red.

Figure 3.19 Homology modeling structure of HRCD66B6

Structure was visualized by Rasmol version 2.7.4.2. Green color indicates structure deduced from CatDChi66 sequences and yellow color indicates structure deduced from ChiB sequences. Conserved motif in family 18 active site (FDGVDLDWE) is displayed in red.

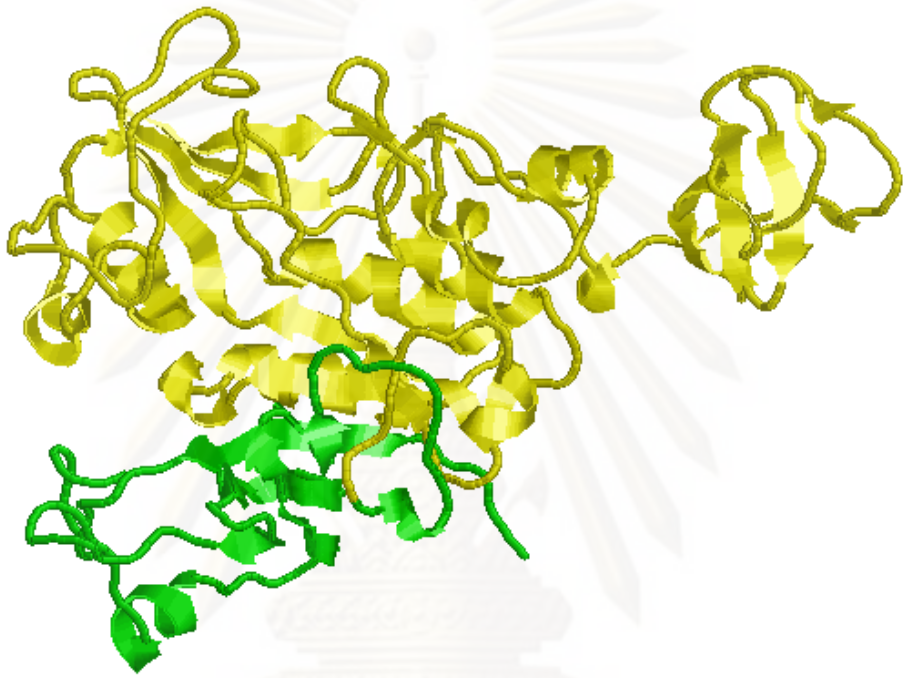


Figure 3.20 Homology modeling structure of HRCD66B15

Structure was visualized by Rasmol version 2.7.4.2. Green color indicates structure deduced from CatDChi66 sequences and yellow color indicates structure deduced from ChiB sequences. Conserved motif in family 18 active site (FDGVDLDWE) is not observed in HRCD66B15.

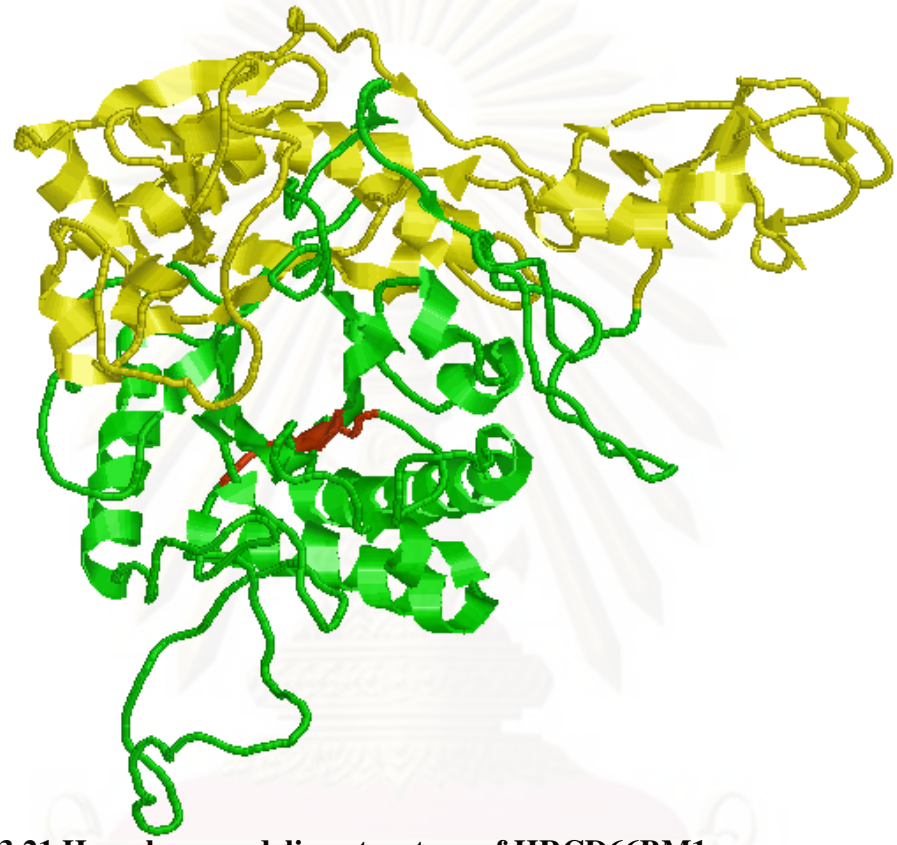


Figure 3.21 Homology modeling structure of HRCD66BM1

Structure was visualized by Rasmol version 2.7.4.2. Green color indicates structure deduced from CatDChi66 sequences and yellow color indicates structure deduced from ChiB sequences. Conserved motif in family 18 active site (FDGVDLDWE) is displayed in red.

respectively. Part of CatDChi66 structure is colorized in green and part of ChiB structure is colorized in yellow. The conserved active site motif is shown in red color which is absent in HRCD66B15.

4. Expression of HRCD66B

pHRCD66B1, pHRCD66B6, pHRCD66B15 and pHRCD66BM1 were retransformed into *E. coli* DH5α and cultured in LB broth containing 100 μg/ml ampicillin for 3 days in incubator shaker at 37°C. Since Chi66 has N-terminal signal peptide, chimeric chitinases were supposed to be expressed and transported into supernatant. Chitinases in supernatant were collected by centrifugation at 5,000 g for 10 minutes at 4°C. Figure 3.22 shows protein expression of HRCD66B1, HRCD66B6, HRCD66B15 and HRCD66BM1 analyzed by SDS-PAGE.

5. Chitinase activity assay of HRCD66B

5.1 Determination of chitinase activity by measuring reducing sugar

Chitinolytic activity of HRCD66B1, HRCD66B6, HRCD66B15 and HRCD66BM1 was assayed as described by Imoto and Yagishita (1971). With transformants of pCD66B as positive control and pBS/SK$^-$ as negative control, activity was first assayed under phosphate buffer pH 6.0 and incubated at 37°C for 60 min using PNAC as soluble chitin substrate and Colloidal chitin as insoluble substrate. Unfortunately, all chimeras showed no chitinolytic activity under this condition. The buffer system was changed to citrate buffer pH 4.0 and Tris-HCl buffer pH 8.0, but their activity were still not observed.

Changing of expression host to *E. coli* Origami (DE3) was also performed without success. This was confirmed by increasing the amount of enzyme and incubation time from 60 min to 120 min and no difference was observed.

5.2 Determination of chitinase activity of HRCD66B by TLC

Chitinase activity of HRCD66B1, HRCD66B6, HRCD66B15 and HRCD66BM1 were assayed in phosphate buffer pH 6 with chitooligosaccharides as substrates and products of reaction were detected by TLC. Figure 3.23 shows products of chitinase reaction using NAG_3 (A.) and NAG_4 (B.) as substrates which were

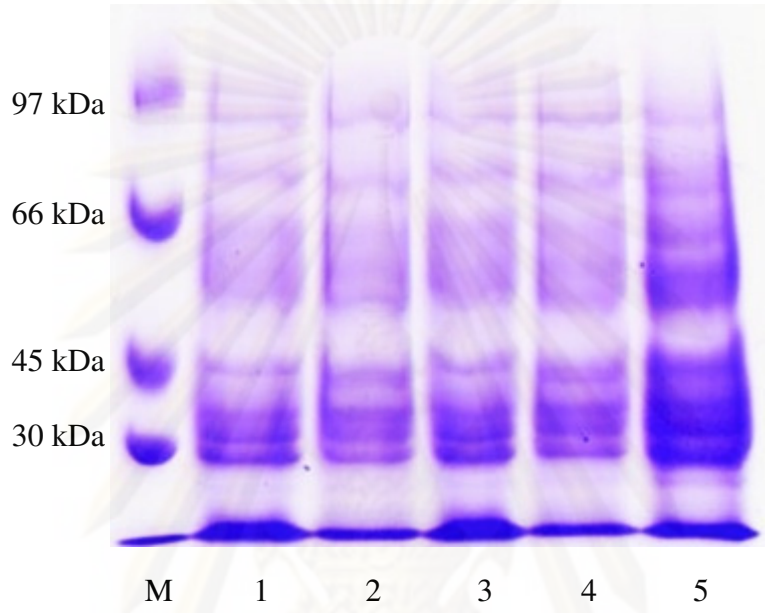


Figure 3.22 SDS-PAGE analysis of crude HRCD66B1, HRCD66B6, HRCD66B15 and HRCD66BM1

Lane M Standard protein marker

Lane 1 HRCD66B1

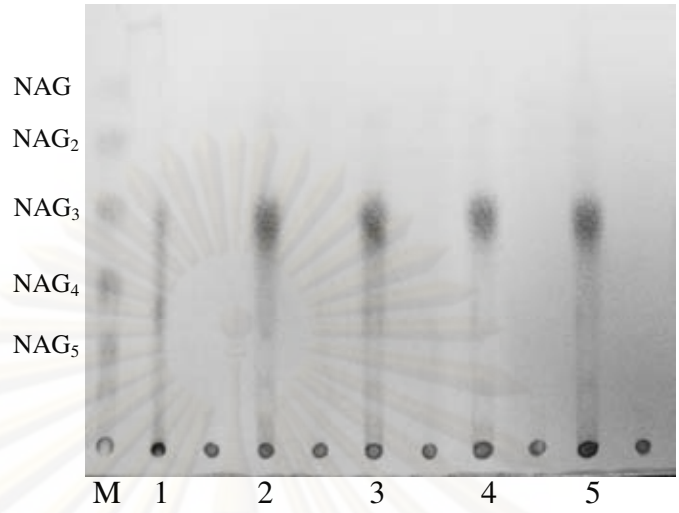
Lane 2 HRCD66B6

Lane 3 HRCD66B15

Lane 4 HRCD66BM1

Lane 5 pBS/SK$^-$ in *E. coli* DH5α

A



B

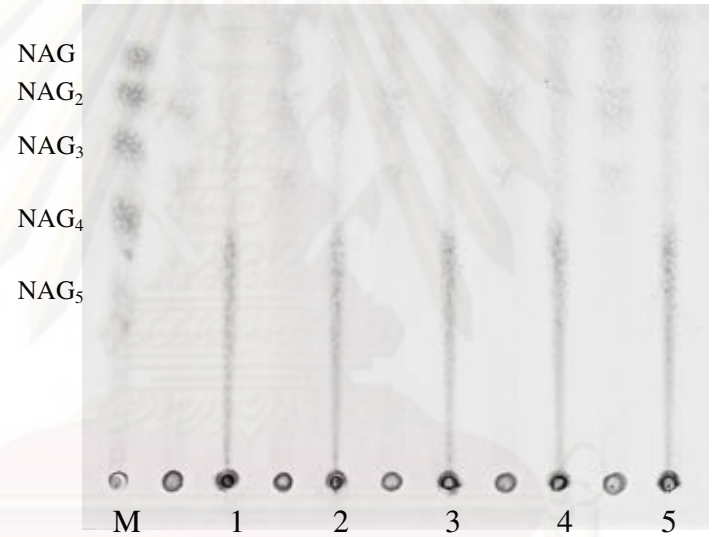


Figure 3.23 Hydrolytic products of HRCD66B1, HRCD66B6, HRCD66B15 and HRCD66BM1 determined by TLC using NAG_3 and NAG_4 as substrates

Hydrolytic products from reactions with NAG_3 (A) and NAG_4 (B) as substrates were detected by TLC.

Lane M Standard mixture of NAG, NAG_2, NAG_3, NAG_4 and NAG_5

Lane 1 Reaction of pBS/SK^- in *E. coli* DH5α

Lane 2 Reaction of HRCD66B1

Lane 3 Reaction of HRCD66B6

Lane 4 Reaction of HRCD66B15

Lane 5 Reaction of HRCD66BM1

detected by TLC. Results indicated that there were no products observed for any of the chimeras.

Construction and expression of HRCHI60B

1. Construction of HRCHI60B

pCHI60B was double digested with *Bst*Z17I and *Hin*dIII and linearized plasmid was purified from 957 bp digested fragment using Qiaquick Gel Extraction Kit. pCHI60B with two knockout genes was transformed into *E. coli* JC8679 by electroporation and screened by the following methods.

1.1 HRCHI60B screening from LB agar plate with ampicillin

One milliliter of transformants culture was spread on LB agar plate containing 100 μg/ml ampicillin and incubated at 37°C for 12-16 hours. The result showed that there were 46 colonies formed on agar plate. Each colony was picked and plasmid was extracted. The extracted plasmids were digested with *Not*I that cleaves at the start of promoter site and *Xho*I that cleaves at stop codon of *chiB* and each digested plasmid was loaded along with the undigested one. From the agarose gel electrophoresis result, there were 26 colonies that contained plasmid with 1.5-2.3 kb chimeric genes which are from colony number 1, 4, 5, 6, 7, 12, 13, 14, 18, 19, 21, 22, 23, 25, 26, 27, 29, 30, 31, 32, 35, 37, 38, 40, 45 and 46. These plasmids were designated as pHRCHI60B1-26.

This screening was repeated and we found 228 of forming colonies. Sixty eight colonies were randomly picked and checked by plasmids extraction. From these results, we observed 49 more plasmids extracted from colony number 1, 2, 3, 6, 7, 13, 15, 16, 18, 19, 20, 21, 24, 25, 26, 28, 30, 31, 32, 33, 34, 35, 37, 38, 39, 40, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 57, 58, 60, 62, 63, 64, 65, 66 and 67 that contained chimeric gene size 1.5-2.3 kb and designated as pHRCHI60B27-75. The agarose gel electrophoresis analyses of plasmids extracted from all forming colonies are shown in appendix D and E. In order to find chimeras with functional chitinases, the other 160 colonies were screened by roughly measuring hydrolytic activity of supernatant from chimera that was still in *E. coli* JC8679 with PNAC as substrate in citrate buffer pH 5.0. Table 3.2 shows the absorbance differences at 420 nm obtained from chitinase activity assay by colorimethod of colony 69 to 228.

We found that colony number 111 and 227 shows little hydrolytic activity so that plasmids of these two colonies were extracted and designated as pHRCHI60B76 and 77. Figure 3.24 shows the agarose gel electrophoresis analysis of pHRCHI60B76 and 77 that were digested with *Not*I and *Kpn*I.

1.2 HRCHI60B screening from CCMM broth with ampicillin

E. coli Top10 with pCHI60B and pBS/SK$^-$ were used as positive and negative control, respectively. Aliquots of 50 μl from 1 ml transformants were cultured into 18 tubes of 3 ml CCMM broth containing 100 μg/ml ampicillin and incubate at 37°C in incubator shaker at 250 rpm. The cultures were observed for 3 days and found that there were 4 chimeras that could grow on CCMM. Plasmids of these four were extracted and digested with *Not*I and *Xho*I and checked by agarose gel electrophoresis as shown in figure 3.25. This screening was repeated twice. In the second screening, 60 μl of aliquots were cultured into 16 tubes of 3 ml CCMM broth containing 100 μg/ml ampicillin at 37°C. This time we found 11 tubes of growing cultures including negative control, thus the cultures were further observed for 5 days. Decreasing of colloidal chitin in the culture was observed in only one tube which was then collected and extracted for plasmid. In the third screening, the volume of aliquots were reduced to 30 μl and cultured into 24 tubes of CCMM with ampicillin and negative controls could still grow in CCMM. After 5 days of culture, only one tube was observed to have a decrease in colloidal chitin in the cultures. The culture was then collected and extracted for plasmid. Figure 3.26 shows agarose gel electrophoresis of plasmids from second and third screening that digested with *Not*I and *Xho*I. Plasmids obtained from CCMM screening were designated as pHRCHI60BM1-6.

2. Sequences analysis of pHRCHI60B

From agarose gel electrophoresis results, pHRCHI60B were grouped based on chimeric gene size into 5 groups with gene size of 1.4, 1.5, 1.6, 2.0 and 2.3 kb as shown in table 3.3. pHRCHI60B1, 3, 5, 20, and 24 as the representatives of each group were sequenced. Sequences of each chimera were aligned using ClustalW2 program. Figure 3.27 shows nucleotide sequences alignment of chimeric chitinases gene from pHRCHI60B.

Table 3.2 The absorbance differences at 420 nm obtained from chitinase activity assay by colorimethod of colony 69 to 228

No. of colony	ΔA_{420}	No. of colony	ΔA_{420}	No. of colony	ΔA_{420}	No. of colony	ΔA_{420}	No. of colony	ΔA_{420}
69	0.02	101	0.02	133	0.00	165	0.00	197	0.02
70	0.00	102	0.00	134	0.00	166	0.00	198	0.03
71	0.00	103	0.05	135	0.00	167	0.00	199	0.03
72	0.00	104	0.02	136	0.00	168	0.00	200	0.01
73	0.04	105	0.01	137	0.02	169	0.00	201	0.00
74	0.03	106	0.00	138	0.05	170	0.04	202	0.01
75	0.00	107	0.05	139	0.00	171	0.01	203	0.07
76	0.04	108	0.01	140	0.00	172	0.02	204	0.02
77	0.00	109	0.04	141	0.00	173	0.05	205	0.03
78	0.04	110	0.08	142	0.00	174	0.00	206	0.02
79	0.00	111	0.14	143	0.00	175	0.01	207	0.05
80	0.00	112	0.01	144	0.00	176	0.02	208	0.04
81	0.04	113	0.08	145	0.00	177	0.01	209	0.04
82	0.03	114	0.03	146	0.00	178	0.01	210	0.00
83	0.04	115	0.04	147	0.00	179	0.01	211	0.02
84	0.01	116	0.03	148	0.00	180	0.01	212	0.03
85	0.01	117	0.09	149	0.00	181	0.04	213	0.04
86	0.03	118	0.06	150	0.00	182	0.02	214	0.03
87	0.02	119	0.00	151	0.00	183	0.06	215	0.02
88	0.04	120	0.00	152	0.00	184	0.04	216	0.04
89	0.00	121	0.00	153	0.00	185	0.06	217	0.06
90	0.02	122	0.00	154	0.00	186	0.03	218	0.05
91	0.04	123	0.00	155	0.00	187	0.03	219	0.01
92	0.05	124	0.00	156	0.00	188	0.03	220	0.03
93	0.05	125	0.00	157	0.00	189	0.03	221	0.04
94	0.01	126	0.00	158	0.00	190	0.03	222	0.00
95	0.01	127	0.00	159	0.00	191	0.00	223	0.00
96	0.04	128	0.01	160	0.00	192	0.02	224	0.03
97	0.05	129	0.00	161	0.08	193	0.01	225	0.02
98	0.02	130	0.00	162	0.00	194	0.03	226	0.04
99	0.05	131	0.00	163	0.00	195	0.00	227	0.10
100	0.05	132	0.00	164	0.00	196	0.03	228	0.04

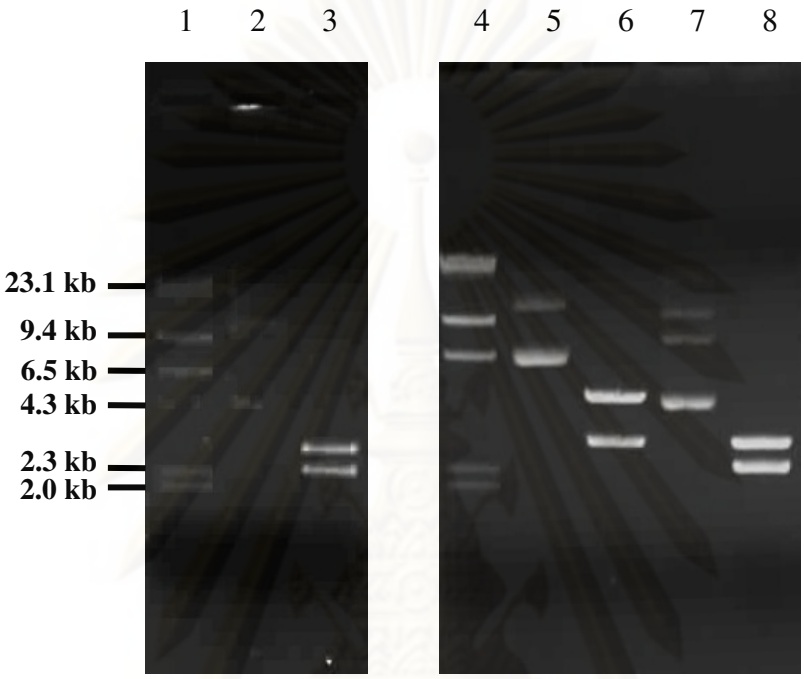


Figure 3.24 Agarose gel electrophoresis analysis of plasmids extracted from colony number 111 and 227 that were screened by hydrolytic activity assay of pHRCHI60B

Lane 1 λ/*Hin*dIII marker
Lane 2 Plasmids from colony 111
Lane 3 Plasmids from colony 111/ *Not*I+ *Xho*I
Lane 4 λ/*Hin*dIII marker
Lane 5 pCHI60B
Lane 6 pCHI60B/ *Not*I+ *Xho*I
Lane 7 Plasmids from colony 227
Lane 8 Plasmids from colony 227/ *Not*I+ *Xho*I

***** Plasmids extracted from colony 111 and 227 were designated as pHRCHI60B76 and 77.

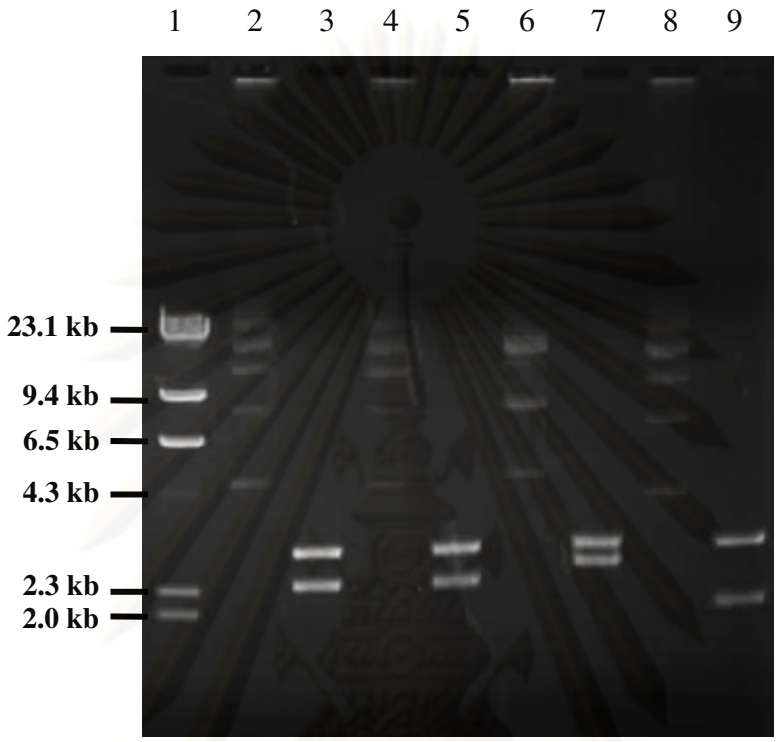


Figure 3.25 Agarose gel electrophoresis analysis of plasmids extracted from cultures of the first pHRCHI60B screening on CCMM broth

Lane 1 λ/*Hin*dIII marker

Lane 2 Plasmids from CCMM tube1

Lane 3 Plasmids from CCMM tube1/ *Not*I+ *Xho*I

Lane 4 Plasmids from CCMM tube2

Lane 5 Plasmids from CCMM tube2/ *Not*I+ *Xho*I

Lane 6 Plasmids from CCMM tube3

Lane 7 Plasmids from CCMM tube3/ *Not*I+ *Xho*I

Lane 8 Plasmids from CCMM tube4

Lane 9 Plasmids from CCMM tube4/ *Not*I+ *Xho*I

*These four plasmids were designated as pHRCHI60BM1-4.

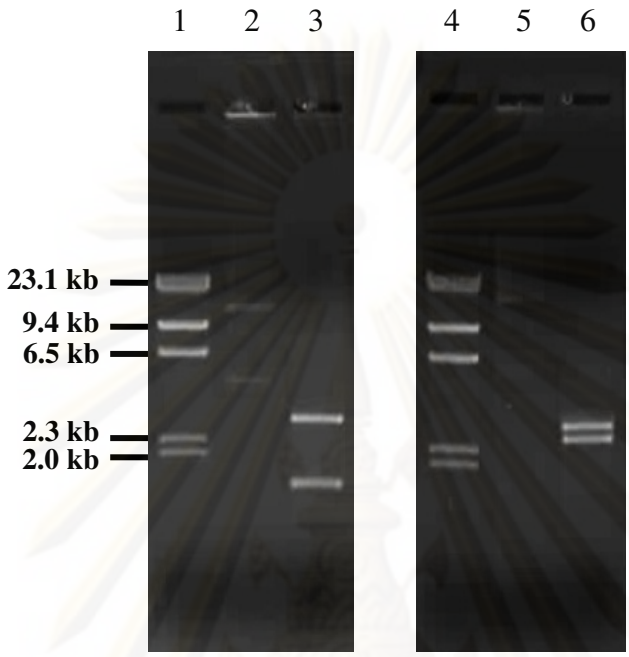


Figure 3.26 Agarose gel electrophoresis analysis of plasmids extracted from cultures of the second and third pHRCHI60B screening by CCMM broth

Lane 1 λ/*Hin*dIII marker

Lane 2 Plasmids from the second CCMM screening

Lane 3 Plasmids from the second CCMM screening/ *Not*I+ *Xho*I

Lane 4 λ/*Hin*dIII marker

Lane 5 Plasmids from the third CCMM screening

Lane 6 Plasmids from the third CCMM screening/ *Not*I+ *Xho*I

*These two plasmids were designated as pHRCHI60BM5 and 6.

Table 3.3 Groups of pHRCHI60B derivatives divided by chimeric gene size

Chimeric gene size	pHRCHI60B
1.4 kb	pHRCHI60B3, 4, 12, 31, 45, 48, 51, 52, 58, 60
1.5 kb	pHRCHI60B24, 35, 55, 58, 59, 65, pHRCHI60BM5
1.6 kb	pHRCHI60B5, 6, 15, 23, 29, 31, 39, 47, pHRCHI60BM4
2.0 kb	pHRCHI60B1, 2, 7, 8, 9, 10, 11, 13, 14, 16, 17, 18, 19, 21, 22, 25, 26, 27, 28, 30, 32, 33, 34, 36, 37, 38, 40, 41, 42, 43, 44, 46, 49, 50, 53, 54, 56, 57, 60, 61, 62, 63, 64, 66, 67, 70, 71, 72, 73, 74, 75, 76, 77, pHRCHI60BM1, 2
2.3 kb	pHRCHI60B20, pHRCHI60BM3, 6

pHRCHI60B3, 24, 5, 1, 20 were chosen to be representatives of plasmids with 1.4, 1.5, 1.6, 2.0 and 2.3 kb chimeric genes, respectively and were sequenced.

```
hrchi60B3       ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
hrchi60B5       ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
hrchi60B20      ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
hrchi60B1       ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
hrchi60B24      ATGCGCAAATTTAATAAACCGCTGTTGGCGCTGTTGATCGGCAGCACGCTGTGTTCCGCG 60
                ************************************************************

hrchi60B3       GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAATACCAAGTTCGCCATC 120
hrchi60B5       GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAATACCAAGTTCGCCATC 120
hrchi60B20      GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAATACCAAGTTCGCCATC 120
hrchi60B1       GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAATACCAAGTTCGCCATC 120
hrchi60B24      GCGCAGGCCGCCGCGCCGGGCAAGCCGACCATCGCCTGGGGCAATACCAAGTTCGCCATC 120
                ************************************************************

hrchi60B3       GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAGTTTGGTGAAGGTAAAAAATGCCGCC 180
hrchi60B5       GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAGTTTGGTGAAGGTAAAAAATGCCGCC 180
hrchi60B20      GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAGTTTGGTGAAGGTAAAAAATGCCGCC 180
hrchi60B1       GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAGTTTGGTGAAGGTAAAAAATGCCGCC 180
hrchi60B24      GTTGAAGTTGACCAGGCGGCTACCGCTTATAATAGTTTGGTGAAGGTAAAAAATGCCGCC 180
                ************************************************************

hrchi60B3       GATGTTTCGGTCTCCTGGAATTTATGGAATGGCGACACCGGTACGACGGCAAAAGTTTTA 240
hrchi60B5       GATGTTTCGGTCTCCTGGAATTTATGGAATGGCGACACCGGTACGACGGCAAAAGTTTTA 240
hrchi60B20      GATGTTTCGGTCTCCTGGAATTTATGGAATGGCGACACCGGTACGACGGCAAAAGTTTTA 240
hrchi60B1       GATGTTTCGGTCTCCTGGAATTTATGGAATGGCGACACCGGTACGACGGCAAAAGTTTTA 240
hrchi60B24      GATGTTTCGGTCTCCTGGAATTTATGGAATGGCGACACCGGTACGACGGCAAAAGTTTTA 240
                ************************************************************

hrchi60B3       TTAAATGGCAAAGAGGCGTGGAGCGGCCCGTCAACCGGTTCTTCCGGTACGGCGAATTTT 300
hrchi60B5       TTAAATGGCAAAGAGGCGTGGAGCGGCCCGTCAACCGGTTCTTCCGGTACGGCGAATTTT 300
hrchi60B20      TTAAATGGCAAAGAGGCGTGGAGCGGCCCGTCAACCGGTTCTTCCGGTACGGCGAATTTT 300
hrchi60B1       TTAAATGGCAAAGAGGCGTGGAGCGGCCCGTCAACCGGTTCTTCCGGTACGGCGAATTTT 300
hrchi60B24      TTAAATGGCAAAGAGGCGTGGAGCGGCCCGTCAACCGGTTCTTCCGGTACGGCGAATTTT 300
                ************************************************************

hrchi60B3       AAAGTCAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
hrchi60B5       AAAGTCAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
hrchi60B20      AAAGTCAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
hrchi60B1       AAAGTCAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
hrchi60B24      AAAGTCAATAAAGGCGGCCGTTATCAAATGCAGGTGGCATTGTGCAATGCCGACGGCTGC 360
                ************************************************************
```

Figure 3.27 Nucleotide sequence alignment of chimeric chitinase genes of pHRCHI60B: hrchi60B1, 3, 5, 20 and 24

```
hrchi60B3       AGCGCCAGCGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
hrchi60B5       AGCGCCAGCGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
hrchi60B20      AGCGCCAGCGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
hrchi60B1       AGCGCCAGCGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
hrchi60B24      AGCGCCAGCGACGCCACCGAAATTGTGGTGGCCGACACCGACGGCAGCCATTTGGCGCCG 420
                ************************************************************

hrchi60B3       TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTCGTC 480
hrchi60B5       TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTCGTC 480
hrchi60B20      TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTCGTC 480
hrchi60B1       TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTCGTC 480
hrchi60B24      TTGAAAGAGCCGCTGCTGGAAAAGAATAAACCGTATAAACAGAACTCCGGCAAAGTCGTC 480
                ************************************************************

hrchi60B3       GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
hrchi60B5       GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
hrchi60B20      GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
hrchi60B1       GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
hrchi60B24      GGTTCTTATTTCGTCGAGTGGGGCGTTTACGGGCGCAATTTCACCGTCGACAAGATCCCG 540
                ************************************************************

hrchi60B3       GCGCAGAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAACGGCATC 600
hrchi60B5       GCGCAGAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAACGGCATC 600
hrchi60B20      GCGCAGAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAACGGCATC 600
hrchi60B1       GCGCAGAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAACGGCATC 600
hrchi60B24      GCGCAGAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTGCGGCGGCAACGGCATC 600
                ************************************************************

hrchi60B3       AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGCTGCAGCGCTCCTGCCAGGGC 660
hrchi60B5       AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGCTGCAGCGCTCCTGCCAGGGC 660
hrchi60B20      AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGCTGCAGCGCTCCTGCCAGGGC 660
hrchi60B1       AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGCTGCAGCGCTCCTGCCAGGGC 660
hrchi60B24      AACGACAGCCTGAAAGAGATCGAAGGCAGCTTCCAGGCGCTGCAGCGCTCCTGCCAGGGC 660
                ************************************************************

hrchi60B3       CGCGAGGACTTCAAAGTCTCGATCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
hrchi60B5       CGCGAGGACTTCAAAGTCTCGATCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
hrchi60B20      CGCGAGGACTTCAAAGTCTCGATCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
hrchi60B1       CGCGAGGACTTCAAAGTCTCGATCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
hrchi60B24      CGCGAGGACTTCAAAGTCTCGATCCACGATCCGTTCGCCGCGCTGCAAAAAGCGCAGAAG 720
                ************************************************************
```

Figure 3.27 Nucleotide sequence alignment of chimeric chitinase genes of pHRCHI60B: hrchi60B1, 3, 5, 20 and 24 (continued)

```
hrchi60B3       GGCGTTACCGCCTGGGATGACCC-CTACAAGGGCAACTTCGGCCAGCTGATG-GCGCTGA 778
hrchi60B5       GGCGTTACCGCCTGGGATGACCC-CTACAAGGGCAACTTCGGCCAGCTGATG-GCGCTGA 778
hrchi60B20      GGCGTTACCGCCTGGGATGACCC-CTACAAGGGCAACTTCGGCCAGCTGATG-GCGCTGA 778
hrchi60B1       GGCGTTACCGCCTGGGATGACCC-CTACAAGGGCAACTTCGGCCAGCTGATG-GCGCTGA 778
hrchi60B24      GGCGTTACCGCCTGGGCCGACCTTCTACAACG--CACTGCGCGAAGCCAATCTGGGCTGG 778
                ****************  ****  ****** *   *** **   ***  **  * ****

hrchi60B3       AACAGGCGCATCCTGACCTGAAAATTCTGCCGTCGATCGGCGGCTGGACGCTGTCCGAC- 837
hrchi60B5       AACAGGCGCATCCTGACCTGAAAATTCTGCCGTCGATCGGCGGCTGGACGCTGTCCGAC- 837
hrchi60B20      AACAGGCGCATCCTGACCTGAAAATTCTGCCGTCGATCGGCGGCTGGACGCTGTCCGAC- 837
hrchi60B1       AACAGGCGCATCCTGACCTGAAAATTCTGCCGTCGATCGGCGGCTGGACGCTGTCCGAC- 837
hrchi60B24      AGCTGGGAAGAGCTGACTCGCGCCTTCCCCAGCCCGTT--CAGCCTGACGGTCGACGCCG 836
                * * **     *****  *    ***  * * *  *   * **  **** *   ** *

hrchi60B3       CCGTTCTTCTTCATG-GGCG-----------------TGCCC------------------ 861
hrchi60B5       CCGTTCTTCTTCATG-GGCGATAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGA 896
hrchi60B20      CCGTTCTTCTTCATG-GGCGATAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGA 896
hrchi60B1       CCGTTCTTCTTCATG-GGCGATAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGA 896
hrchi60B24      CCGTGCAGCAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGC 896
                **** *  *  **   *  *                 **

hrchi60B3       --TTCTACGGCCGCG-----CCTTCAAGGGCGTCAGC----------------------- 891
hrchi60B5       GTTCCTGCAGACCTGGAAGTTCTTCGATGGCGTGGATATCGACTGGGAGTTCCCG----- 951
hrchi60B20      GTTCCTGCAGACCTGGAAGTTCTTCGATGGCGTGGATATCGACTGGGAGTTCCCGGGCGG 956
hrchi60B1       GTTCCTGCAGACCTGGAAGTTCTTCGATGGCGTGGATATCGACTGGGAGTATCCGCAGGC 956
hrchi60B24      CCTTCTACGGCCGCG-----CCTTCAAGGGCGTCAGC----------------------- 928
                  * ** * * *  *      **** * *****

hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      CAAAGGCGCCAACCCGAACCTGGGCAGCCCGCAGGACGGGGAAACCTATGTGCTGCTGAT 1016
hrchi60B1       GGCGGAAGTGGACGGTTTCATCGCCGCGCTGCAGGAGATCCGCACCTTGCTGAACCAGCA 1016
hrchi60B24      ------------------------------------------------------------


hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      GAAGGAGCTGCGGGCGATGCTGGATCAGCTGTCGGCGGAAACCGGCCGCAAATATGAACT 1076
hrchi60B1       AACCATCGCGGACGGCCGCCAGGCGTTGCCGTATCAGCTGACCATCGCCGGCGCCGGCGG 1076
hrchi60B24      ------------------------------------------------------------
```

Figure 3.27 Nucleotide sequence alignment of chimeric chitinase genes of pHRCHI60B: hrchi60B1, 3, 5, 20 and 24 (continued)

```
hrchi60B3       TTCTTCCTGTCGCGCTATTACAGCAAGCTGGCGCAAATCGTCGCGCCACTCGATTACATC 1140
hrchi60B24      ------------------------------------------------------------
hrchi60B1       TTCTTCCTGTCGCGCTATTACAGCAAGCTGGCGCAAATCGTCGCGCCACTCGATTACATC 1140
hrchi60B5       ------------------------------------------------------------
hrchi60B20      TCCGCCATCAGCGCCGGCAAGGACAAGATCGATAAGGTGGCTTACAACGTTGCGCAGAAC 1140

hrchi60B3       AACCTGATGACCTACGATCTGGCCGGCCCCTGGGAGAAGATCACCAACCACCAGGCGGCG 1200
hrchi60B24      ------------------------------------------------------------
hrchi60B1       AACCTGATGACCTACGATCTGGCCGGCCCCTGGGAGAAGATCACCAACCACCAGGCGGCG 1200
hrchi60B5       ------------------------------------------------------------
hrchi60B20      TCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAAGAAC 1200

hrchi60B3       CTGTTCGGCGACGCGGCCGGG---------------------------------------- 1221
hrchi60B24      ------------------------------------------------------------
hrchi60B1       CTGTTCGGCGACGCGGCCGGG---------------------------------------- 1221
hrchi60B5       ------------------------------------------------------------
hrchi60B20      CTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACACCACG 1260

hrchi60B3       ------------------------------------------------------------
hrchi60B24      ------------------------------------------------------------
hrchi60B1       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      GTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAGATCGTGGCTGCA 1320

hrchi60B3       ------------------------------------------------------------
hrchi60B24      ------------------------------------------------------------
hrchi60B1       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      GGAGATCCGCACCTTGCTGAACCAGCAAACCATCGCGGACGGCCGCCAGGCGTTGCCGTA 1380

hrchi60B3       ------------------------------------------------------------
hrchi60B24      ------------------------------------------------------------
hrchi60B1       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      TCAGCTGACCATCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATTACAGCAAGCT 1440
```

Figure 3.27 Nucleotide sequence alignment of chimeric chitinase genes of pHRCHI60B: hrchi60B1, 3, 5, 20 and 24 (continued)

```
hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      GACCTCCGCCATCAGCGCCGGCAAGGACAAGATCGATAAGGTGGCTTACAACGTTGCGCA 1136
hrchi60B1       CGCCTTCTTCCTGTCGCGCTATTACAGCAAGCTGGCGCAAATCGTCGCGCCACTCGATTA 1136
hrchi60B24      ------------------------------------------------------------

hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      GAACTCGATGGATCACATCTTCCTGATGAGCTACGACTTCTATGGCGCCTTCGATCTGAA 1196
hrchi60B1       CATCAACCTGATGACCTACGATCTGGCCGGCCCCTGGGAGAAGATCACCAACCACCAGGC 1196
hrchi60B24      ------------------------------------------------------------

hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      GAACCTGGGGCATCAGACCGCGCTGAATGCGCCGGCCTGGAAGCCGGACACCGCTTACAC 1256
hrchi60B1       GGCGCTGTTCGGCGACGCGGCCGGGCCGACCTTCTACAACGCACTGCGCGAAGCCAATCT 1256
hrchi60B24      ------------------------------------------------------------

hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      CACGGTGAACGGCGTCAATGCGCTGCTGGCGCAGGGCGTCAAGCCGGGCAAGATCGTGGC 1316
hrchi60B1       GGGCTGGAGCTGGGAAGAGCTGACTCGCGCCTTCCCCAGCCCGTTCAGCCTGACGGTCGA 1316
hrchi60B24      ------------------------------------------------------------

hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      TGCAGGAGATCCGCACCTTGCTGAACCAGCAAACCATCGCGGACGGCCGCCAGGCGTTGC 1376
hrchi60B1       CGCCGCCGTGCAGCAGCACCTGATGATGGAAGGCGTGCCGAGCGCCAAAATCGTCATGGG 1376
hrchi60B24      ------------------------------------------------------------

hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      CGTATCAGCTGACCATCGCCGGCGCCGGCGGCGCCTTCTTCCTGTCGCGCTATTACAGCA 1436
hrchi60B1       CGTGCCCTTCTACGGCCGCGCCTTCAAGGGCGTCAGC---------------------- 1413
hrchi60B24      ------------------------------------------------------------
```

Figure 3.27 Nucleotide sequence alignment of chimeric chitinase genes of pHRCHI60B: hrchi60B1, 3, 5, 20 and 24 (continued)

```
hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      AGCTGGCGCAAATCGTCGCGCCACTCGATTACATCAACCTGATGACCTACGATCTGGCCG 1496
hrchi60B1       ------------------------------------------------------------
hrchi60B24      ------------------------------------------------------------

hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      GCCCCTGGGAGAAGATCACCAACCACCAGGCGGCGCTGTTCGGCGACGCGGCCGGGCCGA 1556
hrchi60B1       ------------------------------------------------------------
hrchi60B24      ------------------------------------------------------------

hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      CCTTCTACAACGCACTGCGCGAAGCCAATCTGGGCTGGAGCTGGGAAGAGCTGACTCGCG 1616
hrchi60B1       ------------------------------------------------------------
hrchi60B24      ------------------------------------------------------------

hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      CCTTCCCCAGCCCGTTCAGCCTGACGGTCGACGCCGCCGTGCAGCAGCACCTGATGATGG 1676
hrchi60B1       ------------------------------------------------------------
hrchi60B24      ------------------------------------------------------------

hrchi60B3       ------------------------------------------------------------
hrchi60B5       ------------------------------------------------------------
hrchi60B20      AAGGCGTGCCGAGCGCCAAAATCGTCATGGGCGTGCCCTTCTACGGCCGCGCCTTCAAGG 1736
hrchi60B1       ------------------------------------------------------------
hrchi60B24      ------------------------------------------------------------

hrchi60B3       --------GGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACGCCGGGCGAAGATCCGT 943
hrchi60B5       --------GGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACGCCGGGCGAAGATCCGT 1003
hrchi60B20      GCGTCAGCGGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACGCCGGGCGAAGATCCGT 1796
hrchi60B1       --------GGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACGCCGGGCGAAGATCCGT 1465
hrchi60B24      --------GGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACGCCGGGCGAAGATCCGT 980
                        **************************************************
```

Figure 3.27 Nucleotide sequence alignment of chimeric chitinase genes of pHRCHI60B: hrchi60B1, 3, 5, 20 and 24 (continued)

```
hrchi60B3       ATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTGCGCGACAAGGATCCGC 1003
hrchi60B5       ATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTGCGCGACAAGGATCCGC 1063
hrchi60B20      ATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTGCGCGACAAGGATCCGC 1856
hrchi60B1       ATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTGCGCGACAAGGATCCGC 1525
hrchi60B24      ATCCGAACGCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTGCGCGACAAGGATCCGC 1040
                ************************************************************

hrchi60B3       GCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAACTACGGCTATCAGCGGT 1063
hrchi60B5       GCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAACTACGGCTATCAGCGGT 1123
hrchi60B20      GCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAACTACGGCTATCAGCGGT 1916
hrchi60B1       GCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAACTACGGCTATCAGCGGT 1585
hrchi60B24      GCATCGCCTCCTATCGCCAGCTGGAGCAGATGCTGCAGGGCAACTACGGCTATCAGCGGT 1100
                ************************************************************

hrchi60B3       TGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCCCAGAACGGGCTGTTTGTCA 1123
hrchi60B5       TGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCCCAGAACGGGCTGTTTGTCA 1183
hrchi60B20      TGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCCCAGAACGGGCTGTTTGTCA 1976
hrchi60B1       TGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCCCAGAACGGGCTGTTTGTCA 1645
hrchi60B24      TGTGGAACGATAAGACCAAAACCCCGTATCTGTATCATGCCCAGAACGGGCTGTTTGTCA 1160
                ************************************************************

hrchi60B3       CCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATCAAGCAGCAGCAGCTGG 1183
hrchi60B5       CCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATCAAGCAGCAGCAGCTGG 1243
hrchi60B20      CCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATCAAGCAGCAGCAGCTGG 2036
hrchi60B1       CCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATCAAGCAGCAGCAGCTGG 1705
hrchi60B24      CCTATGACGATGCCGAGAGCTTCAAATACAAAGCGAAGTACATCAAGCAGCAGCAGCTGG 1220
                ************************************************************

hrchi60B3       GCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGCGATCTGCTGGCCGCGC 1243
hrchi60B5       GCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGCGATCTGCTGGCCGCGC 1303
hrchi60B20      GCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGCGATCTGCTGGCCGCGC 2096
hrchi60B1       GCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGCGATCTGCTGGCCGCGC 1765
hrchi60B24      GCGGCGTAATGTTCTGGCATTTGGGGCAAGACAACCGCAACGGCGATCTGCTGGCCGCGC 1280
                ************************************************************

hrchi60B3       TGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTGGATATGGGCACCGGCC 1303
hrchi60B5       TGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTGGATATGGGCACCGGCC 1363
hrchi60B20      TGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTGGATATGGGCACCGGCC 2156
hrchi60B1       TGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTGGATATGGGCACCGGCC 1825
hrchi60B24      TGGATCGCTATTTCAACGCCGCAGACTACGACGACAGCCAGCTGGATATGGGCACCGGTC 1340
                ************************************************************ *
```

Figure 3.27 Nucleotide sequence alignment of chimeric chitinase genes of pHRCHI60B: hrchi60B1, 3, 5, 20 and 24 (continued)

```
hrchi60B3       TGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACCGCGCCGGCCTATGTGC 1363
hrchi60B5       TGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACCGCGCCGGCCTATGTGC 1423
hrchi60B20      TGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACCGCGCCGGCCTATGTGC 2216
hrchi60B1       TGCGATATACCGGCGTCGGCCCCGGCAATCTGCCGATCATGACCGCGCCGGCCTATGTGC 1885
hrchi60B24      TGCGATATACCGGCGTCGGCCCCGGCAACCTGCCGATCATGACCGCGCCGGCCTATGTGC 1400
                *************************** ******************************

hrchi60B3       CGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGCTACGTCTGGCAGACCA 1423
hrchi60B5       CGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGCTACGTCTGGCAGACCA 1483
hrchi60B20      CGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGCTACGTCTGGCAGACCA 2276
hrchi60B1       CGGGAACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGCTACGTCTGGCAGACCA 1945
hrchi60B24      CGGGCACCACTTACGCCCAGGGCGCGCTGGTGTCCTACCAAGGCTACGTCTGGCAGACCA 1460
                **** ****************************************************

hrchi60B3       AGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGGCTGAAGGTGGGCCGCC 1483
hrchi60B5       AGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGGCTGAAGGTGGGCCGCC 1543
hrchi60B20      AGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGGCTGAAGGTGGGCCGCC 2336
hrchi60B1       AGTGGGGTTATATCACCTCGGCGCCCGGCTCAGACAGCGCCTGGCTGAAGGTGGGCCGCC 2005
hrchi60B24      AGTGGGGTTATATCACCTCGGCGCCCGGCTCAGGCAGCGCCTGGCTGAAGGTGGGCCGCC 1520
                ******************************** *************************

hrchi60B3       TGGCGTAA 1491
hrchi60B5       TGGCGTAA 1551
hrchi60B20      TGGCGTAA 2344
hrchi60B1       TGGCGTAA 2013
hrchi60B24      TGGCGTAA 1528
                ********
```

Figure 3.27 Nucleotide sequence alignment of chimeric chitinase genes of pHRCHI60B: hrchi60B1, 3, 5, 20 and 24 (continued)

Nucleotide sequences of pHRCHI60B1, 3, 5, 20 and 24 were analyzed to find recombining site by using BLAST. Figure 3.28 shows the graphical view of recombination site between *chi60* and *chiB* of each chimera. After that, gene sequences were then translated to amino acid sequences using EMBOSS Transeq program which showed that pHRCHI60B20 and pHRCHI60B24 had single base frame-shifted and could not translate through the entire gene. Amino acid sequences of HRCHI60B1, 3 and 5 were also sent to BLAST to find recombining regions. Recombined regions obtained from nucleotide blast and protein blast of HRCHI60B1, 3 and 5 are shown in figure 3.29, 3.30 and 3.31, respectively.

3. Three dimensional structure prediction of HRCHI60B1, HRCHI60B3 and HRCHI60B5

Amino acid sequences of HRCHI60B1, HRCHI60B3 and HRCHI60B5 were submitted to HHpred interactive server for structure prediction. Predicted 3D structure of HRCHI60B3, HRCHI60B5 and HRCHI60B1 were visualized by Rasmol version 2.7.4.2 as shown in figure 3.32, 3.33 and 3.34 respectively. Part of Chi60 structure is displayed in blue and part of ChiB is displayed in yellow. The conserved active site motif of family 18 chitinase is shown in red color which is not observed in HRCHI60B3.

4. Expression of HRCHI60B

pHRCHI60B1-77 and pHRCHI60BM1-6 were retransformed into *E. coli* DH5α and cultured in LB broth containing 100 μg/ml ampicillin for 3 days in incubator shaker at 37°C. Chitinases in supernatant were collected by centrifugation at 5,000 g for 10 minutes at 4°C and prepared for chitinase activity assay. Figure 3.35 shows protein expression of HRCHI60B3, HRCHI60B5 and HRCHI60B1 analyzed by SDS-PAGE.

5. Chitinase activity assay of HRCHI60B

5.1 Determination of chitinase activity by measuring reducing sugar

Chitinolytic activity of all chimeras was assayed as described by Imoto and Yagishita (1971). With transformants of pCHI60B as positive control and pBS/SK$^-$ as negative control, activity was first assayed under phosphate buffer pH 6.0

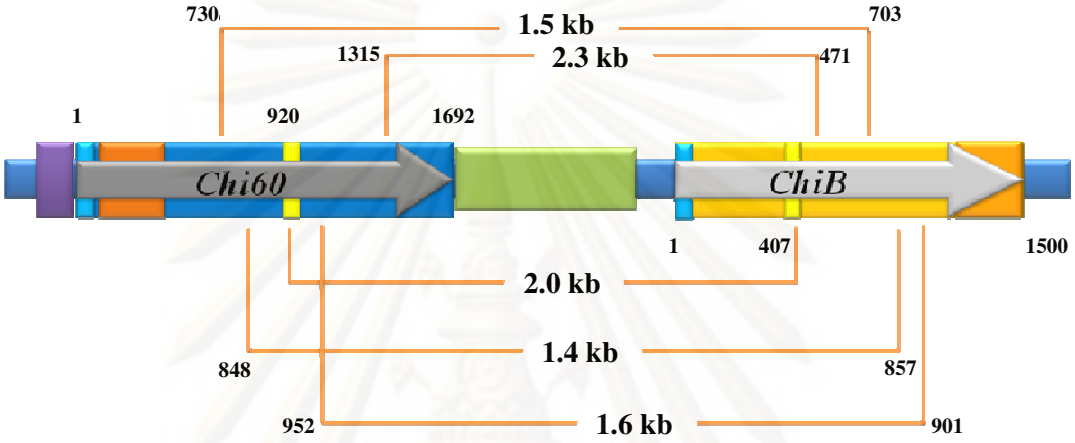


Figure 3.28 Graphical view of recombination site between *chi60* and *chiB* of each chimeric gene size

represents promoter of chi60

represents coding sequence of signal peptide

represents coding sequence of active site

Query: hrchi60B1 Molecule type: nucleic acid Query Length: 2013

Serratia sp. TU09 chitinase (Chi60) gene, complete cds

Length=2847

```
Query  841   TTCTTCTTCATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1170  TTCTTCTTCATGGGCGATAAGGTGAAGCGCGATCGCTTCGTCGGTTCGGTGAAAGAGTTC  1229

Query  901   CTGCAGACCTGGAAGTTCTTCGATGGCGTGGATATCGACTGGGAGTATCCGCAGGCGGC  959
             |||||||||||||||||||||||||||||||||||||||||||| ||| | ||||||
Sbjct  1230  CTGCAGACCTGGAAGTTCTTCGATGGCGTGGATATCGACTGGGAGT-TCC-CGGGCGGC  1286
```

Serratia marcescens chiB gene for chitinase B (EC 3.2.1.14)

Length=1500

```
Query  918   CTTCGATGGCGTGGATATCGACTGGGAGTATCCGCAGGCGGCGGAAGTGGACGGTTTCAT  977
             |||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  405   CTTCGACGGCGTGGACATCGACTGGGAGTATCCGCAGGCGGCGGAAGTGGACGGTTTCAT  464

Query  978   CGCCGCGCTGCAGGAGATCCGCACCTTGCTGAACCAGCAAACCATCGCGGACGGCCGCCA  1037
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  465   CGCCGCGCTGCAGGAGATCCGCACCTTGCTGAACCAGCAAACCATCGCGGACGGCCGCCA  524
```

Query: HRCHI60B1 Molecule type: amino acid Query Length: 670

Chitinase [Serratia sp. TU09]Length=563

```
Query  241  GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF  300
            GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF
Sbjct  241  GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF  300

Query  301  LQTWKFFDGVDIDWEYP----------QAAEVDGFIAALQEIRTLLNQQTIADGRQALPY  350
            LQTWKFFDGVDIDWE+P             + + ++  ++E+R +L+Q +   GR+   Y
Sbjct  301  LQTWKFFDGVDIDWEFPGGKGANPNLGSPQDGETYVLLMKELRAMLDQLSAETGRK---Y  357
```

Chitinase B [Serratia marcescens]Length=499

```
Query  304  WKF------------FDGVDIDWEYPQAAEVDGFIAALQEIRTLLNQQTIADGRQALPYQ  351
             KF            FDGVDIDWEYPQAAEVDGFIAALQEIRTLLNQQTI DGRQALPYQ
Sbjct  120  AKFAQSCVRIMKDYGFDGVDIDWEYPQAAEVDGFIAALQEIRTLLNQQTITDGRQALPYQ  179

Query  352  LTIAGAGGAFFLSRYYSKLAQIVAPLDYINLMTYDLAGPWEKITNHQAALFGDAAGPTFY  411
            LTIAGAGGAFFLSRYYSKLAQIVAPLDYINLMTYDLAGPWEK+TNHQAALFGDAAGPTFY
Sbjct  180  LTIAGAGGAFFLSRYYSKLAQIVAPLDYINLMTYDLAGPWEKVTNHQAALFGDAAGPTFY  239
```

>HRCHI60B1

MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNSLVKVKNAADVSVSWNLWNGDTGTT
AKVLLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGCSASDATEIVVADTDGSHLAPLKEPLLEKNKPY
KQNSGKVVGSYFVEWGVYGRNFTVDKIPAQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSI
HDPFAALQKAQKGVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEFLQTW
KFFDGVDIDWEYPQAAEVDGFIAALQEIRTLLNQQTIADGRQALPYQLTIAGAGGAFFLSRYYSKLAQIVAPLDYI
NLMTYDLAGPWEKITNHQAALFGDAAGPTFYNALREANLGWSWEELTRAFPSPFSLTVDAAVQQHLMMEGVPSAKI
VMGVPFYGRAFKGVSGGNGGQYSSHSTPGEDPYPNADYWLVGCDECVRDKDPRIASYRQLEQMLQGNYGYQRLWND
KTKTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQQLGGVMFWHLGQDNRNGDLLAALDRYFNAADYDDSQLDMGTG
LRYTGVGPGNLPIMTAPAYVPGTTYAQGALVSYQGYVWQTKWGYITSAPGSDSAWLKVGRLA

Figure 3.29 Recombination site of HRCHI60B1

Fasta format of deduced amino acid sequence is displayed below. The underlined sequences are recombination site between Chi60 and light grey highlighted ChiB. The dark grey highlighted sequences with white alphabets represent ChBD of ChiB.

Query: hrchi60B3 Molecule type: nucleic acid Query Length: 1491

Serratia sp. TU09 chitinase (Chi60) gene, complete cds

Length=2847

```
Query  781   CAGGCGCATCCTGACCTGAAAATTCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCG  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1110  CAGGCGCATCCTGACCTGAAAATTCTGCCGTCGATCGGCGGCTGGACGCTGTCCGACCCG  1169

Query  841   TTCTTCTTCATGGGCG  856
             ||||||||||||||||
Sbjct  1170  TTCTTCTTCATGGGCG  1185
```

Serratia marcescens chiB gene for chitinase B (EC 3.2.1.14)

Length=1700

```
Query  848   TCATGGGCGTGCCCTTCTACGGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCC  907
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  857   TCATGGGCGTGCCCTTCTACGGCCGCGCCTTCAAGGGCGTCAGCGGCGGCAACGGCGGCC  916

Query  908   AGTACAGCAGCCACAGCACGCCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGG  967
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  917   AGTACAGCAGCCACAGCACGCCGGGCGAAGATCCGTATCCGAACGCCGATTACTGGCTGG  976
```

Query: HRCHI60B3 Molecule type: amino acid Query Length: 496

Chitinase [Serratia sp. TU09]Length=563

```
Query  181  AQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDPFAALQKAQK  240
            AQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDPFAALQKAQK
Sbjct  181  AQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSIHDPFAALQKAQK  240

Query  241  GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMG  285
            GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMG
Sbjct  241  GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMG  285
```

Chitinase B [Serratia marcescens]Length=499

```
Query  284  MGVPFYGRAFKGVSGGNGGQYSSHSTPGEDPYPNADYWLVGCDECVRDKDPRIASYRQLE  343
            MGVPFYGRAFKGVSGGNGGQYSSHSTPGEDPYPNADYWLVGCDECVRDKDPRIASYRQLE
Sbjct  287  MGVPFYGRAFKGVSGGNGGQYSSHSTPGEDPYPNADYWLVGCDECVRDKDPRIASYRQLE  346

Query  344  QMLQGNYGYQRLWNDKTKTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQQLGGVMFWHLG  403
            QMLQGNYGYQRLWNDKTKTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQQLGGVMFWHLG
Sbjct  347  QMLQGNYGYQRLWNDKTKTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQQLGGVMFWHLG  406
```

>HRCHI60B3

MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNSLVKVKNAADVSVSWNLWNGDTGTT
AKVLLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGCSASDATEIVVADTDGSHLAPLKEPLLEKNKPY
KQNSGKVVGSYFVEWGVYGRNFTVDKIPAQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSI
HDPFAALQKAQKGVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGVPFYGRAFKGVSGGNGGQY
SSHSTPGEDPYPNADYWLVGCDECVRDKDPRIASYRQLEQMLQGNYGYQRLWNDKTKTPYLYHAQNGLFVTYDDAE
SFKYKAKYIKQQQLGGVMFWHLGQDNRNGDLLAALDRYFNAADYDDSQLDMGTGLRYTGVGPGNLPIMTAPAYVPG
TTYAQGALVSYQGYVWQTKWGYITSAPGSDSAWLKVGRLA

Figure 3.30 Recombination site of HRCHI60B3

Fasta format of deduced amino acid sequence is displayed below. The underlined sequences are recombination site between Chi60 and light grey highlighted ChiB. The dark grey highlighted sequences with white alphabets represent ChBD of ChiB.

Query: hrchi60B5 Molecule type: nucleic acid Query Length: 1551

Serratia sp. TU09 chitinase (Chi60) gene, complete cds

Length=2847

```
Query  901   CTGCAGACCTGGAAGTTCTTCGATGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1230  CTGCAGACCTGGAAGTTCTTCGATGGCGTGGATATCGACTGGGAGTTCCCGGGCGGCAAA  1289

Query  961   GGCGGCCA  968
             |||| |||
Sbjct  1290  GGCG-CCA  1296
```

Serratia marcescens chiB gene for chitinase B (EC 3.2.1.14)

Length=1700

```
Query  952   GGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACGCCGGGCGAAGATCCGTATCCGAAC  1011
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   GGCGGCAACGGCGGCCAGTACAGCAGCCACAGCACGCCGGGCGAAGATCCGTATCCGAAC  960

Query  1012  GCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTGCGCGACAAGGATCCGCGCATCGCC  1071
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   GCCGATTACTGGCTGGTGGGCTGCGACGAGTGCGTGCGCGACAAGGATCCGCGCATCGCC  1020
```

Query: HRCHI60B5 Molecule type: amino acid Query Length: 516

Chitinase [Serratia sp. TU09]Length=563

```
Query  241  GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF  300
            GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF
Sbjct  241  GVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEF  300

Query  301  LQTWKFFDGVDIDWEFPGGNG  321
            LQTWKFFDGVDIDWEFPGG G
Sbjct  301  LQTWKFFDGVDIDWEFPGGKG  321
```

Chitinase B [Serratia marcescens]Length=499

```
Query  318  GGNGGQYSSHSTPGEDPYPNADYWLVGCDECVRDKDPRIASYRQLEQMLQGNYGYQRLWN  377
            GGNGGQYSSHSTPGEDPYP+ DYWLVGC+ECVRDKDPRIASYRQLEQMLQGNYGYQRLWN
Sbjct  301  GGNGGQYSSHSTPGEDPYPSTDYWLVGCEECVRDKDPRIASYRQLEQMLQGNYGYQRLWN  360

Query  378  DKTKTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQQLGGVMFWHLGQDNRNGDLLAALDR  437
            DKTKTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQQLGGVMFWHLGQDNRNGDLLAALDR
Sbjct  361  DKTKTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQQLGGVMFWHLGQDNRNGDLLAALDR  420
```

>HRCHI60B5

MRKFNKPLLALLIGSTLCSAAQAAAPGKPTIAWGNTKFAIVEVDQAATAYNSLVKVKNAADVSVSWNLWNGDTGTT
AKVLLNGKEAWSGPSTGSSGTANFKVNKGGRYQMQVALCNADGCSASDATEIVVADTDGSHLAPLKEPLLEKNKPY
KQNSGKVVGSYFVEWGVYGRNFTVDKIPAQNLTHLLYGFIPICGGNGINDSLKEIEGSFQALQRSCQGREDFKVSI
HDPFAALQKAQKGVTAWDDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVGSVKEFLQTW
KFFDGVDIDWEFPGGNGGQYSSHSTPGEDPYPNADYWLVGCDECVRDKDPRIASYRQLEQMLQGNYGYQRLWNDKT
KTPYLYHAQNGLFVTYDDAESFKYKAKYIKQQQLGGVMFWHLGQDNRNGDLLAALDRYFNAADYDDSQLDMGTGLR
YTGVGPGNLPIMTAPAYVPGTTYAQGALVSYQGYVWQTKWGYITSAPGSDSAWLKVGRLA

Figure 3.31 Recombination site of HRCHI60B5

Fasta format of deduced amino acid sequence is displayed below. The underlined sequences are recombination site between Chi60 and light grey highlighted ChiB. The dark grey highlighted sequences with white alphabets represent ChBD of ChiB.

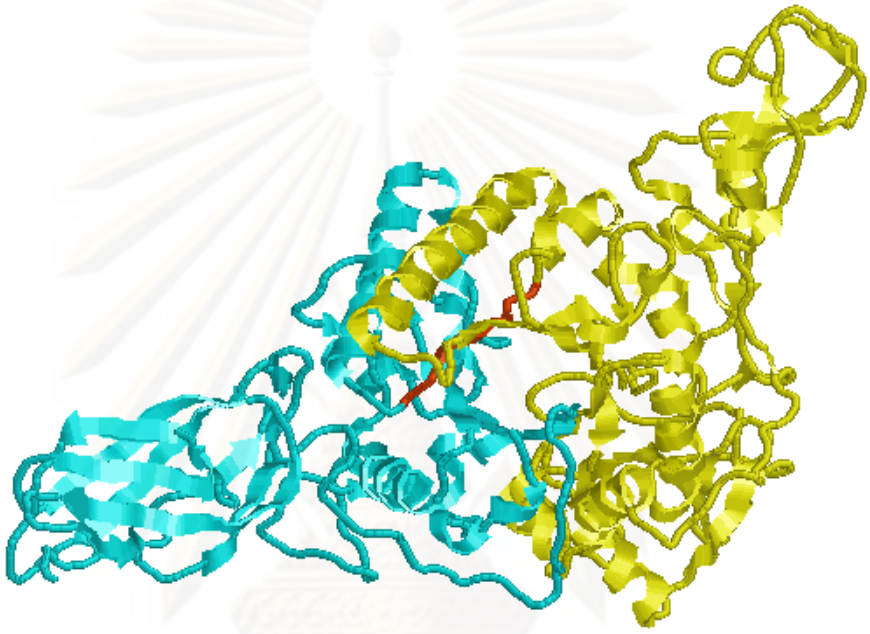


Figure 3.32 Homology modeling structure of HRCHI60B1

Structure was visualized by Rasmol version 2.7.4.2. Blue color indicates structure deduced from Chi60 sequences and yellow color indicates structure deduced from ChiB sequences. Conserved motif in family 18 active site (FDGVDIDWE) is displayed in red.

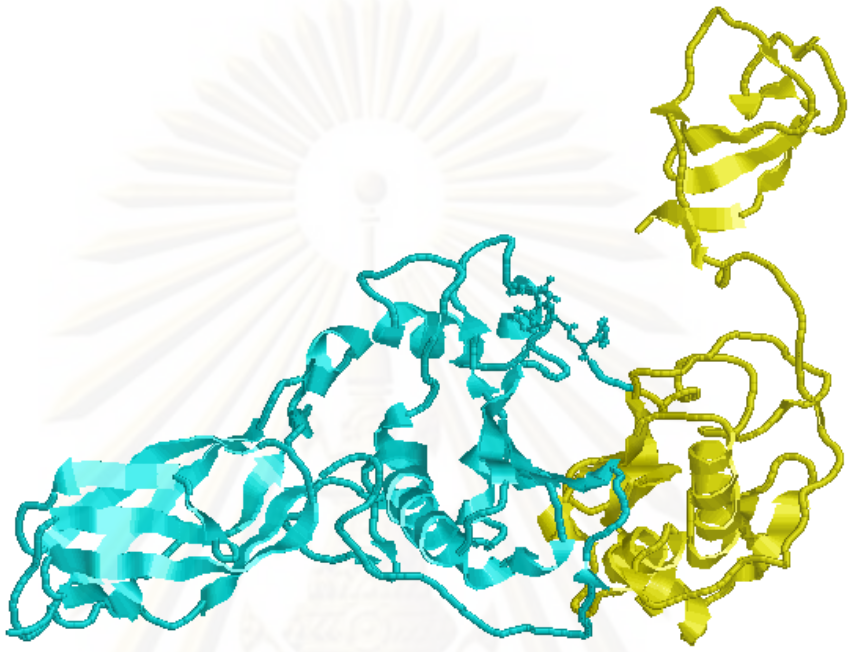


Figure 3.33 Homology modeling structure of HRCHI60B3

Structure was visualized by Rasmol version 2.7.4.2. Blue color indicates structure deduced from Chi60 sequences and yellow color indicates structure deduced from ChiB sequences. Conserved motif in family 18 active site (FDGVDIDWE) is absent in HRCHI60B3.

Figure 3.34 Homology modeling structure of HRCHI60B5

Structure was visualized by Rasmol version 2.7.4.2. Blue color indicates structure deduced from Chi60 sequences and yellow color indicates structure deduced from ChiB sequences. Conserved motif in family 18 active site (FDGVDIDWE) is displayed in red

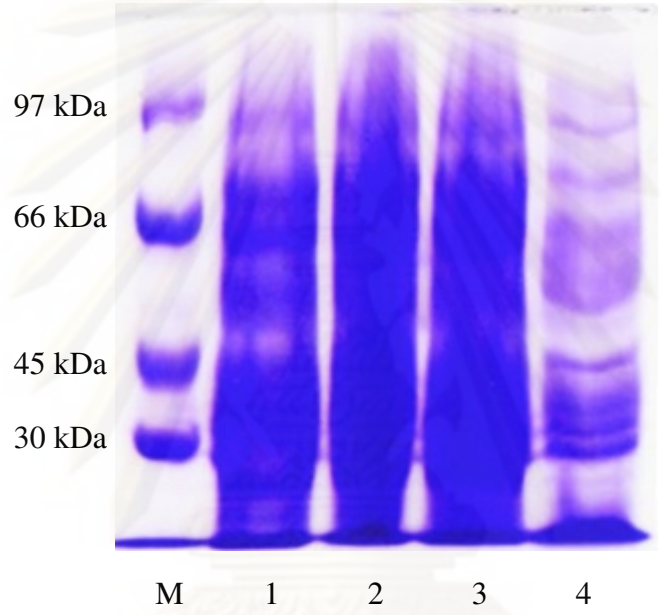


Figure 3.35 SDS-PAGE analysis of crude HRCHI60B3, HRCHI60B5 and HRCHI60B1

Lane M Standard protein marker

Lane 1 HRCHI60B3

Lane 2 HRCHI60B5

Lane 3 HRCHI60B1

Lane 5 pBS/SK$^-$ in *E. coli* DH5α

and incubated at 37°C for 60 min using PNAC as soluble chitin substrate and colloidal chitin as insoluble substrate. Unfortunately, all chimeras showed no chitinolytic activity under this condition including HRCHI60B76 and 77 that were screened by hydrolytic activity assay. Buffering system was also changed to citrate buffer pH 4.0 and Tris-HCl buffer pH 8.0 and changing of expression host to *E. coli* Origami (DE3) was also tried, but there were still no activity observed.

5.2 Determination of chitinase activity of HRCHI60B by TLC

Chitinase activity with chitooligosaccharides substrate of HRCHI60B3, HRCHI60B5 and HRCHI60B1 were assayed in phosphate buffer pH 6.0. Products of reaction were detected by TLC which indicated that there was no product observed as shown in figure 3.36.

Mutation mediated by ultraviolet radiation of HRCD66B1

With an attempt to find functional chimeric chitinase, pHRCD66B1 was transformed into *E. coli* DH5α and 100 μl aliquots were spread onto 6 plates of CCMM agar plate with ampicillin. Cultured plates were then radiated with UV for 0, 5, 10, 30, 50, and 80 seconds and incubated at 37°C for 12-16 hours. Result showed that only cultured plate radiated with UV for 5 seconds was found to have 5 forming colonies but no clear zone was observed. These five colonies were then expressed and assay for chitinolytic activity by the same procedure as previously mentioned. Unfortunately, all mutants still showed no chitinolytic activity.

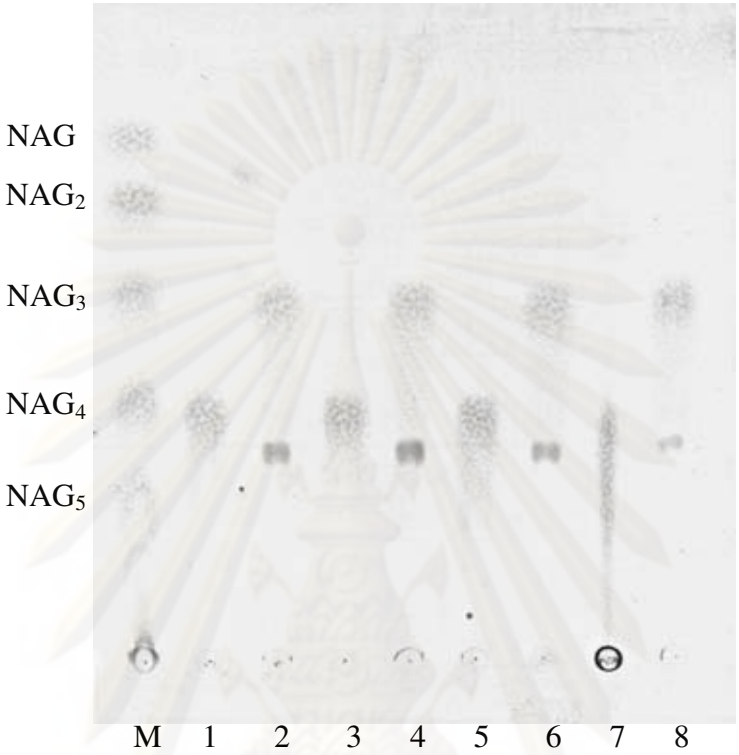


Figure 3.36 Hydrolytic products of HRCHI60B3, HRCHI60B5 and HRCHI60B1 determined by TLC method

Lane M Standard mixture of NAG, NAG_2, NAG_3, NAG_4 and NAG_5

Lane 1 Reaction of pBS/SK^- in *E. coli* DH5α with NAG_4

Lane 2 Reaction of pBS/SK^- in *E. coli* DH5α with NAG_3

Lane 3 Reaction of HRCHI60B3 with NAG_4

Lane 4 Reaction of HRCHI60B3 with NAG_3

Lane 5 Reaction of HRCHI60B5 with NAG_4

Lane 6 Reaction of HRCHI60B5 with NAG_3

Lane 7 Reaction of HRCHI60B1 with NAG_4

Lane 8 Reaction of HRCHI60B1 with NAG_3

CHAPTER IV

DISCUSSION

Construction of chimeric chitinases

1. HRCD66B screened from LB agar plate with ampicillin

Screening of chimeras with this method is a phenotypic screening that forced transformants to repair linearized plasmid with ampicillin resistance gene in order to survive on ampicillin media. At the first screening, we found 12 out of 39 colonies that contained appropriated size of chimeric chitinases genes and four of these appeared to be wild-type pCD66B that might be an incompletely digested background from the linearized pCD66B preparation step. At second screening, we observed a lot more 86 colonies that could survive on LB with ampicillin, but most of them contained unrecognizable plasmids as shown in appendix B and C. Only 8 colonies were found to have plasmids with appropriated gene size and one of them was pCD66B. These results suggested that around 1 μg of linearized plasmid could produce around 8 forming colonies with proper chimeric genes. Further nucleotide sequencing and sequences alignment of these chimeras (figure 3.12) revealed that there were actually 5 individuals with gene size of 1.34, 1.57, 1.65, 1.67 and 1.68 kb as shown in group in table 3.1.

2. HRCD66B screened from CCMM broth with ampicillin

Screening with CCMM method was carried out for more specifically phenotypic screening. Compared with positive and negative control, 2 out of 12 aliquots clearly showed a potential to grow in CCMM broth with ampicillin. One of these contained unrecognizable plasmid while another one contained pHRCD66BM1 which had chimeric gene size of 1.76 kb. Interestingly, nucleotide sequence of hrcd66Bm1, which was also aligned with other hrcd66Bm shown in figure 3.12, was found to be unique from those screened by LB agar plate screening method. This type of recombination might be involved in the ability to grow in CCMM media so that analysis of sequences was studied.

3. HRCHI60B screened from LB agar plate with ampicillin

Screening of HRCHI60B with this method has significant differences compare with HRCD66B screening. At the first screening, we found 26 out of 46 colonies that contained appropriated size of chimeric chitinases genes with no background of pCHI60B but at the second screening, we observed a lot more 228 colonies that could survive on LB with ampicillin from the same amount of around 1 µg of linearized plasmid. At first glance we thought that most of them should be the undigested pCHI60B background, so that 68 colonies were randomly picked and plasmids were extracted. Surprisingly, 49 of them were found to have plasmids with appropriated gene size. The difference in ratio of forming colony might occur because of the decreasing in efficiency of *E. coli* JC8679 competent cell. Strategy for screening the other 160 colonies was then changed in order to find chimeras with functional chitinases. Result from roughly measuring hydrolytic activity of supernatant from 160 colonies in table 3.2 shows that ΔA_{420} value of colony 111 and 227 were 0.14 and 0.10. The activity assays were then checked by measuring 3 repetitive of ΔA_{420} values and results still showed significant differences. Plasmids from this step of screening were collected and grouped by similarity of gene size.

4. HRCHI60B screened from CCMM broth with ampicillin

Screening of HRCHI60B with CCMM method was carried out and also found to have some problems in screening. Compared with positive and negative control, 4 out of 18 aliquots could grow in CCMM broth with ampicillin. But at the second and third screening, negative control which is the transformant of pBS/SK$^-$ in *E. coli* Top10 could grow in CCMM broth containing ampicillin. This result suggested that not only tansformants with functional chitinase but also transformants with ampicillin resistance could grow in CCMM broth containing ampicillin. Thus, the cultures were further incubated for 5 days to observe the decreasing of colloidal chitin which will indicate the culture tubes with functional chitinase. Unfortunately, positive control itself could only grow in CCMM broth containing ampicillin but no significant decrease in colloidal observed. These all results suggested that screening for HRCHI60B by CCMM with ampicillin method could only screen for chimeras with recombined plasmid with ampicillin resistance gene and this might affect the CCMM screening of HRCD66B too. Nevertheless, pHRCHIO60BM1-6 were

extracted and grouped together with pHRCHI60B1-75 screened from LB agar with ampicillin.

Sequences analysis of pHRCD66B

Nucleotide sequences and translated amino acid sequences of HRCD66B1, 6, 15 and M1 were analyzed to find recombining site by using BLAST and these results showed that some of homologous recombination occurred at homologous regions that we didn't expect. Figure 4.1 shows pairwise alignment of nucleotide sequences between CatDChi66 and CatDChiB and recombination site of each chimera was highlighted. HRCD66B1, a representative of HRCD66B3, 9, 10, 13, 14, 17, 19 and 20 that have 1.67 kb gene size, has recombined region highlighted in yellow. This region appeared to be in the most similar region between CatDChi66 and CatDChiB, moreover, these homologous sequences are encoded to FDGVDLDWE which is the active site motif of family 18 chitinase. By all means, HRCD66B1 is a chimeric chitinase constructed from homologous recombination between CatDChi66 and ChiB at the active site. Predicted structure of HRCD66B1 was successfully achieved as shown in figure 3.18 which we can see that green color of CatDChi66 are connected to yellow color of ChiB through orange color of active site and the overall structure of catalytic domain could still retain the $(\beta/\alpha)_8$ barrel structure.

Next, HRCD66B6 and 8 which have 1.68 kb gene size have recombined region highlighted in green. This region is one of the regions with high homology as detected by pairwise alignment. From protein BLAST result, this region is found to be located on C-terminal side of the active site which is clearly displayed in the predicted structure (figure 3.19). As we can see from the figure, most part of catalytic domain derived from CatDChi66 (green). This structure also shows that the overall structure of catalytic domain could still retain the $(\beta/\alpha)_8$ barrel structure too, but there is an additional loop which used to folded into N-terminal chitin binding loop in original CatDChi66. This loop might be caused by prediction program that based on the folding of CatDChiB and this might corrupt the actual folding of catalytic domain.

Next one is HRCD66B15 with 1.34 gene size. Recombined region of HRCD66B15 highlighted in red was not found in the homologous region computed

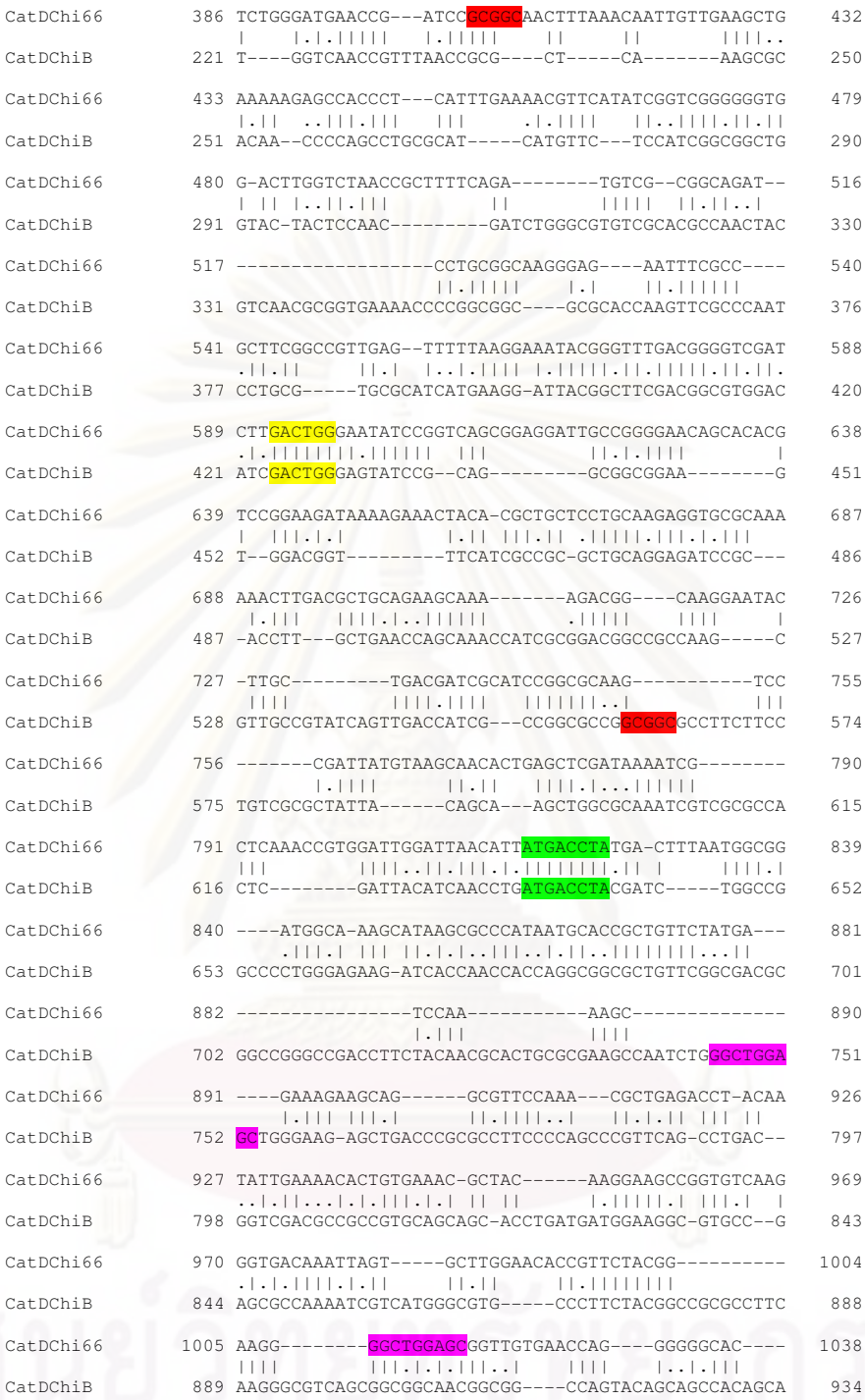


Figure 4.1 Pairwise alignment of nucleotide sequences between CatDChi66 and CatDChiB

The alignment was achieved using EMBOSS Pairwise Alignment with EMBOSS: water (local) method. Recombined region of HRCD66B1 is highlighted in yellow, recombined region of HRCD66B6 is highlighted in green, recombined region of HRCD66B15 is highlighted in red and recombined region of HRCD66BM1 is highlighted in pink.

by pairwise alignment. From the sequences alignment in figure 4.1 together with protein BLAST in figure 3.16, this recombination occurred at N-terminal of CatDChi66 and recombined with C-terminal of CatDChiB which skipped the major part including active site of the catalytic domain, resulting in the improper folding of predicted structure shown in figure 3.20.

The last one screened on CCMM is HRCD66BM1. Recombined region of HRCD66BM1 highlighted in pink was not found in the homologous region computed by pairwise alignment neither but the major part of catalytic domain was still present in CatDChi66 part. From figure 4.1, recombined region of HRCD66BM1 is located near the HRCD66B6's but the sequences of *catDchi66* at bp 1009-1017 are recombined to sequences of *chiB* at bp 745-753. This recombination leads to structure that has N-terminal loop similar to HRCD66B6 and the extra loop connected between catalytic domain and ChBD of ChiB caused by recombination as shown in figure 3.21. The $(\beta/\alpha)_8$ barrel core structure of catalytic domain seems to be properly folded with extra loop placed on top of the domain which is clearly showed by side viewed display in figure 4.2.

Sequences analysis of pHRCHI60B

Chimeric genes of pHRCHI60B1-75 and pHRCHI60BM1-6 were grouped into 5 groups with gene size of 1.4, 1.5, 1.6, 2.0 and 2.3 kb and pHRCHI60B1, 3, 5, 20 and 24 as the representatives of each group were sent for nucleotide sequencing of chimeric genes. Nucleotide sequences alignment of each chimera confirmed that each hrchi60B has its own recombined region. Since pHRCHI60B20 and pHRCHI60B24 were found to have single base frame-shifted, nucleotide sequences and translated amino acid sequences of HRCHI60B1, 3 and 5 were analyzed to find recombining site by using BLAST. Recombined region of each chimera were compared to a pairwise alignment of nucleotide sequences between CatDChi60 and CatDChiB shown in figure 4.3. HRCHI60B1 as a representative of the largest group of HRCHI60B was firstly analyzed. Recombination site of HRCHI60B1 which is highlighted in yellow appeared to be in a region encoded for active site motif of family 18 chitinase (FDGVDIDWE). Hence HRCHI60B1 is a chimeric chitinase constructed by homologous recombination between Chi60 and ChiB which recombines at the active

Figure 4.2 Three dimensional structure of HRCD66BM1

A side viewed structure was visualized by Rasmol version 2.7.4.2. Green color indicates structure deduced from CatDCHI60 sequences and yellow color indicates structure deduced from ChiB sequences. Conserved motif in family 18 active site (FDGVDLDWE) is displayed in red.

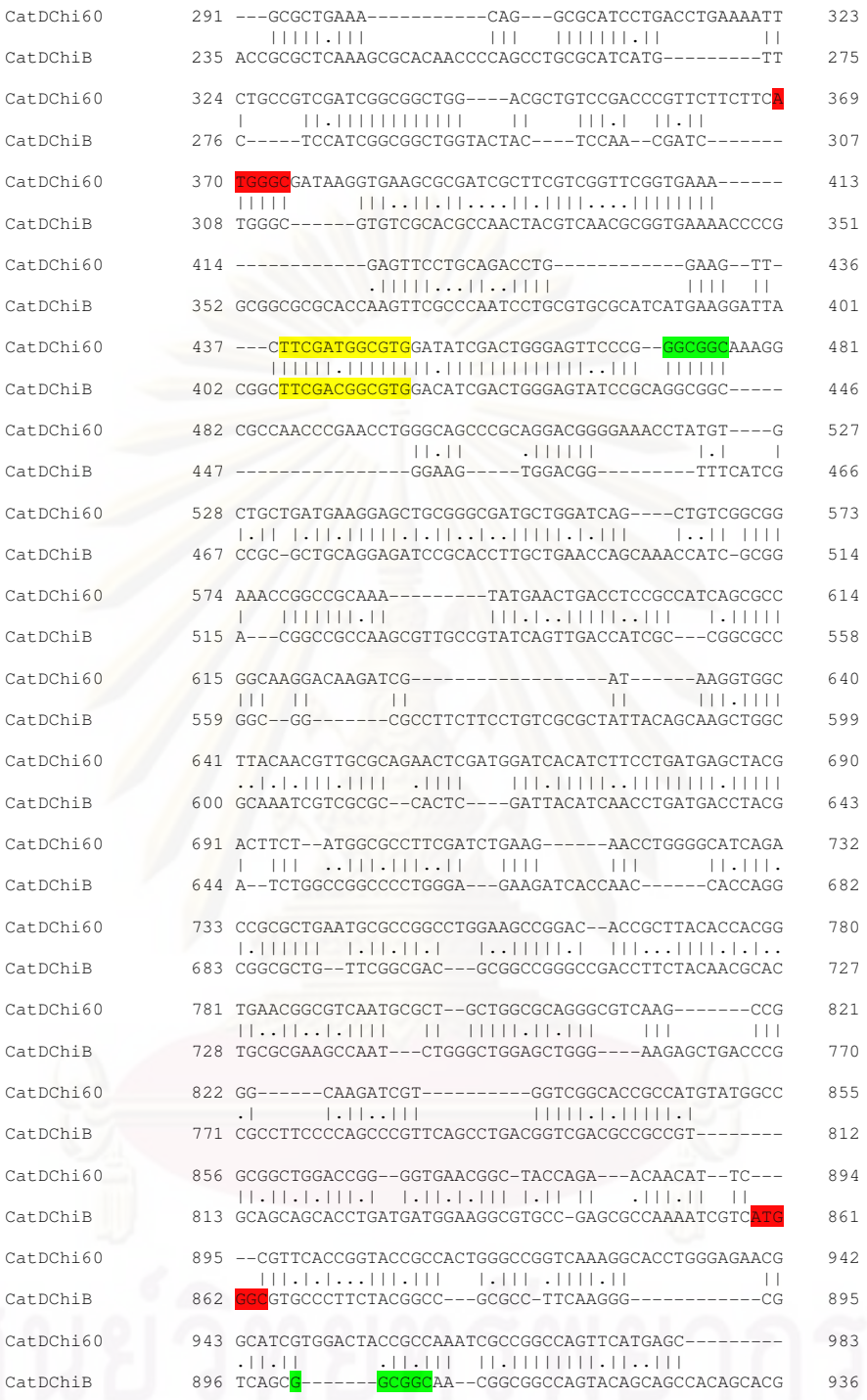


Figure 4.3 Pairwise alignment of nucleotide sequences between CatDChi60 and CatDChiB

The alignment was achieved using EMBOSS Pairwise Alignment with EMBOSS: water (local) method. Recombined region of HRCHI60B1 is highlighted in yellow, recombined region of HRCHI60B3 is highlighted in red and recombined region of HRCHI60B5 is highlighted in green.

site. Predicted structure of HRCHI60B1 shown in figure 3.32 clearly demonstrates the recombination of Chi60 (blue) and ChiB (yellow) at the active site motif (orange). The overall structure of catalytic domain could still retain the $(\beta/\alpha)_8$ barrel structure.

Next, HRCHI60B3 as a representative of a group that has 1.4 kb gene size was analyzed. Results from nucleotide BLAST combined with pairwise alignment in figure 4.3 showed that HRCHI60B3, similar to HRCD66B15, has a recombined region at the site that was not found in the homologous region computed by pairwise alignment. This region highlighted in red is the recombination of N-terminal side of CatDChi60 with C-terminal side of CatDChiB which leads to chimeric chitinase that lacks the catalytic core including active site. Predicted structure of HRCHI60B3 displayed in figure 3.33 clearly shows the incomplete folding of catalytic domain.

HRCHI60B5 as a representative of a group with 1.5 kb gene size also has a recombined region at the site that was not found in the homologous region computed by pairwise alignment. As shown in figure 4.3, this recombined region highlighted in green is located near the active site toward C-terminal side of CatDChi60 which recombined with a very far C-terminal side of CatDChiB. Thus, this chimera also has incomplete $(\beta/\alpha)_8$ barrel catalytic domain as shown in figure 3.34.

Expression and chitinase activity assay of chimeric chitinase

Protein expression of both HRCD66B and HRCHI60B was not observed. Chimeric chitinases, if successfully expressed, should have protein size ranging from 55 to 65 kDa based on deduced amino acid sequences. Results from SDS-PAGE shown in figure 3.22 and 3.35 stated that there was no significant difference observed compared with negative control. Reasons for these results should be that (1) there was low level of expression under promoter of Chi60, or (2) there was a problem in protein folding of chimeras that might cause protein translation to stop or degrade rapidly. Anyhow, chitinase activity of the chimeras was still assayed. For HRCD66B chimeras, chitinolytic activity of HRCD66B1, HRCD66B6, HRCD66B15 and HRCD66BM1 whose chimeric genes were already analyzed to have proper coding sequences were assayed. Unfortunately, result suggested that all HRCD66B showed no chitinase activity on any substrates including HRCD66BM1 that were screened from CCMM method. To avoid missing of active chimeras, all chimeric plasmids

pHRCHI60B1-77 and pHRCHI60BM1-6 were expressed and assayed for chitinase activity, since nucleotide sequences of only representatives has been studied. Unluckily, all the chimeras still showed no activity including HRCHI60B76 and 77 that were screened hydrolytic activity assay and HRCHI60BM1-6 that were screened by CCMM method.

The Attempts to find functional chimeric chitinase

1. Mutation mediated by ultraviolet radiation of HRCD66B1

Since HRCD66B1 is the chimeric chitinase constructed from CatDChi66 and ChiB which recombined at exactly the active site and the predicted structure showed the most possible folding to be functional chitinase, it was chosen to try random mutation mediated by UV radiation. We hypothesized that some natural mutation on the nutrient stress condition should force this chimera to function in order to survive, thus phenotypic screening on CCMM agar plate with ampicillin was used. After UV radiation for 5 seconds, there were 5 small forming colonies that could survive on CCMM agar plate with ampicillin but still no clear zone was observed. Further expression and chitinolytic activity assay were achieved and no activity observed. This result together with the evidence of HRCHI60B screening by CCMM method implied that bacterial transformants could still grow in minimum media with just little yeast extract as carbon source.

2. Expression system changing of HRCD66B1

pHRCD66B1 was constructed from pBS/SK$^-$ as base vector with promoter of Chi60 as a strong promoter of chitinase gene which consequently made this expression system uncontrollable. The approach to control the expression is to exchange hrcd66B1 gene to other expression vector. Firstly, we tried pTrcHis2C system which contains inducible *lac* operon and His tag for further purification. hrcd66B1 gene was successfully subcloned into pTrcHis2C at restriction sites of *Nco*I and *Kpn*I but the size of vector was decreased from 4.4 kb to 3 kb (data not shown). Hence, the expression vector was changed to pET19b vector which also contains inducible *lac* operon and His tag. Primers were designed for subcloning of hrcd66B1 into pET19b at restriction sites of *Nde*I and *Xho*I. Ligation reaction of hrcd66B1 into

pET19b via *Nde*I and *Xho*I sites was tried out but the effort was unsuccessful (data not shown).

3. Construction of chimeric chitinase by genetic engineering

The attempt to construct a new chimera by genetic engineering at specific site was conducted since we observed many non-function recombinants from the *in vivo* homologous recombination. Figure 4.4 shows parts of protein and nucleotide sequences alignment of between Chi60 and ChiB with designed recombining site highlighted in pink color. This region was chosen since it lined in the turn loop of catalytic domain at non-homology sequences near the active site thus, this recombination might not interfere the folding of catalytic domain. This was confirmed by 3D structure prediction as shown in figure 4.5 where pink color indicated recombination site. Primers shown in table 4.1 are designed for recombination by silence mutation of nucleotide sequences that encoded for Lys Ile in *chi60* and Lys Leu in *chiB* into *Hin*dIII restriction site. Strategy for recombination by genetic engineering of Chi60 and ChiB starts with cloning of *chi60* part with forward primer specific to start codon of *chi60* and designed reversed primer. This *chi60* piece will be ligated into pET19b vector. Then, *chiB* part will be cloned using designed forward primer and reversed primer specific to stop codon of *chiB*. Finally, *chiB* piece will be subcloned into pET19b containing *chi60* part at the designed *Hin*dIII site and this recombinant plasmid will be expressed and further characterized.

A

```
chi60          246 DDPYKGNFGQLMALKQAHPDLKILPSIGGWTLSDPFFFMGDKVKRDRFVG    295
                   .:|.|||..:|.|:|:.|||||..|:.   :|  |....:|.
ChiB            76 --------NRLTALKAHNPSLRIMFSIGGWYYSND---LG--VSHANYVN    112

chi60          296 SVKEFLQTWKF------------FDGVDIDWEFPGGKGANPNLGSPQDGE    333
                   :||......||            |||||||||:|        ..:..||
ChiB           113 AVKTPAARTKFAQSCVRIMKDYGFDGVDIDWEYP--------QAAEVDG-    153

chi60          334 TYVLLMKELRAMLDQLSAETGRK---YELTSAISAGKDKIDKVAYNVAQ-    379
                    ::..::|:|.:|:|.:...|||:   |:||.|.:.|...:.:...:||
ChiB           154 -FIAALQEIRTLLNQQTIADGRQALPYQLTIAGAGGAFFLSRYYSKLAQI    202

chi60          380 -NSMDHIFLMSYDFYGAFDLKNLGHQTALNAPAWKPDTAYTTVNGVNA--    426
                    ..:|:|.||:||..|.:: |...||.||...|..| |.|..:...|.
ChiB           203 VAPLDYINLMTYDLAGPWE-KITNHQAALFGDAAGP-TFYNALREANLGW    250
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B

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chi60         1026 GCGGGCGATGCTGGATCAG----CTGTCGGCGGAAACCGGCCGCAAA---   1068
                   .||..|..|||||.|.|||    |..|| |||||   |||||||.||
ChiB           483 CCGCACCTTGCTGAACCAGCAAACCATC-GCGGA---CGGCCGCCAAGCG    528

chi60         1069 ------TATGAACTGACCTCCGCCATCAGCGCCGGCAAGGACAAGATCG-   1111
                       |||.|..||||||..|||   |.||||||||  ||       ||
ChiB           529 TTGCCGTATCAGTTGACCATCGC---CGGCGCCGGC--GG-------CGC    566

chi60         1112 -----------------AT------AAGGTGGCTTACAACGTTGCGCAGA   1138
                                   ||      |||.||||..|.|.|||.||||  .
ChiB           567 CTTCTTCCTGTCGCGCTATTACAGCAAGCTGGCGCAAATCGTCGCGC--C    614

chi60         1139 ACTCGATGGATCACATCTTCCTGATGAGCTACGACTTCT--ATGGCGCCT   1186
                   ||||    |||.||||||.|||||||.||||||  |||  ..|||.|||
ChiB           615 ACTC----GATTACATCAACCTGATGACCTACGA--TCTGGCCGGCCCCT    658
```

Figure 4.4 Amino acid sequence and nucleotide sequence alignments between Chi60 and ChiB

The alignment of amino acid sequence (A) and nucleotide sequence (B) were achieved using EMBOSS Pairwise Alignment with EMBOSS: water (local) method The designed recombining site are highlighted in pink and the active site motif of family 18 chitinase is highlighted in yellow.

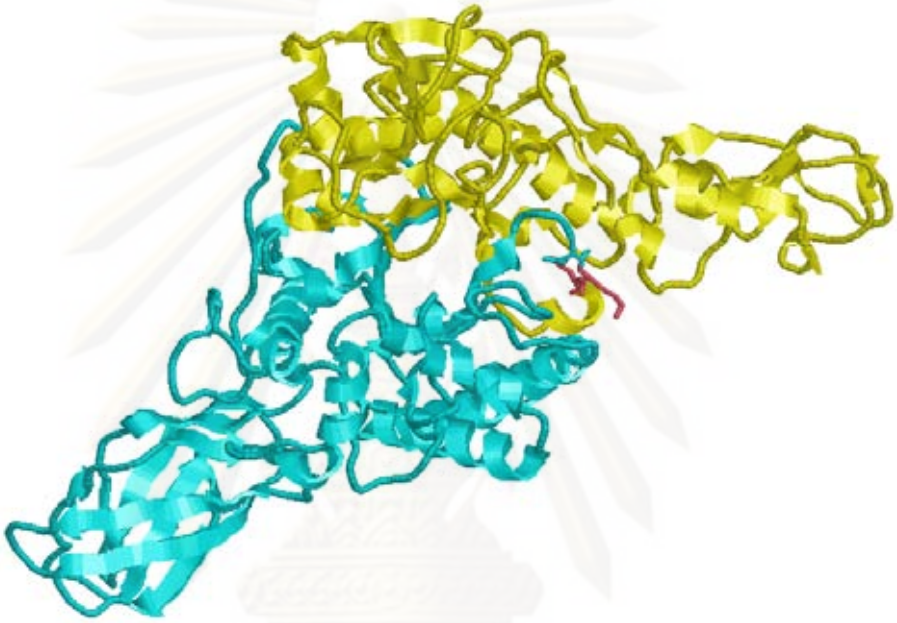


Figure 4.5 Homology modeling structure of chimeric chitinase between Chi60 and ChiB by site specific recombination

Structure was visualized by Rasmol version 2.7.4.2. Blue color indicates structure deduced from Chi60 sequences and yellow color indicates structure deduced from ChiB sequences. Recombination site is displayed in pink color.

Table 4.1 Primers designed for recombination of Chi60 and ChiB by genetic engineering

Primer name	Nucleotide sequence
ChiBfusion Forward primer	5’ AAG CTT GCG CAA ATC GTC GC HindIII
Chi60fusion Reversed Primer	5’ AAG CTT ATC GAT CTT GTC CTT GCC HindIII

CHAPTER V

CONCLUSION

Chimeric chitinases HRCD66B were constructed by *in vivo* homologous recombinations between CatDChi66 and ChiB which are screened from LB agar plate containing ampicillin method and CCMM broth containing ampicillin method. We observed 6 chimeras with individual chimeric genes size of 1.34, 1.57, 1.65, 1.67, 1.68 and 1.76 kb and only 4 of them with gene size of 1.67, 1.68, 1.34 and 1.76 kb have in frame recombination and could be translated to amino acid sequences. From sequences analysis, we found that homologous recombination mostly occurred at the region encoding for active site which contributes to chimera with 1.67 kb and encoded for 555 amino acid residues.

HRCHI60B were also constructed by screening with LB agar plate containing ampicillin method and CCMM broth containing ampicillin method. We have 83 of chimeras which are grouped into 5 groups with gene size of 1.4, 1.5, 1.6, 2.0 and 2.3 kb. Recombined regions of each group were analyzed and found that only 3 of them with gene size of 1.49, 1.55 and 2.0 kb have in frame recombination and could be translated to amino acid sequences. 55 of chimeras with 2.0 kb gene size were found that the homologous recombination occurred at the region encoding for active site.

We concluded that chimeric chitinase constructed by *in vivo* homologous recombination mostly have recombined region at the active site which has the highest homology between ChiB and both CatDChi66 and Chi60. Other HRCD66B and HRCHI60B recombination resulted in frame shift that either yielded internal deletion, lacking the active site, or C-terminal truncated protein. Chitinolytic activity of chimeric chitinases on colloidal chitin, PNAC and chitooligosaccharides was not observed.

We observed that, high homology regions in the chitinase genes are not the same homology regions in the 3D structure of the translated protein products, which might causes most of the recombinant chitinases to misfold and lost their activity.

REFERENCES

Andersen, M. D., Jensen, A., Robertus, J. D., Leah, R., and Skriver, K. Heterologous Expression and Characterization of Wild-Type and Mutant Forms of a 26 kDa Endochitinase from Barley (*Hordeum vulgare* L.). Biochemical Journal 322 (1997): 815-22.

Blackwell, J. Structure of Beta-Chitin or Parallel Chain Systems of Poly-Beta-(1-4)-*N*-Acetyl-D-Glucosamine. Biopolymers 7,3 (1969): 281-98.

Brameld, K. A., and Goddard, W. A. The Role of Enzyme Distortion in the Single Displacement Mechanism of Family 19 Chitinases. In Proceedings of the National Academy of Sciences of the United States of America, pp.4276-81. 1998a.

Brameld, K. A., and Goddard, W. A. Substrate Distortion to a Boat Conformation at Subsite -1 Is Critical in the Mechanism of Family 18 Chitinases. Journal of the American Chemical Society 120,15 (1998b): 3571-80.

Brameld, K. A., Shrader, W. D., Imperiali, B., and Goddard Iii, W. A. Substrate Assistance in the Mechanism of Family 18 Chitinases: Theoretical Studies of Potential Intermediates and Inhibitors. Journal of Molecular Biology 280,5 (1998): 913-23.

Brurberg, M. B., Nes, I. F., and Eijsink, V. G. H. Comparative Studies of Chitinases A and B from *Serratia marcescens*. Microbiology 142,7 (1996): 1581-89.

Carbone, M. N., and Arnold, F. H. Engineering by Homologous Recombination: Exploring Sequence and Function within a Conserved Fold. Current Opinion in Structural Biology 17,4 (2007): 454-59.

Choi, W. S., Ahn, K. J., Lee, D. W., Byun, M. W., and Park, H. J. Preparation of Chitosan Oligomers by Irradiation. Polymer Degradation and Stability 78,3 (2002): 533-38.

Dykhuizen, D. E., and Green, L. Recombination in *Escherichia coli* and the Definition of Biological Species. Journal of Bacteriology 173,22 (1991): 7257-68.

Hernandez-Munoz, P., Almenar, E., Valle, V. D., Velez, D., and Gavara, R. Effect of Chitosan Coating Combined with Postharvest Calcium Treatment on Strawberry (*Fragaria ananassa*) Quality During Refrigerated Storage. Food Chemistry 110,2 (2008): 428-35.

Ikegami, T., et al. Solution Structure of the Chitin-Binding Domain of *Bacillus circulans* Wl-12 Chitinase A1. Journal of Biological Chemistry 275,18 (2000): 13654-61.

Imoto, T., and Yagishita, K. A Simple Activity Measurement of Lysozyme. Agricultural and Biological Chemistry 35,7 (1971): 1154-56.

Jeuniaux, C., Elizabeth, F. N., and Victor, G. Chitinases. In Methods in Enzymology, pp.644-50. Academic Press, 1966.

John Hart, P., Pfluger, H. D., Monzingo, A. F., Hollis, T., and Robertus, J. D. The Refined Crystal Structure of an Endochitinase from *Hordeum vulgare* L. Seeds at 1.8 Å Resolution. Journal of Molecular Biology 248,2 (1995): 402-13.

Jung, W. J., Souleimanov, A., Park, R. D., and Smith, D. L. Enzymatic Production of *N*-Acetyl Chitooligosaccharides by Crude Enzyme Derived from *Paenibacillus illioisensis* Kja-424. Carbohydrate Polymers 67,2 (2007): 256-59.

Kachanechai, T., Jantawat, P., and Pichyangkura, R. The Influence of Chitosan on Physico-Chemical Properties of Chicken Salt-Soluble Protein Gel. Food Hydrocolloids 22,1 (2008): 74-83.

Kananont, N., Pichyangkura, R., Chanprame, S., Chadchawan, S., and Limpanavech, P. Chitosan Specificity for the in Vitro Seed Germination of Two Dendrobium Orchids (*Asparagales orchidaceae*). Scientia Horticulturae 124,2 (2010): 239-47.

Keadsin, A. Construction of Chimeric Cyclodextrin Glucanotransferases by Homologous Recombination and Study of Their Activities. Master's Thesis, Department of Biochemistry Faculty of Science Chulalongkorn University, 2003.

Keim, P., and Lark, K. G. The RecE Recombination Pathway Mediates Recombination between Partially Homologous DNA Sequences : Structural Analysis of Recombination Products. Journal of structural biology 104 (1990): 97-106.

Knoor, D. Use of Chitinous Polymers in Food – A Challenge for Food Research & Development. Food Technology 38 (1984): 85-97.

Koga, D., Mitsutomi, M., Kono, M., and Matsumiya, M. Biochemistry of Chitinases. In P. Jollès and R. A. A. Muzzarelli (eds.), Chitin and Chitinases, pp.111-23. Basel; Boston: Birkhäuser Verlag, 1999.

Kudan, S. Chitinase from Thermotolerant Bacteria : Enzyme Characterization and Gene Cloning. Master's Thesis, Department of Biochemistry Faculty of Science Chulalongkorn University, 2001.

Kudan, S. Protein Engineering of Bacterial Chitinase for *N*-Acetylchitooligosaccharide Production. Doctoral dissertation, Department of Biochemistry Faculty of Science Chulalongkorn University, 2004.

Kuttiyawong, K. Cloning and Nucleotide Sequencing of Chitinase Gene from *Burkholderia cepacia* TU09. Master's Thesis, Department of Biochemistry Faculty of Science Chulalongkorn University, 2001.

Kuttiyawong, K., Nakapong, S., and Pichyangkura, R. The Dual Exo/Endo-Type Mode and the Effect of Ionic Strength on the Mode of Catalysis of Chitinase 60 (Chi60) from *Serratia* sp. TU09 and Its Mutants. Carbohydrate Research 343,16 (2008): 2754-62.

Lloyd, L. L., Kennedy, J. F., Methacanon, P., Paterson, M., and Knill, C. J. Carbohydrate Polymers as Wound Management Aids. Carbohydrate Polymers 37,3 (1998): 315-22.

Muzzarelli, R. A. A., Weckx, M., Filippini, O., and Sigon, F. Removal of Trace Metal Ions from Industrial Waters, Nuclear Effluents and Drinking Water, with the Aid of Cross-Linked *N*-Carboxymethyl Chitosan. Carbohydrate Polymers 11,4 (1989): 293-306.

Papanikolau, Y., et al. High Resolution Structural Analyses of Mutant Chitinase A Complexes with Substrates Provide New Insight into the Mechanism of Catalysis. Biochemistry 40,38 (2001): 11338-43.

Perrakis, A., et al. Crystal Structure of a Bacterial Chitinase at 2.3 Å Resolution. Structure 2,12 (1994): 1169-80.

Rao, V., Guan, C., and Roey, P. V. Crystal Structure of Endo-[Beta]-*N*-Acetylglucosaminidase H at 1.9 Å Resolution: Active-Site Geometry and Substrate Recognition. Structure 3,5 (1995): 449-57.

Roey, P. V., Rao, V., Plummer, T. H., and Tarentino, A. Crystal Structure of Endo-B-*N*-Acetylglucosaminidase F1, an A/B-Barrel Enzyme Adapted for a Complex Substrate. Biochemistry 33,47 (1994): 13989-96.

Sambrook, J., and Russell, D. W. Molecular Cloning : A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, 2001.

Shibuya, N., and Minami, E. Oligosaccharide Signalling for Defence Responses in Plant. Physiological and Molecular Plant Pathology 59,5 (2001): 223-33.

Shu, X. Z., Zhu, K. J., and Song, W. Novel Ph-Sensitive Citrate Cross-Linked Chitosan Film for Drug Controlled Release. International Journal of Pharmaceutics 212,1 (2001): 19-28.

Soding, J., Biegert, A., and Lupas, A. N. The HHpred Interactive Server for Protein Homology Detection and Structure Prediction. Nucleic Acids Research 33,2 (2005): W244-48.

Suzuki, K., et al. Antitumor Effect of Hexa-*N*-Acetylchitohexaose and Chitohexaose. Carbohydrate Research 151 (1986): 403-08.

Tanaka, T., et al. A Unique Chitinase with Dual Active Sites and Triple Substrate Binding Sites from the Hyperthermophilic archaeon *Pyrococcus kodakaraensis* Kod1. Applied and Environmental Microbiology 65,12 (1999): 5338-44.

Terwisscha van Scheltinga, A. C., et al. Stereochemistry of Chitin Hydrolysis by a Plant Chitinase/Lysozyme and X-Ray Structure of a Complex with Allosamidin Evidence for Substrate Assisted Catalysis. Biochemistry 34,48 (1995): 15619-23.

Terwisscha van Scheltinga, A. C., Kalk, K. H., Beintema, J. J., and Dijkstra, B. W. Crystal Structures of Hevamine, a Plant Defence Protein with Chitinase and Lysozyme Activity, and Its Complex with an Inhibitor. Structure 2,12 (1994): 1181-89.

Tharanathan, R. N., and Kittur, F. S. Chitin — The Undisputed Biomolecule of Great Potential. Critical Reviews in Food Science and Nutrition 43,1 (2003): 61-87.

Toratani, T., Kezuka, Y., Nonaka, T., Hiragi, Y., and Watanabe, T. Structure of Full-Length Bacterial Chitinase Containing Two Fibronectin Type III Domains Revealed by Small Angle X-Ray Scattering. Biochemical and Biophysical Research Communications 348,3 (2006): 814-18.

van Aalten, D. M. F., et al. Structure of a Two-Domain Chitotriosidase from *Serratia marcescens* at 1.9Å Resolution. In Proceedings of the National Academy of Sciences of the United States of America, pp.5842-47. 2000.

Walker-Simmons, M., Hadwiger, L., and Ryan, C. A. Chitosans and Pectic Polysaccharides Both Induce the Accumulation of the Antifungal Phytoalexin Pisatin in Pea Pods and Antinutrient Proteinase Inhibitors in Tomato Leaves. Biochemical and Biophysical Research Communications 110,1 (1983): 194-99.

Wang, Q., Graham, R. W., Trimbur, D., Warren, R. A. J., and Withers, S. G. Changing Enzymic Reaction Mechanisms by Mutagenesis: Conversion of a Retaining Glucosidase to an Inverting Enzyme. Journal of the American Chemical Society 116,25 (1994): 11594-95.

Watanabe, T., et al. The Roles of the C-Terminal Domain and Type III Domains of Chitinase A1 from *Bacillus circulans* Wl-12 in Chitin Degradation. Journal of Bacteriology 176,15 (1994): 4465-72.

Watanabe, T., et al. Identification of Glutamic Acid 204 and Aspartic Acid 200 in Chitinase A1 of *Bacillus circulans* Wl-12 as Essential Residues for Chitinase Activity. Journal of Biological Chemistry 268,25 (1993): 18567-72.

Weber, K., and Osborn, M. The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Journal of Biological Chemistry 244,16 (1969): 4406-12.

Yamada, H., and Imoto, T. A Convenient Synthesis of Glycolchitin, a Substrate of Lysozyme. Carbohydrate Research 92,1 (1981): 160-62.

Yoshida, H., Kojima, K., Witarto, A. B., and Sode, K. Engineering a Chimeric Pyrroloquinoline Quinone Glucose Dehydrogenase: Improvement of EDTA Tolerance, Thermal Stability and Substrate Specificity. Protein Engineering 12,1 (1999): 63-70.

Zhang, Y., Buchholz, F., Muyrers, J. P. P., and Stewart, A. F. A New Logic for DNA Engineering Using Recombination in *Escherichia coli*. Nature Genetics 20,2 (1998): 123-28.

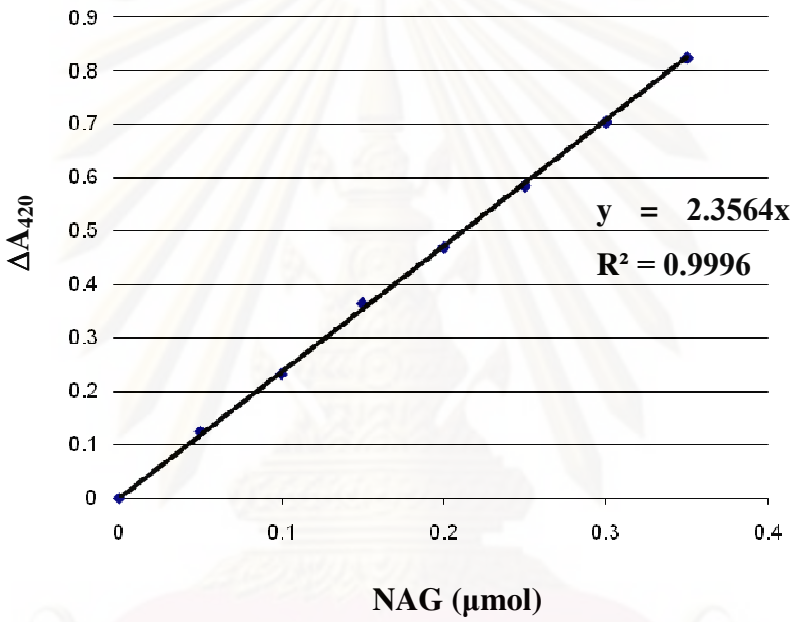
Zhang, Y., Muyrers, J. P. P., Testa, G., and Stewart, A. F. DNA Cloning by Homologous Recombination in *Escherichia coli*. Nature Biotechnology 18,12 (2000): 1314-17.

APPENDICES

APPENDIX A

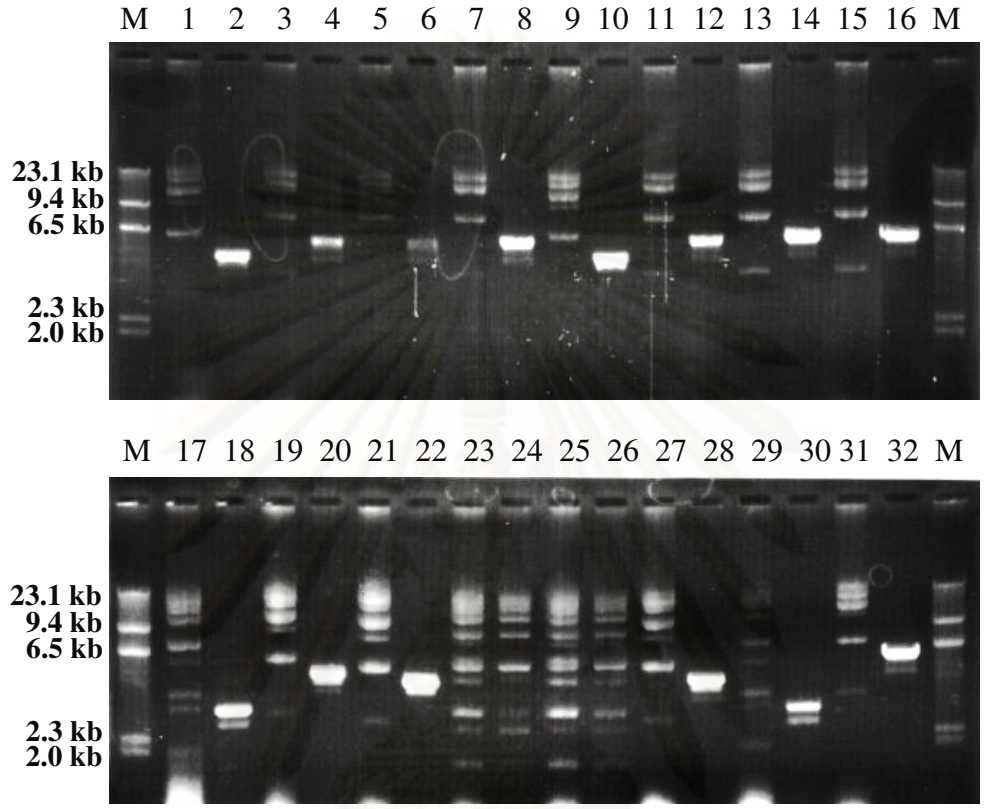
Standard curve for NAG determination by colorimetric method.

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



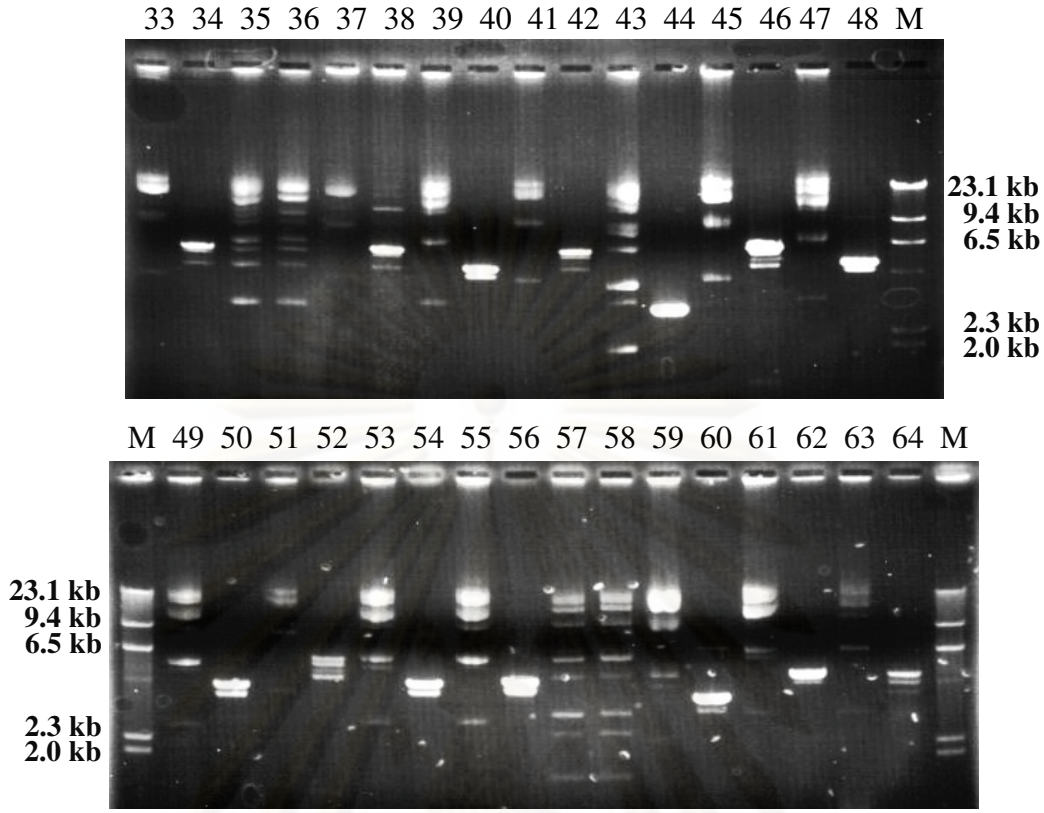
APPENDIX B

Agarose gel electrophoresis analysis of plasmids extracted from transformants of pCD66B that were first screened by LB agar plate with ampicillin



Lanes M contain λ/*Hin*dIII marker.

Lane 1 Plasmid from colony1
Lane 2 Plasmid from colony1/ *Kpn*I
Lane 3 Plasmid from colony2
Lane 4 Plasmid from colony2/ *Kpn*I
Lane 5 Plasmid from colony3
Lane 6 Plasmid from colony3/ *Kpn*I
Lane 7 Plasmid from colony4
Lane 8 Plasmid from colony4/ *Kpn*I
Lane 9 Plasmid from colony5
Lane 10 Plasmid from colony5/ *Kpn*I
Lane 11 Plasmid from colony6
Lane 12 Plasmid from colony6/ *Kpn*I
Lane 13 Plasmid from colony7
Lane 14 Plasmid from colony7/ *Kpn*I
Lane 15 Plasmid from colony8
Lane 16 Plasmid from colony8/ *Kpn*I
Lane 17 Plasmid from colony9
Lane 18 Plasmid from colony9/ *Kpn*I
Lane 19 Plasmid from colony10
Lane 20 Plasmid from colony10/ *Kpn*I
Lane 21 Plasmid from colony11
Lane 22 Plasmid from colony11/ *Kpn*I
Lane 23 Plasmid from colony12
Lane 24 Plasmid from colony12/ *Kpn*I
Lane 25 Plasmid from colony13
Lane 26 Plasmid from colony13/ *Kpn*I
Lane 27 Plasmid from colony14
Lane 28 Plasmid from colony14/ *Kpn*I
Lane 29 Plasmid from colony15
Lane 30 Plasmid from colony15/ *Kpn*I
Lane 31 Plasmid from colony16
Lane 32 Plasmid from colony16/ *Kpn*I

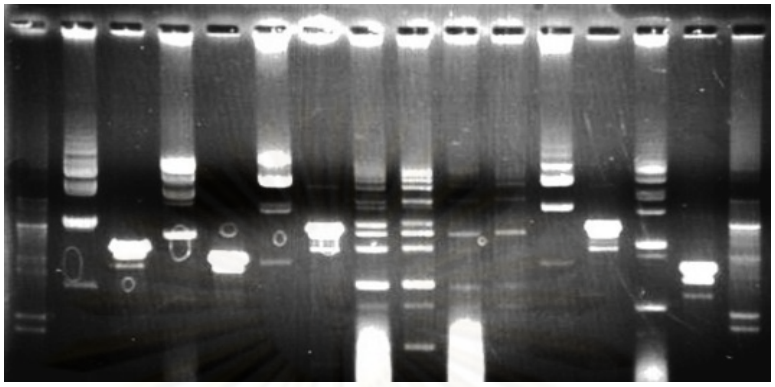


Lanes M contain λ/*Hin*dIII marker.

Lane 33 Plasmid from colony17
Lane 34 Plasmid from colony17/ *Kpn*I
Lane 35 Plasmid from colony18
Lane 36 Plasmid from colony18/ *Kpn*I
Lane 37 Plasmid from colony19
Lane 38 Plasmid from colony19/ *Kpn*I
Lane 39 Plasmid from colony20
Lane 40 Plasmid from colony20/ *Kpn*I
Lane 41 Plasmid from colony21
Lane 42 Plasmid from colony21/ *Kpn*I
Lane 43 Plasmid from colony22
Lane 44 Plasmid from colony22/ *Kpn*I
Lane 45 Plasmid from colony23
Lane 46 Plasmid from colony23/ *Kpn*I
Lane 47 Plasmid from colony24
Lane 48 Plasmid from colony24/ *Kpn*I

Lane 49 Plasmid from colony25
Lane 50 Plasmid from colony25/ *Kpn*I
Lane 51 Plasmid from colony26
Lane 52 Plasmid from colony26/ *Kpn*I
Lane 53 Plasmid from colony27
Lane 54 Plasmid from colony27/ *Kpn*I
Lane 55 Plasmid from colony28
Lane 56 Plasmid from colony28/ *Kpn*I
Lane 57 Plasmid from colony29
Lane 58 Plasmid from colony29/ *Kpn*I
Lane 59 Plasmid from colony30
Lane 60 Plasmid from colony30/ *Kpn*I
Lane 61 Plasmid from colony31
Lane 62 Plasmid from colony31/ *Kpn*I
Lane 63 Plasmid from colony32
Lane 64 Plasmid from colony32/ *Kpn*I

M 65 66 67 68 69 70 71 72 73 74 75 76 77 78 M



Lanes M contain λ/*Hin*dIII marker.

Lane 65 Plasmid from colony33

Lane 66 Plasmid from colony33/ *Kpn*I

Lane 67 Plasmid from colony34

Lane 68 Plasmid from colony34/ *Kpn*I

Lane 69 Plasmid from colony35

Lane 70 Plasmid from colony35/ *Kpn*I

Lane 71 Plasmid from colony36

Lane 72 Plasmid from colony36/ *Kpn*I

Lane 73 Plasmid from colony37

Lane 74 Plasmid from colony37/ *Kpn*I

Lane 75 Plasmid from colony38

Lane 76 Plasmid from colony38/ *Kpn*I

Lane 77 Plasmid from colony39

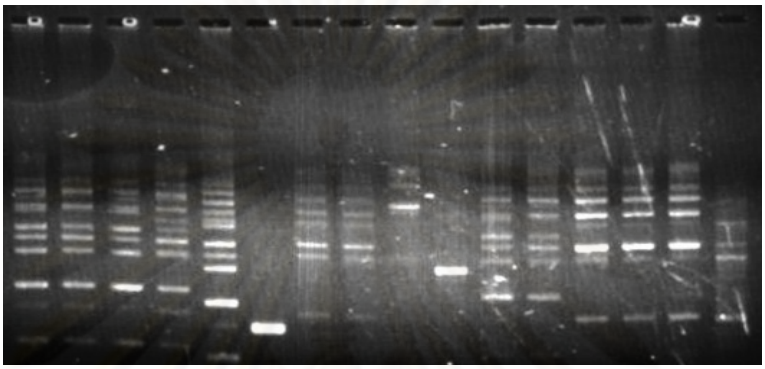
Lane 78 Plasmid from colony39/ *Kpn*I

*Plasmids from colony 1, 5, 10, 17, 20, 24, 26, 31, 32, 33, 35, and 38 were designated as pHRCD66B1-12.

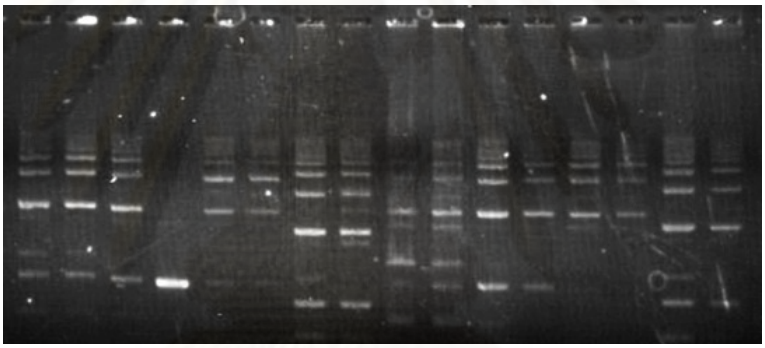
APPENDIX C

Agarose gel electrophoresis analysis of plasmids extracted from transformants of pCD66B that were screened for the second time on LB agar plate with ampicillin

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16



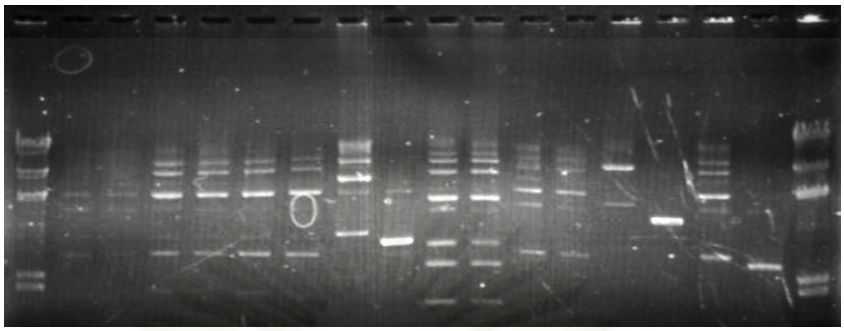
17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32



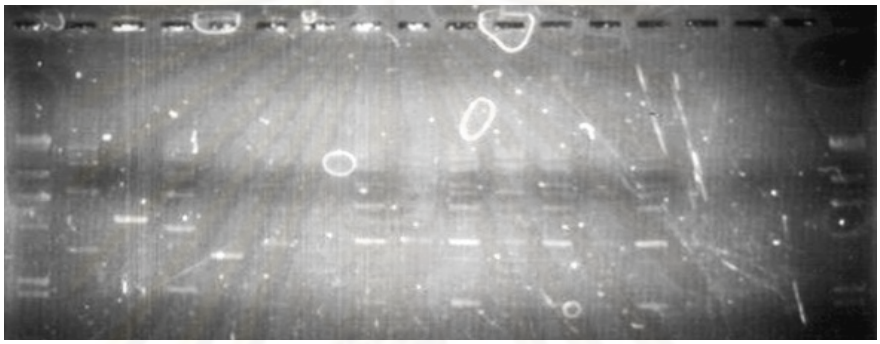
Lane 1 Plasmid from colony1
Lane 2 Plasmid from colony1/ *Kpn*I
Lane 3 Plasmid from colony2
Lane 4 Plasmid from colony2/ *Kpn*I
Lane 5 Plasmid from colony3
Lane 6 Plasmid from colony3/ *Kpn*I
Lane 7 Plasmid from colony4
Lane 8 Plasmid from colony4/ *Kpn*I
Lane 9 Plasmid from colony5
Lane 10 Plasmid from colony5/ *Kpn*I
Lane 11 Plasmid from colony6
Lane 12 Plasmid from colony6/ *Kpn*I
Lane 13 Plasmid from colony7
Lane 14 Plasmid from colony7/ *Kpn*I
Lane 15 Plasmid from colony8
Lane 16 Plasmid from colony8/ *Kpn*I

Lane 17 Plasmid from colony9
Lane 18 Plasmid from colony9/ *Kpn*I
Lane 19 Plasmid from colony10
Lane 20 Plasmid from colony10/ *Kpn*I
Lane 21 Plasmid from colony11
Lane 22 Plasmid from colony11/ *Kpn*I
Lane 23 Plasmid from colony12
Lane 24 Plasmid from colony12/ *Kpn*I
Lane 25 Plasmid from colony13
Lane 26 Plasmid from colony13/ *Kpn*I
Lane 27 Plasmid from colony14
Lane 28 Plasmid from colony14/ *Kpn*I
Lane 29 Plasmid from colony15
Lane 30 Plasmid from colony15/ *Kpn*I
Lane 31 Plasmid from colony16
Lane 32 Plasmid from colony16/ *Kpn*I

M 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 M

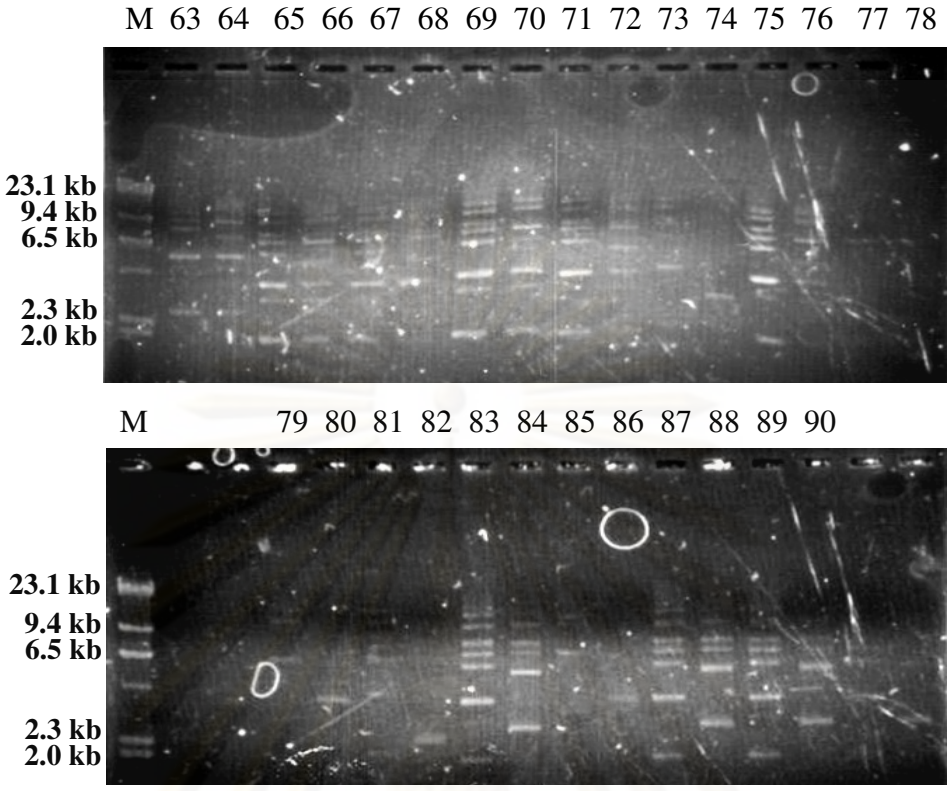


M 49 50 51 52 53 54 55 56 57 58 59 60 61 62 M

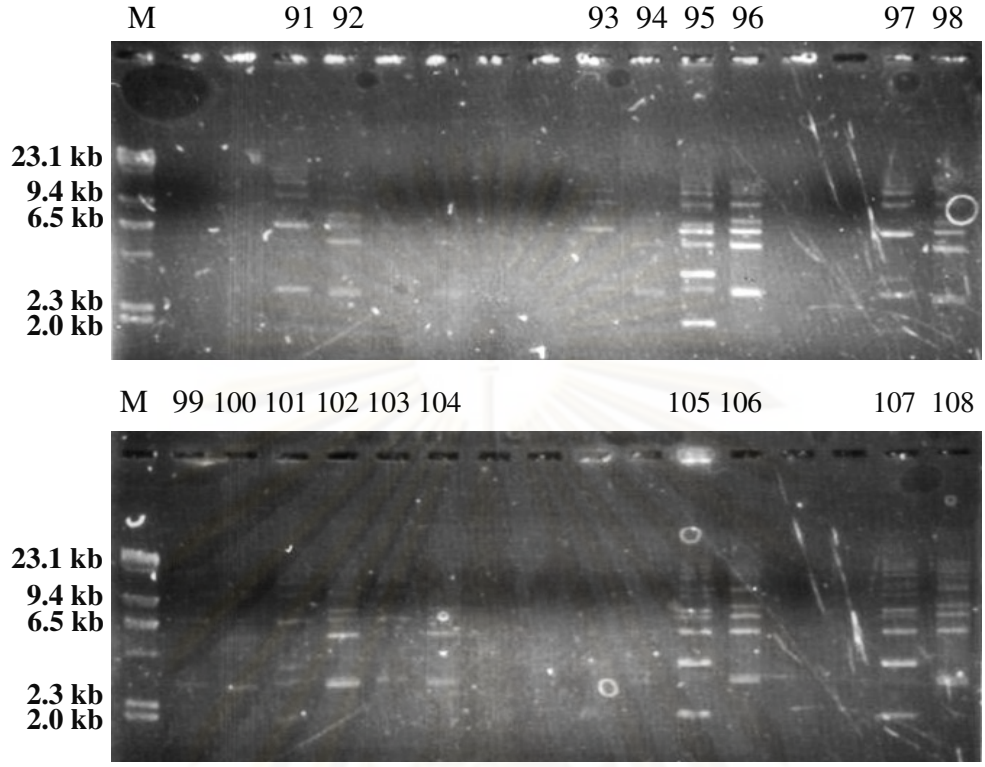


Lane 33 Plasmid from colony17
Lane 34 Plasmid from colony17/ *Kpn*I
Lane 35 Plasmid from colony18
Lane 36 Plasmid from colony18/ *Kpn*I
Lane 37 Plasmid from colony19
Lane 38 Plasmid from colony19/ *Kpn*I
Lane 39 Plasmid from colony20
Lane 40 Plasmid from colony20/ *Kpn*I
Lane 41 Plasmid from colony21
Lane 42 Plasmid from colony21/ *Kpn*I
Lane 43 Plasmid from colony22
Lane 44 Plasmid from colony22/ *Kpn*I
Lane 45 Plasmid from colony23
Lane 46 Plasmid from colony23/ *Kpn*I
Lane 47 Plasmid from colony24
Lane 48 Plasmid from colony24/ *Kpn*I

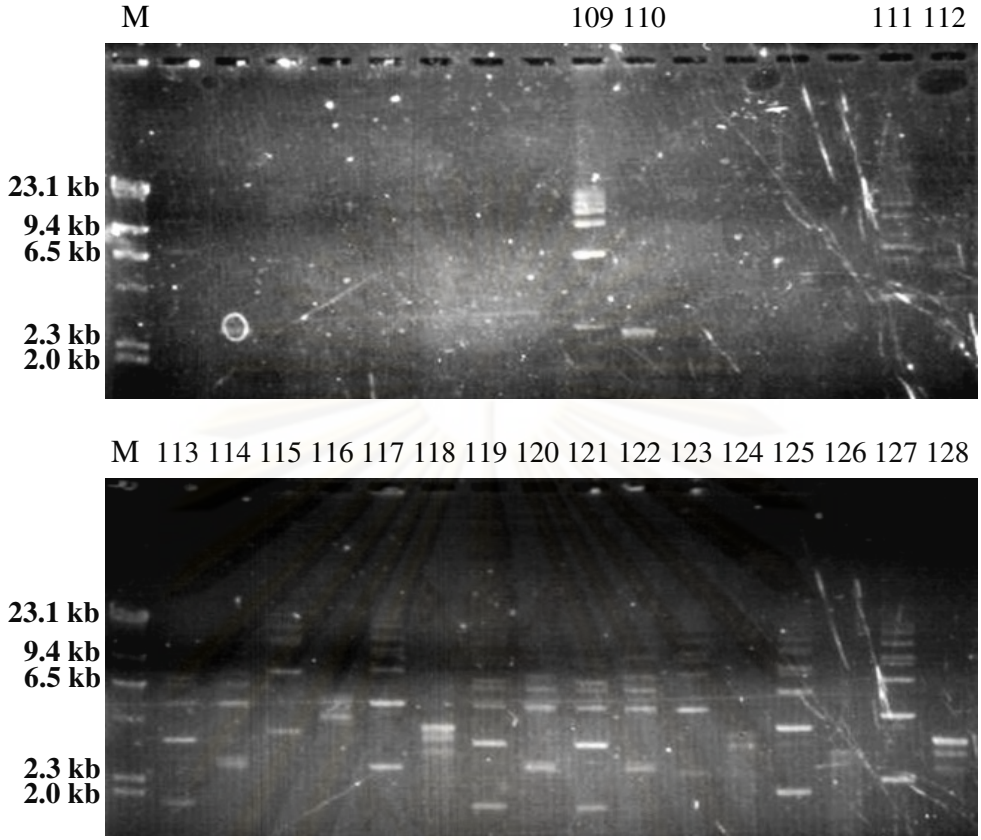
Lane 49 Plasmid from colony25
Lane 50 Plasmid from colony25/ *Kpn*I
Lane 51 Plasmid from colony26
Lane 52 Plasmid from colony26/ *Kpn*I
Lane 53 Plasmid from colony27
Lane 54 Plasmid from colony27/ *Kpn*I
Lane 55 Plasmid from colony28
Lane 56 Plasmid from colony28/ *Kpn*I
Lane 57 Plasmid from colony29
Lane 58 Plasmid from colony29/ *Kpn*I
Lane 59 Plasmid from colony30
Lane 60 Plasmid from colony30/ *Kpn*I
Lane 61 Plasmid from colony31
Lane 62 Plasmid from colony31/ *Kpn*I
Lanes M λ/*Hin*dIII marker



Lane 63 Plasmid from colony32
Lane 64 Plasmid from colony32/ *Kpn*I
Lane 65 Plasmid from colony33
Lane 66 Plasmid from colony33/ *Kpn*I
Lane 67 Plasmid from colony34
Lane 68 Plasmid from colony34/ *Kpn*I
Lane 69 Plasmid from colony35
Lane 70 Plasmid from colony35/ *Kpn*I
Lane 71 Plasmid from colony36
Lane 72 Plasmid from colony36/ *Kpn*I
Lane 73 Plasmid from colony37
Lane 74 Plasmid from colony37/ *Kpn*I
Lane 75 Plasmid from colony38
Lane 76 Plasmid from colony38/ *Kpn*I
Lane 77 Plasmid from colony39
Lane 78 Plasmid from colony39/ *Kpn*I
Lane 79 Plasmid from colony40
Lane 80 Plasmid from colony40/ *Kpn*I
Lane 81 Plasmid from colony41
Lane 82 Plasmid from colony41/ *Kpn*I
Lane 83 Plasmid from colony42
Lane 84 Plasmid from colony42/ *Kpn*I
Lane 85 Plasmid from colony43
Lane 86 Plasmid from colony43/ *Kpn*I
Lane 87 Plasmid from colony44
Lane 88 Plasmid from colony44/ *Kpn*I
Lane 89 Plasmid from colony45
Lane 90 Plasmid from colony45/ *Kpn*I
Lanes M λ/*Hin*dIII marker



Lane 91 Plasmid from colony46
Lane 92 Plasmid from colony46/ *Kpn*I
Lane 93 Plasmid from colony47
Lane 94 Plasmid from colony47/ *Kpn*I
Lane 95 Plasmid from colony48
Lane 96 Plasmid from colony48/ *Kpn*I
Lane 97 Plasmid from colony49
Lane 98 Plasmid from colony49/ *Kpn*I
Lane 99 Plasmid from colony50
Lane 100 Plasmid from colony50/ *Kpn*I
Lane 101 Plasmid from colony51
Lane 102 Plasmid from colony51/ *Kpn*I
Lane 103 Plasmid from colony52
Lane 104 Plasmid from colony52/ *Kpn*I
Lane 105 Plasmid from colony53
Lane 106 Plasmid from colony53/ *Kpn*I
Lane 107 Plasmid from colony54
Lane 108 Plasmid from colony54/ *Kpn*I
Lanes M λ/*Hin*dIII marker



Lane 109 Plasmid from colony55

Lane 110 Plasmid from colony55/ *Kpn*I

Lane 111 Plasmid from colony56

Lane 112 Plasmid from colony56/ *Kpn*I

Lane 113 Plasmid from colony57

Lane 114 Plasmid from colony57/ *Kpn*I

Lane 115 Plasmid from colony58

Lane 116 Plasmid from colony58/ *Kpn*I

Lane 117 Plasmid from colony59

Lane 118 Plasmid from colony59/ *Kpn*I

Lane 119 Plasmid from colony60

Lane 120 Plasmid from colony60/ *Kpn*I

Lane 121 Plasmid from colony61

Lane 122 Plasmid from colony61/ *Kpn*I

Lane 123 Plasmid from colony62

Lane 124 Plasmid from colony62/ *Kpn*I

Lane 125 Plasmid from colony63

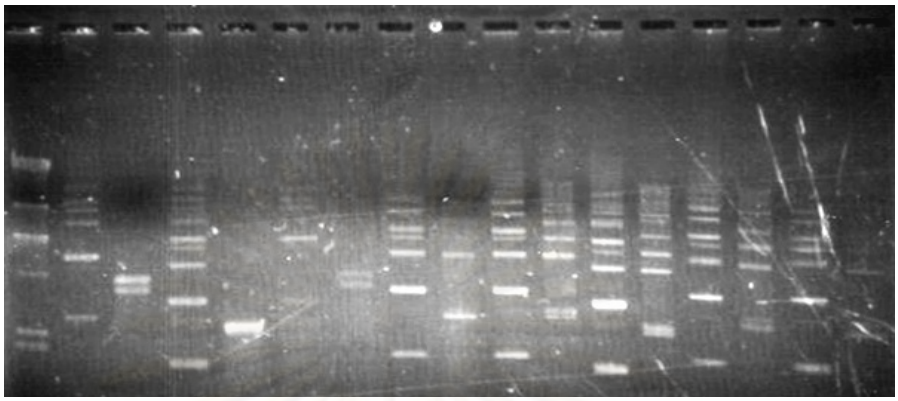
Lane 126 Plasmid from colony63/ *Kpn*I

Lane 127 Plasmid from colony64

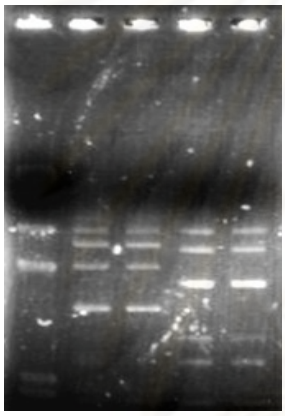
Lane 128 Plasmid from colony64/ *Kpn*I

Lanes M λ/*Hin*dIII marker

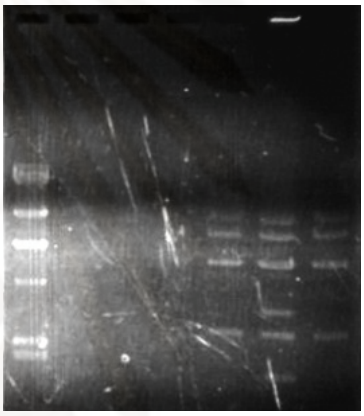
M 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144



M 145 146 147 148

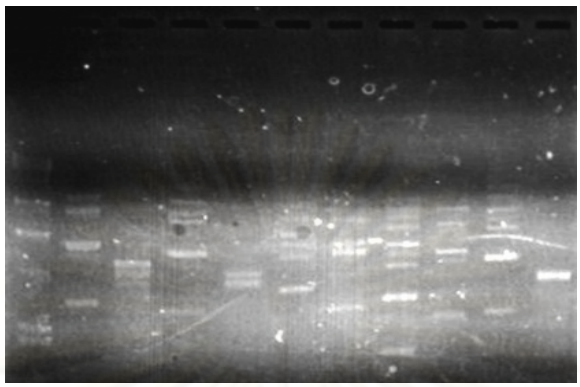


M 149 150 151 152



Lane 129 Plasmid from colony65
Lane 130 Plasmid from colony65/ *Kpn*I
Lane 131 Plasmid from colony66
Lane 132 Plasmid from colony66/ *Kpn*I
Lane 133 Plasmid from colony67
Lane 134 Plasmid from colony67/ *Kpn*I
Lane 135 Plasmid from colony68
Lane 136 Plasmid from colony68/ *Kpn*I
Lane 137 Plasmid from colony69
Lane 138 Plasmid from colony69/ *Kpn*I
Lane 139 Plasmid from colony70
Lane 140 Plasmid from colony70/ *Kpn*I
Lane 141 Plasmid from colony71
Lane 142 Plasmid from colony71/ *Kpn*I
Lane 143 Plasmid from colony72
Lane 144 Plasmid from colony72/ *Kpn*I
Lane 145 Plasmid from colony73
Lane 146 Plasmid from colony73/ *Kpn*I
Lane 147 Plasmid from colony74
Lane 148 Plasmid from colony74/ *Kpn*I
Lane 149 Plasmid from colony75
Lane 150 Plasmid from colony75/ *Kpn*I
Lane 151 Plasmid from colony76
Lane 152 Plasmid from colony76/ *Kpn*I
Lanes M λ/*Hin*dIII marker

M 153 154 155 156 157 158 159 160 161 162

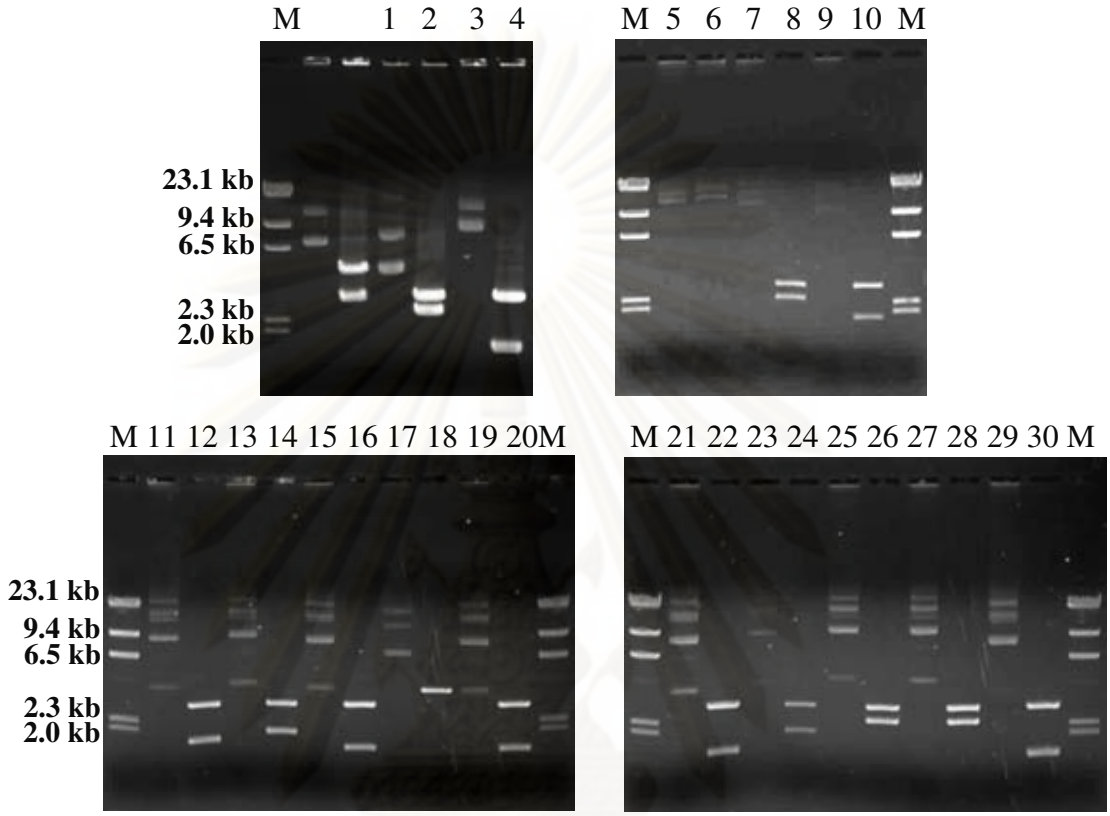


Lane 153 Plasmid from colony77
Lane 154 Plasmid from colony78/ *Kpn*I
Lane 155 Plasmid from colony79
Lane 156 Plasmid from colony80/ *Kpn*I
Lane 157 Plasmid from colony81
Lane 158 Plasmid from colony82/ *Kpn*I
Lane 159 Plasmid from colony83
Lane 160 Plasmid from colony84/ *Kpn*I
Lane 161 Plasmid from colony85
Lane 162 Plasmid from colony86/ *Kpn*I
Lanes M λ/*Hin*dIII marker

*Plasmids from colony 7, 23, 25, 40, 43, 58, 67 and 77 were designated as pHRCD66B13-20.

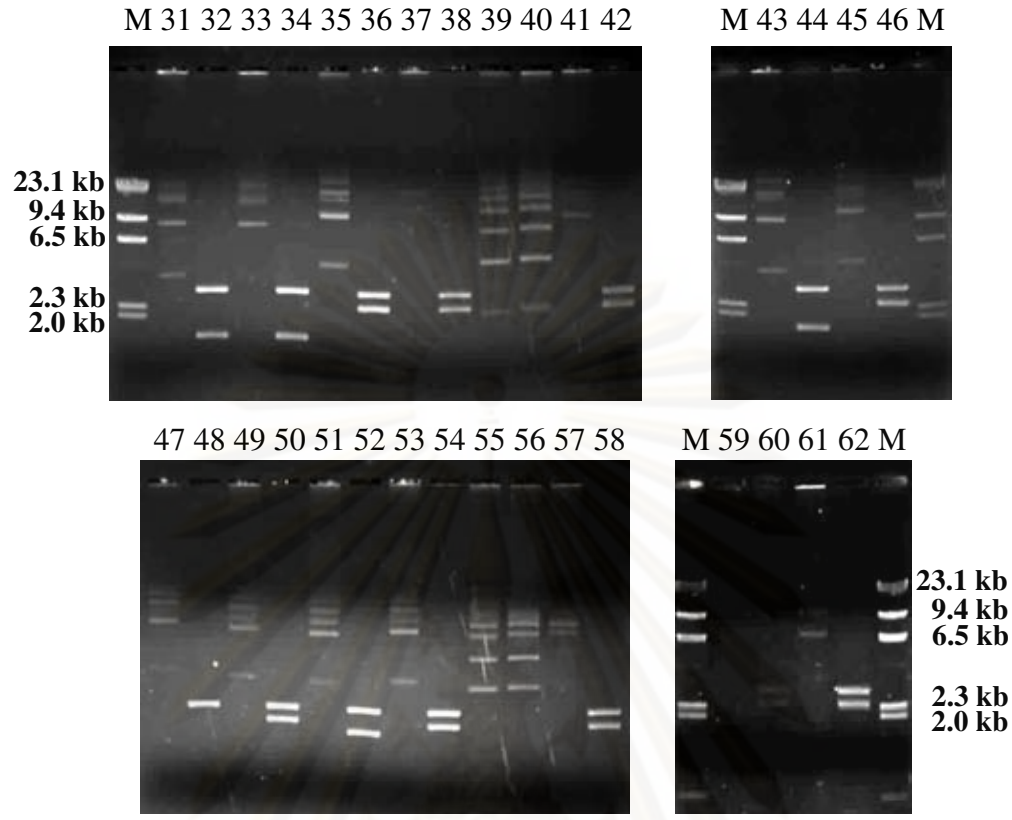
APPENDIX D

Agarose gel electrophoresis analysis of plasmids extracted from transformants of pCHI60B that were first screened on LB agar plate with ampicillin



Lanes M contain λ/*Hin*dIII marker.

Lane 1 From colony1
Lane 2 From colony1/ *Not*I+*Xho*I
Lane 3 From colony2
Lane 4 From colony2/ *Not*I+*Xho*I
Lane 5 From colony3
Lane 6 From colony3/ *Not*I+*Xho*I
Lane 7 From colony4
Lane 8 From colony4/ *Not*I+*Xho*I
Lane 9 From colony5
Lane 10 From colony5/ *Not*I+*Xho*I
Lane 11 From colony6
Lane 12 From colony6/ *Not*I+*Xho*I
Lane 13 From colony7
Lane 14 From colony7/ *Not*I+*Xho*I
Lane 15 From colony8
Lane 16 From colony8/ *Not*I+*Xho*I
Lane 17 From colony9
Lane 18 From colony9/ *Not*I+*Xho*I
Lane 19 From colony10
Lane 20 From colony10/ *Not*I+*Xho*I
Lane 21 From colony11
Lane 22 From colony11/ *Not*I+*Xho*I
Lane 23 From colony12
Lane 24 From colony12/ *Not*I+*Xho*I
Lane 25 From colony13
Lane 26 From colony13/ *Not*I+*Xho*I
Lane 27 From colony14
Lane 28 From colony14/ *Not*I+*Xho*I
Lane 29 From colony15
Lane 30 From colony15/ *Not*I+*Xho*I



Lanes M contain λ/*Hin*dIII marker.

Lane 31 From colony16

Lane 32 From colony16/ *Not*I+*Xho*I

Lane 33 From colony17

Lane 34 From colony17/ *Not*I+*Xho*I

Lane 35 From colony18

Lane 36 From colony18/ *Not*I+*Xho*I

Lane 37 From colony19

Lane 38 From colony19/ *Not*I+*Xho*I

Lane 39 From colony20

Lane 40 From colony20/ *Not*I+*Xho*I

Lane 41 From colony21

Lane 42 From colony21/ *Not*I+*Xho*I

Lane 43 From colony22

Lane 44 From colony22/ *Not*I+*Xho*I

Lane 45 From colony23

Lane 46 From colony23/ *Not*I+*Xho*I

Lane 47 From colony24

Lane 48 From colony24/ *Not*I+*Xho*I

Lane 49 From colony25

Lane 50 From colony25/ *Not*I+*Xho*I

Lane 51 From colony26

Lane 52 From colony26/ *Not*I+*Xho*I

Lane 53 From colony27

Lane 54 From colony27/ *Not*I+*Xho*I

Lane 55 From colony28

Lane 56 From colony28/ *Not*I+*Xho*I

Lane 57 From colony29

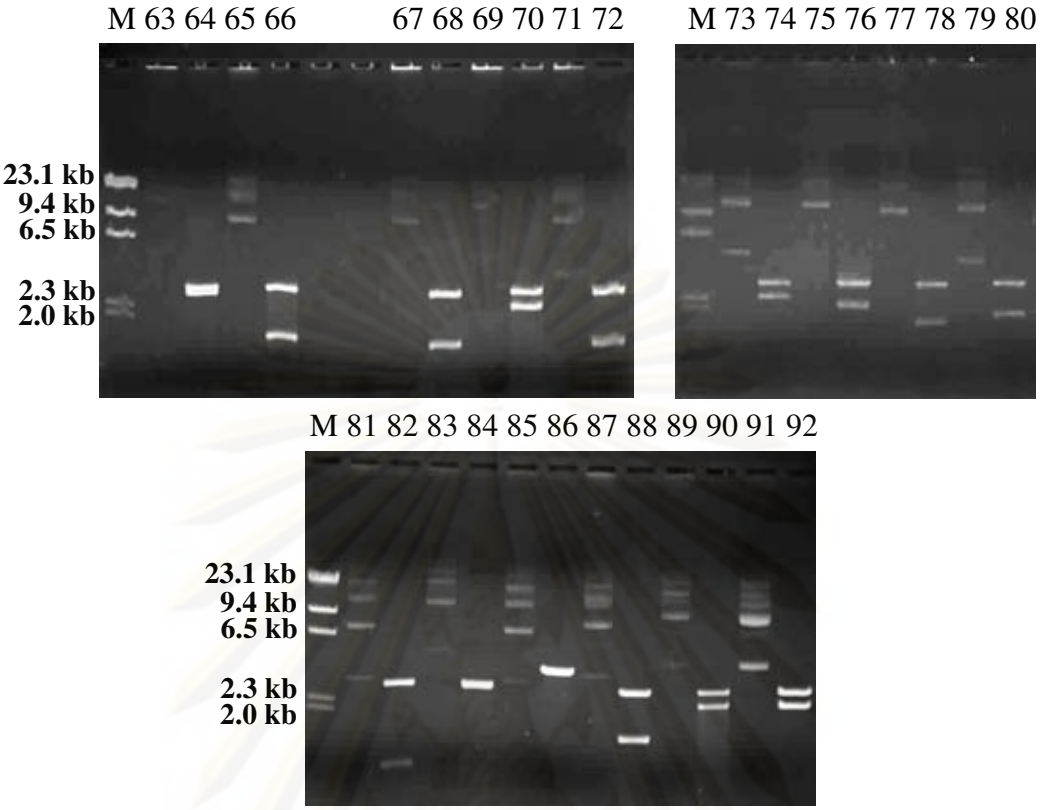
Lane 58 From colony29/ *Not*I+*Xho*I

Lane 59 From colony30

Lane 60 From colony30/ *Not*I+*Xho*I

Lane 61 From colony31

Lane 62 From colony31/ *Not*I+*Xho*I



Lanes M contain λ/*Hin*dIII marker.

Lane 63 From colony32

Lane 64 From colony32/ *Not*I+*Xho*I

Lane 65 From colony33

Lane 66 From colony33/ *Not*I+*Xho*I

Lane 67 From colony34

Lane 68 From colony34/ *Not*I+*Xho*I

Lane 69 From colony35

Lane 70 From colony35/ *Not*I+*Xho*I

Lane 71 From colony36

Lane 72 From colony36/ *Not*I+*Xho*I

Lane 73 From colony37

Lane 74 From colony37/ *Not*I+*Xho*I

Lane 75 From colony38

Lane 76 From colony38/ *Not*I+*Xho*I

Lane 77 From colony39

Lane 78 From colony39/ *Not*I+*Xho*I

Lane 79 From colony40

Lane 80 From colony40/ *Not*I+*Xho*I

Lane 81 From colony41

Lane 82 From colony41/ *Not*I+*Xho*I

Lane 83 From colony42

Lane 84 From colony42/ *Not*I+*Xho*I

Lane 85 From colony43

Lane 86 From colony43/ *Not*I+*Xho*I

Lane 87 From colony44

Lane 88 From colony44/ *Not*I+*Xho*I

Lane 89 From colony45

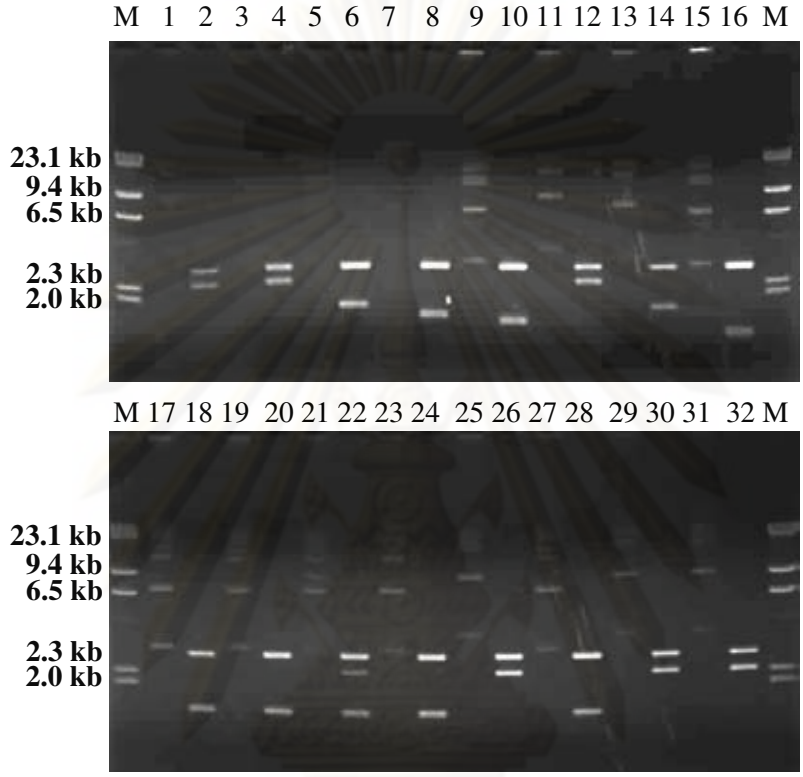
Lane 90 From colony45/ *Not*I+*Xho*I

Lane 91 From colony46

Lane 92 From colony46/ *Not*I+*Xho*I

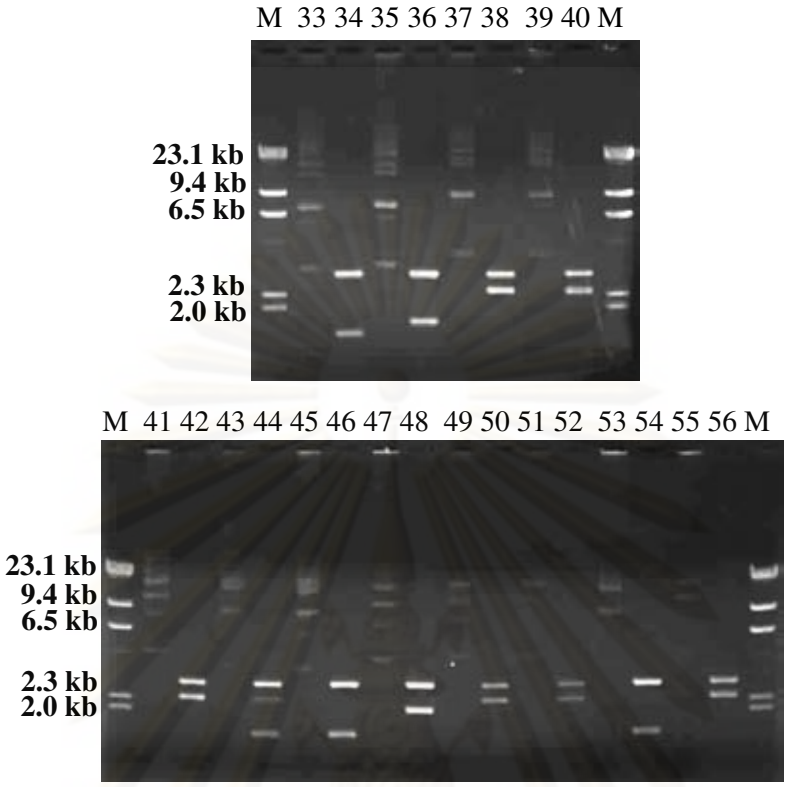
APPENDIX E

Agarose gel electrophoresis analysis of plasmids extracted from transformants of pCHI60B that were screened for the second time on LB agar plate with ampicillin



Lanes M contain λ/*Hin*dIII marker.

Lane 1 From colony1
Lane 2 From colony1/ *Not*I+*Xho*I
Lane 3 From colony2
Lane 4 From colony2/ *Not*I+*Xho*I
Lane 5 From colony3
Lane 6 From colony3/ *Not*I+*Xho*I
Lane 7 From colony4
Lane 8 From colony4/ *Not*I+*Xho*I
Lane 9 From colony5
Lane 10 From colony5/ *Not*I+*Xho*I
Lane 11 From colony6
Lane 12 From colony6/ *Not*I+*Xho*I
Lane 13 From colony7
Lane 14 From colony7/ *Not*I+*Xho*I
Lane 15 From colony8
Lane 16 From colony8/ *Not*I+*Xho*I
Lane 17 From colony9
Lane 18 From colony9/ *Not*I+*Xho*I
Lane 19 From colony10
Lane 20 From colony10/ *Not*I+*Xho*I
Lane 21 From colony11
Lane 22 From colony11/ *Not*I+*Xho*I
Lane 23 From colony12
Lane 24 From colony12/ *Not*I+*Xho*I
Lane 25 From colony13
Lane 26 From colony13/ *Not*I+*Xho*I
Lane 27 From colony14
Lane 28 From colony14/ *Not*I+*Xho*I
Lane 29 From colony15
Lane 30 From colony15/ *Not*I+*Xho*I
Lane 31 From colony16
Lane 32 From colony16/ *Not*I+*Xho*I



Lanes M contain λ/*Hin*dIII marker.

Lane 33 From colony17

Lane 34 From colony17/ *Not*I+*Xho*I

Lane 35 From colony18

Lane 36 From colony18/ *Not*I+*Xho*I

Lane 37 From colony19

Lane 38 From colony19/ *Not*I+*Xho*I

Lane 39 From colony20

Lane 40 From colony20/ *Not*I+*Xho*I

Lane 41 From colony21

Lane 42 From colony21/ *Not*I+*Xho*I

Lane 43 From colony22

Lane 44 From colony22/ *Not*I+*Xho*I

Lane 45 From colony23

Lane 46 From colony23/ *Not*I+*Xho*I

Lane 47 From colony24

Lane 48 From colony24/ *Not*I+*Xho*I

Lane 49 From colony25

Lane 50 From colony25/ *Not*I+*Xho*I

Lane 51 From colony26

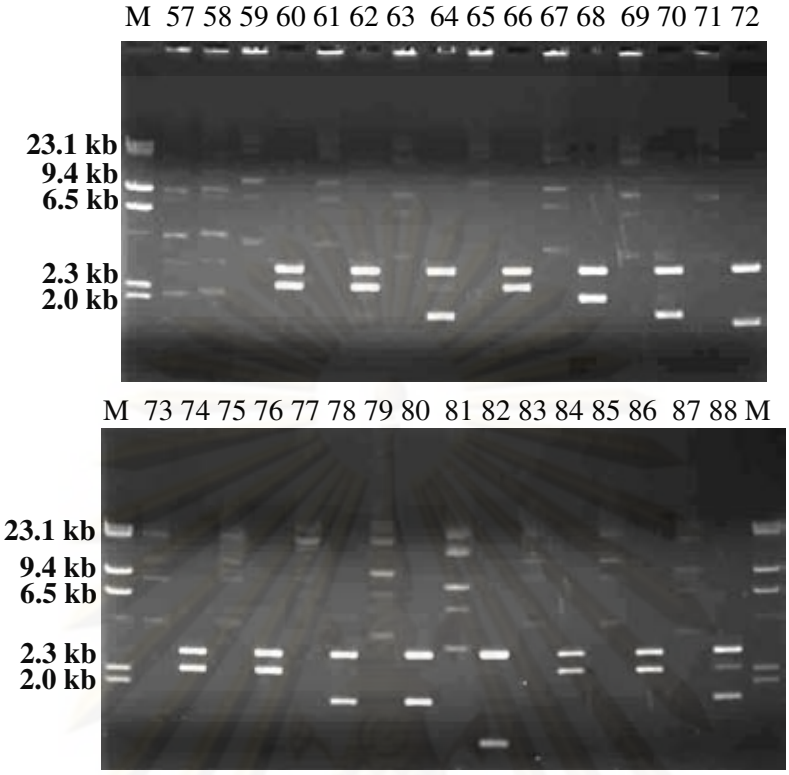
Lane 52 From colony26/ *Not*I+*Xho*I

Lane 53 From colony27

Lane 54 From colony27/ *Not*I+*Xho*I

Lane 55 From colony28

Lane 56 From colony28/ *Not*I+*Xho*I



Lanes M contain λ/*Hin*dIII marker.

Lane 57 From colony29

Lane 58 From colony29/ *Not*I+*Xho*I

Lane 59 From colony30

Lane 60 From colony30/ *Not*I+*Xho*I

Lane 61 From colony31

Lane 62 From colony31/ *Not*I+*Xho*I

Lane 63 From colony32

Lane 64 From colony32/ *Not*I+*Xho*I

Lane 65 From colony33

Lane 66 From colony33/ *Not*I+*Xho*I

Lane 67 From colony34

Lane 68 From colony34/ *Not*I+*Xho*I

Lane 69 From colony35

Lane 70 From colony35/ *Not*I+*Xho*I

Lane 71 From colony36

Lane 72 From colony36/ *Not*I+*Xho*I

Lane 73 From colony37

Lane 74 From colony37/ *Not*I+*Xho*I

Lane 75 From colony38

Lane 76 From colony38/ *Not*I+*Xho*I

Lane 77 From colony39

Lane 78 From colony39/ *Not*I+*Xho*I

Lane 79 From colony40

Lane 80 From colony40/ *Not*I+*Xho*I

Lane 81 From colony41

Lane 82 From colony41/ *Not*I+*Xho*I

Lane 83 From colony42

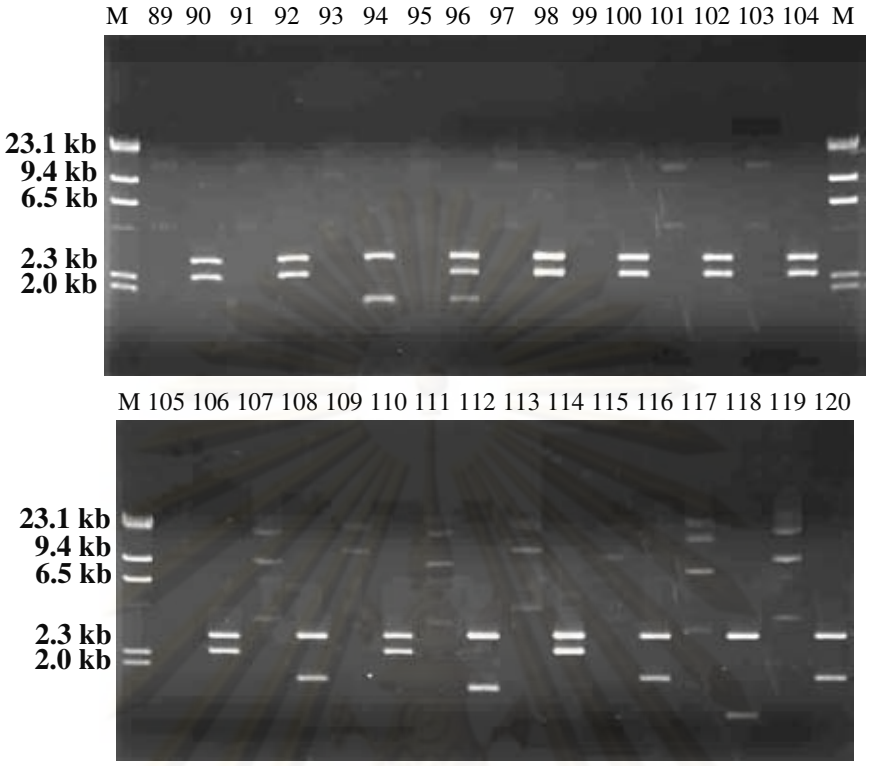
Lane 84 From colony42/ *Not*I+*Xho*I

Lane 85 From colony43

Lane 86 From colony43/ *Not*I+*Xho*I

Lane 87 From colony44

Lane 88 From colony44/ *Not*I+*Xho*I



Lanes M contain λ/*Hin*dIII marker.

Lane 89 From colony45	Lane 105 From colony53
Lane 90 From colony45/ *Not*I+*Xho*I	Lane 106 From colony53/ *Not*I+*Xho*I
Lane 91 From colony46	Lane 107 From colony54
Lane 92 From colony46/ *Not*I+*Xho*I	Lane 108 From colony54/ *Not*I+*Xho*I
Lane 93 From colony47	Lane 109 From colony55
Lane 94 From colony47/ *Not*I+*Xho*I	Lane 110 From colony55/ *Not*I+*Xho*I
Lane 95 From colony48	Lane 111 From colony56
Lane 96 From colony48/ *Not*I+*Xho*I	Lane 112 From colony56/ *Not*I+*Xho*I
Lane 97 From colony49	Lane 113 From colony57
Lane 98 From colony49/ *Not*I+*Xho*I	Lane 114 From colony57/ *Not*I+*Xho*I
Lane 99 From colony50	Lane 115 From colony58
Lane 100 From colony50/ *Not*I+*Xho*I	Lane 116 From colony58/ *Not*I+*Xho*I
Lane 101 From colony51	Lane 117 From colony59
Lane 102 From colony51/ *Not*I+*Xho*I	Lane 118 From colony59/ *Not*I+*Xho*I
Lane 103 From colony52	Lane 119 From colony60
Lane 104 From colony52/ *Not*I+*Xho*I	Lane 120 From colony60/ *Not*I+*Xho*I

Lanes M contain λ/*Hin*dIII marker.

Lane 121 From colony61

Lane 122 From colony61/ *Not*I+*Xho*I

Lane 123 From colony62

Lane 124 From colony62/ *Not*I+*Xho*I

Lane 125 From colony63

Lane 126 From colony63/ *Not*I+*Xho*I

Lane 127 From colony64

Lane 128 From colony64/ *Not*I+*Xho*I

Lane 129 From colony65

Lane 130 From colony65/ *Not*I+*Xho*I

Lane 131 From colony66

Lane 132 From colony66/ *Not*I+*Xho*I

Lane 133 From colony67

Lane 134 From colony67/ *Not*I+*Xho*I

Lane 135 From colony68

Lane 136 From colony68/ *Not*I+*Xho*I

BIOGRAPHY

Mr. Thanakorn Theerapanon was born on October, 22nd 1983 in Saraburi province of Thailand. He graduated with a Bachelor Degree in Biochemistry, Faculty of Science, Chulalongkorn University in 2005. He has enrolled in the Master Degree in Biochemistry, Faculty of Science, Chulalongkorn University in 2006. He participated in the 2nd Biochemistry and Molecular Biology (BMB) Conference on Biochemistry and Molecular Biology for Regional Sustainable Development on May, 7th -8th 2009 and his proceeding was published in the topic of Construction of chimeric chitinase from *Bacillus licheniformis* SK-1 chitinase 66 and *Serratia marcescens* chitinase B by *in vivo* homologous recombination.