PRODUCTION AND CHARACTERIZATION OF RECOMBINANT *BACILLUS SUBTILIS* CHITOSANASE

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

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การผลิต และศึกษาคุณสมบัติของ ไคโตซานเนสจาก *บาซิลลัส สับติลิส* ที่ผ่านการดัดแปลงพันธุกรรม

นางสาวพรศิริ เพชรศรีช่วง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PRODUCTION AND CHARACTERIZATION OF

RECOMBINANT *BACILLUS SUBTILIS* CHITOSANASE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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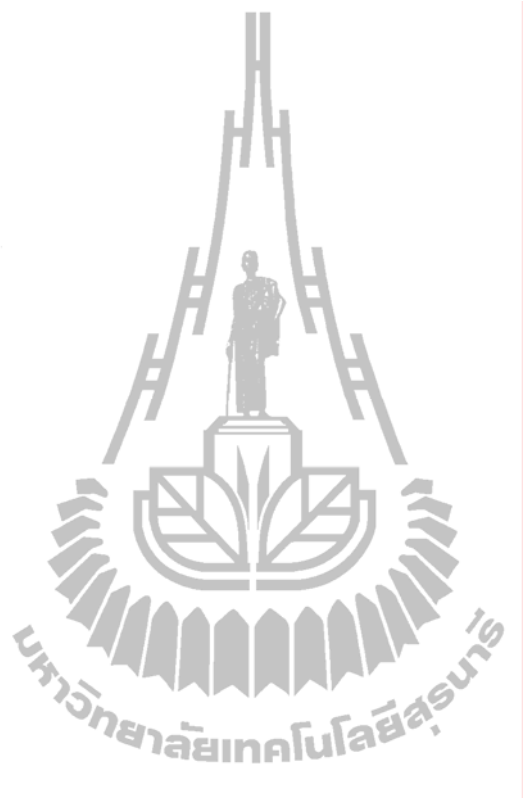
PHORNSIRI PECHSRICHUANG : PRODUCTION AND CHARACTERIZATION OF RECOMBINANT *BACILLUS SUBTILIS* CHITOSANASE. THESIS ADVISOR : ASSOC. PROF. MONTAROP YAMABHAI, Ph.D., 97 PP.

CLONING/RECOMBINANT CHITOSANASE/EXPRESSION/ SECRETION/CHITOOLIGOSACCHARIDE/*BACILLUS SUBTILIS*

Chitosanases (Csn) are enzymes that catalyse the hydrolysis of β-1,4 glycosidic bond of chitosan. One of the most important applications of chitosanases is for the bioconversion of chitosan into chitooligosaccharides (COS), which have various applications in medical, agricultural, or food industries. The objectives of this study were to clone, express, purify, and characterize the recombinant chitosanase from *Bacillus subtilis* 168. Two forms of recombinant chitosanase (Csn) were constructed by PCR-based cloning into pMY202 expression vector and overexpressed in *Escherichia coli* (*E. coli*) to compare the secretion efficiency and specific activity. For the first construct (CsnOmpApMY202), the native signal peptide of *B. subtilis* Csn was replaced with that of *E. coli* OmpA signal peptide by sub-cloning the DNA inserts into *Hind*III and *Bgl*II sites of the expression vector (OmpA-Csn). For the second construct (CsnNativepMY202), the native signal peptide was retained by sub-cloning the entire gene of *B. subtilis* Csn into *Nde*I and *Bgl*II sites of pMY202 vector (Native-Csn). Both of the recombinant Csn constructs were fused with C-terminal deca-histidine tag to facilitate one-step affinity purification using immobilized metal affinity chromatography (IMAC). The gene was overexpressed in *E. coli* TOP10 under the control of *tac* promoter. The specific activities of both recombinant

enzymes in three different compartments, i.e., cytoplasm, periplasmic space, and culture broth were analyzed. Our result demonstrated that the yield and specificity of the recombinant OmpA-Csn were higher than those of Native-Csn in all three compartments. Most importantly, at 20 h after induction, the amount of OmpA-Csn that was secreted into the culture broth was approximately 5-fold higher than that obtained from Native-Csn. Therefore, the construct CsnOmpApMY202 was used for the subsequent experiments. The enzyme was overexpressed by induction with 0.1 mM IPTG, and purified to homogeneity by Ni-NTA affinity column chromatography, and characterized. A total activity of approximately 45,000 U from culture supernatant and 12,000 U from cell lysate (periplasmic and cytosol) could be obtained from 1-L shake flask cultures. The specific activity of the purified enzyme when using low MW chitosan as substrate was 904.8 units/mg. The optimal pH of the enzyme was between pH 5.0-6.0, whereas the optimal temperature was between 40-50 ˚C. The recombinant Csn was stable within pH 2-11 after incubation for 3 hr, and within pH 2-9 after incubation for 24 hr, at 30˚C. In an absence of substrate, the enzyme was stable up to 40^{o}C for 30 min, and completely inactivated at 50^{o}C. However in the presence of substrates, the enzyme was thermostable with a half-life time of activity ($\tau 1/2$) of approximately 19 hr at 50˚C and pH 5.5. Analysis of hydrolytic products by thin layer chromatography (TLC) revealed that the recombinant *B. subtilis* chitosanase is an endo-chitosanase that prefers substrate longer than G3. Analysis of hydrolytic products using various types of chitosan by thin layer chromatography indicated that the enzyme could be used efficiently for the production of various lengths of chitooligosaccharides, ranging from dimer to hexamer (G2-G6). In conclusion, this research reports an efficient *E. coli* expression system for the expression, secretion,

and purification of a relatively thermo- and pH stable chitosanase from *B. subtilis*, suitable for various biotechnological applications.



School of Biotechnology Student's Signature_____

Academic Year 2011 Advisor's Signature_____

พรศิริ เพชรศรีช่วง : การผลิต และศึกษาคุณสมบัติของไคโตซานเนสจาก *บาซิลลัส สับติลิส* ที่ผ่านการดัดแปลงพันธุกรรม (PRODUCTION AND CHARACTERIZATION OF RECOMBINANT *BACILLUS SUBTILIS* CHITOSANASE)
อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.มณฑารพ ยมาภัย, ๙๗ หน้า.

เอนไซม์ไคโตซานเนส คือเอนไซม์ที่ทำหน้าที่เร่งปฏิกิริยาการย่อยสลายพันธะไกลโคซิดิคของไคโตซานในสภาวะที่มีน้ำ การประยุกต์ใช้เอนไซม์ไคโตซานเนสที่สำคัญที่สุดประการหนึ่งคือการใช้ย่อยไคโตซานด้วยวิธีทางชีวภาพ ให้เป็นไคโตโอลิโกแซคคาไรด์ หรือ คอซ ซึ่งสามารถนำมาใช้ประโยชน์ในด้านต่างๆได้อย่างหลากหลาย ทั้งทางด้านการแพทย์ การเกษตร การอาหาร วัตถุประสงค์ของการศึกษาวิจัยนี้คือ การโคลน การแสดงออก การทำให้บริสุทธิ์ และการศึกษาคุณสมบัติของเอนไซม์ไคโตซานเนส จาก*บาซิลลัส สับติสิส* (*Bacillus subtilis*) สายพันธุ์ ๑๖๘ ที่สร้างขึ้นจากการดัดแปลงพันธุกรรม โดยในขั้นแรกเอนไซม์ไคโตซานเนสที่เกิดจากการดัดแปลงพันธุกรรมนี้ ถูกสร้างขึ้นมาเป็นสองรูปแบบ เพื่อเปรียบเทียบคุณสมบัติในการปลดปล่อยเอนไซม์ออกสู่ภายนอกเซลล์ การสร้างเอนไซม์ทำโดยใช้ปฏิกิริยาลูกโซ่พีซีอาร์ในการเพิ่มปริมาณดีเอ็นเอแล้วจึงโคลนยีนเข้าสู่ดีเอ็นเอพาหะ ชื่อว่า pMY202 เพื่อให้สามารถแสดงออกได้ในแบคทีเรีย *เอสเชอริเชีย โคไล* หรือ *อี. โคไล* (*Escherichia coli* : *E. coli*) เอนไซม์รูปแบบแรก (CsnOmpA pMY202) สร้างขึ้นโดยตัดเปปไทด์ส่งสัญญาณของไคโตซานเนสที่ใช้ใน*บาซิลลัส สับติลิส* ออกแล้วแทนที่ด้วยเปปไทด์ส่งสัญญาณของ *อี. โคไล* ชื่อว่า OmpA จากนั้นโคลนชิ้นยีนเข้าสู่ดีเอ็นเอพาหะที่ตำแหน่งของเอนไซม์ตัดจำเพาะ *Hind*III และ *Bgl*II เรียกเอนไซม์ที่ผ่านการดัดแปลงพันธุกรรมนี้ว่า OmpA-Csn สำหรับการสร้างเอนไซม์ในรูปแบบที่สอง (CsnNativepMY202) สร้างโดยการโคลนยีนไคโตซานเนสที่ยังมีเปปไทด์ส่งสัญญาณของ *บาซิลลัส สับติลิส อยู่* เข้าสู่ดีเอ็นเอพาหะ pMY202 ที่ตำแหน่งของเอนไซม์ตัดจำเพาะ *Nde*I และ *Bgl*II เรียกเอนไซม์ที่ผ่านการดัดแปลงพันธุกรรมนี้ว่า Native-Csn ซึ่งเอนไซม์ไคโตซานเนสทั้งสองรูปแบบสามารถแยกให้บริสุทธิ์ได้ในขั้นตอนเดียว ด้วยวิธีการโครมาโตกราฟฟีคอลัมน์แบบจับจำเพาะกับโลหะที่ตรึงอยู่กับที่ (IMAC) เนื่องจากมีกรดอะมิโนฮิสทิดีนจำนวนสิบโมเลกุลต่ออยู่ที่ปลายคาร์บอกซีของโปรตีน จากนั้นยีนไคโตซานเนสได้ถูกทำให้แสดงออกเป็นจำนวนมากใน *อี. โคไล* สายพันธุ์ทอปเทน แล้วทำการวิเคราะห์กิจกรรมจำเพาะของเอนไซม์ทั้งสองรูปแบบที่สกัดจากสามส่วนคือ ไซโตพลาสซึมบริเวณช่องว่างระหว่างผนังเซลล์ และในน้ำเลี้ยงเชื้อ ผลการทดลองพบว่าปริมาณเอนไซม์และกิจกรรมจำเพาะของ OmpA-Csn สูงกว่า Native-Csn ในทั้งสามส่วน โดยเฉพาะอย่างยิ่งหลังจากเหนี่ยวนำให้เกิดการแสดงออกเป็นเวลา ๒๐ ชั่วโมง OmpA-Csn สามารถถูกปลดปล่อยออกมา

สู่ภายนอกเซลล์แบคทีเรียไปยังอาหารเลี้ยงเชื้อได้มากกว่า Native-Csn ประมาณ ๕ เท่า ดังนั้นจึงเลือกใช้ CsnOmpApMY202 ในการทดลองขั้นต่อไป โดยจากการหาสภาวะที่เหมาะสมในการผลิตพบว่า OmpA-Csn ถูกเหนี่ยวนำให้เกิดการแสดงออกของโปรตีนในปริมาณมากที่สุด เมื่อใช้ IPTG ความเข้มข้น ๐.๑ มิลลิโมล่าร์ และกระตุ้นให้แสดงออกเป็นเวลา ๒๐ ชั่วโมง จากนั้นได้ทำการแยกเอนไซม์ให้บริสุทธิ์ด้วยวิธีการโครมาโตกราฟฟีคอลัมน์แบบจับจำเพาะ (Ni-NTA) ผลจากการวิเคราะห์ความสามารถในการทำกิจกรรมของเอนไซม์ พบว่าเมื่อทำการเลี้ยงเซลล์ *อี. โคไล* ที่มียีนไคโตซานเนส ในอาหารเลี้ยงเชื้อปริมาณ ๑ ลิตร ให้กิจกรรมเอนไซม์จากน้ำเลี้ยงเชื้อประมาณ ๔๕,๐๐๐ หน่วย และในสารสกัดจากเซลล์ประมาณ ๑๒,๐๐๐ หน่วย ค่ากิจกรรมจำเพาะของเอนไซม์บริสุทธิ์มีค่า ๘๐๔.๘ หน่วยต่อมิลลิกรัมเอนไซม์ ค่าความเป็นกรดด่างที่เหมาะสมต่อการทำงานของเอนไซม์อยู่ในช่วง ๕ ถึง ๖ ขณะที่อุณหภูมิที่เหมาะสมอยู่ในช่วงอุณหภูมิ ๔๐ ถึง ๕๐ องศาเซลเซียส ส่วนเสถียรภาพของเอนไซม์ พบว่าเมื่อบ่มเอนไซม์ที่อุณหภูมิ ๓๐ องศาเซลเซียส เอนไซม์สามารถทนต่อค่าความเป็นกรดเป็นด่างได้ในช่วงค่าความเป็นกรดด่าง ๒ ถึง ๑๑ เป็นเวลา ๓ ชั่วโมง และที่ค่าความเป็นกรดด่าง ๒ ถึง ๙ เป็นเวลา ๒๔ ชั่วโมง ส่วนเสถียรภาพของเอนไซม์ที่อุณหภูมิต่างๆ พบว่าเอนไซม์มีเสถียรภาพที่อุณหภูมิ ๔๐ องศาเซลเซียส เป็นเวลา ๓๐ นาที และถูกยับยั้งกิจกรรมโดยสมบูรณ์ที่อุณหภูมิ ๕๐ องศาเซลเซียส อย่างไรก็ตาม เมื่อบ่มเอนไซม์กับสารตั้งต้น เอนไซม์จะมีความทนต่ออุณหภูมิสูงขึ้น โดยมีค่าครึ่งชีวิตที่ประมาณ ๑๙ ชั่วโมง ที่อุณหภูมิ ๕๐ องศาเซลเซียส และค่าความเป็นกรดด่าง ๕.๕ จากการศึกษาผลิตภัณฑ์ที่เกิดจากปฏิกิริยาการย่อยสลายในสภาวะที่มีน้ำของเอนไซม์ด้วยวิธีโครมาโตกราฟฟีแบบแผ่นบาง (TLC) พบว่าไคโตซานเนสจาก *บาซิลลัส สับติลิส* ที่ผ่านการดัดแปลงพันธุกรรม เป็นเอนไซม์ที่ตัดจากภายใน และมีความจำเพาะต่อไคโตโอลิโกแซคคาไรด์ที่มีสายยาวมากกว่า ๓ หน่วยย่อย (G3) ทั้งนี้จากการศึกษาความสามารถในการย่อยสลายเมื่อใช้ไคโตซานชนิดต่างๆ เป็นสารตั้งต้นนั้น พบว่าเอนไซม์มีประสิทธิภาพในการผลิตไคโตโอลิโกแซคคาไรด์ในช่วงความยาว ๒ ถึง ๖ หน่วย (G2-G6) ดังนั้นจึงเห็นได้ว่า งานวิจัยนี้ได้แสดงถึงประสิทธิภาพในการใช้ระบบการแสดงออกของยีนใน *อี. โคไล* เพื่อการผลิตเอนไซม์ที่มีประสิทธิภาพสูง รวมทั้งยังแสดงถึงประสิทธิภาพในการใช้ระบบนี้ในการหลั่งเอนไซม์ที่ต้องการออกสู่ภายนอกเซลล์ และวิธีที่มีประสิทธิภาพในการแยกเอนไซม์ให้บริสุทธิ์ ซึ่งเอนไซม์ไคโตซานเนสที่สร้างจากการดัดแปลงทางพันธุกรรมนี้มีคุณสมบัติที่ค่อนข้างทนต่ออุณหภูมิ และค่าความเป็นกรดเป็นด่างสูงจึงความเหมาะสมต่อการประยุกต์ใช้ในงานทางเทคโนโลยีชีวภาพด้านต่างๆ ต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2554 ลายมือชื่ออาจารย์ที่ปรึกษา_____

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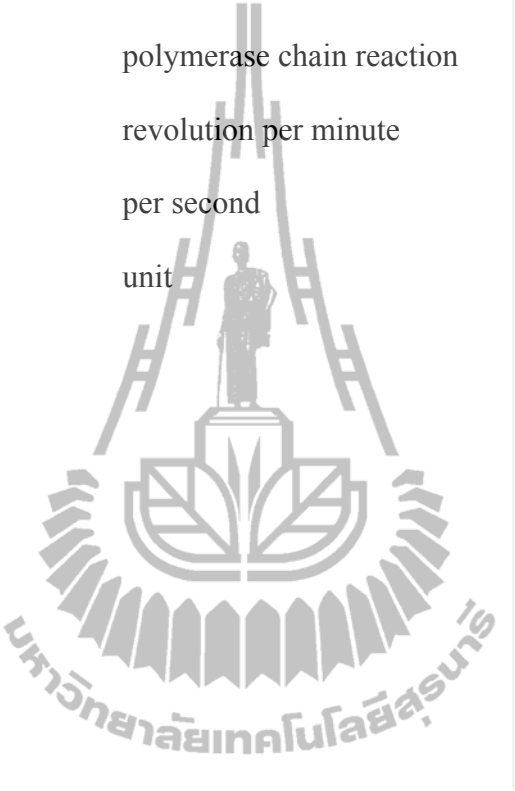
LIST OF ABBREVIATIONS

^{o}C	=	degree celcius
µg	=	microgram
µL	=	microlitre
µmole	=	micromole
bp	=	base pair
DNA	=	deoxyribonucleic acid
hr	=	hour
et. al.	=	Et alia (and other)
g	=	gram
kb	=	Kilobase
kDa	=	kilodalton
L	=	litre
M	=	molarity
mg	=	milligram
mM	=	millimolar
mL	=	milliliter
PCR	=	polymerase chain reaction
rpm	=	revolution per minute
s^{-1}	=	per second
U	=	unit
mg	=	milligram

LIST OF ABBREVIATIONS (Continued)

mM	=	millimolar
mL	=	milliliter
PCR	=	polymerase chain reaction
rpm	=	revolution per minute
s^{-1}	=	per second
U	=	unit



CHAPTER I

INTRODUCTION

1.1 Significant of this study

Chitin is the second most abundant biopolymer on earth after cellulose. Exoskeleton of insects, cell walls of various fungi, crab and shrimp wastes are the main sources of chitin (Ravi Kumar, 2000). It is a β-(1,4)-linked polymer of *N*-acetyl D-Glucosamine (GlcNAc) (Haki & Rakshit, 2003). Chitosan, a D-glucosamine polymer (GlcN), is completely or partially deacetylated derivative of chitin (San-Lang Wang et al., 2006). It is usually obtained by the artificial deacetylation of chitin in the presence of alkaline.

The biodegradation of chitin in crustacean shell waste is very slow, so that accumulation of large quantities of discards seafood processing industry has become a major environmental concern. The amount of worldwide shrimp and prawn production was 6,091,869 tons in 2005 (FAO, 2008). The wastes are abundantly dumped into the sea, whereas a small part is dried and used as chicken feed (Xu et al., 2008). Disposal of shellfish processing discards has, thus, been a challenge for most of the shellfish-producing countries. Out of the different species of crustaceans, shrimp and crab shell wastes have been widely used for the isolation of chitin. The production of value-added products such as chitin and their derivatives and application in different fields are therefore of utmost interest. The oligosaccharide of chitin and chitosan have various potential application in the field of food

(Fernandes et al., 2008), agricultural (Hadwiger et al., 1984), and pharmaceutical (Dou et al., 2009) industries. Today, commercial interest in the conversion of chitin or chitosan to bioactive chito-oligosaccharides (COS), using enzymatic reaction such as chitosanase has been increased. This is because many problems exist in chemical processes such as the production of a large amount of short chain oligosaccharides, low yields of oligosaccharides, high cost in separation, and environmental pollution (Su et al., 2006). Thus, chitosanase hydrolysis has become more popular in recent years.

Recent work in my laboratory revealed that endo-chitinase (ChiA) from *Bacillus licheniformis* DSM8785 has suitable property for industrial application. However, when the enzyme was incubate with colloidal chitin in difference pH at 50^{o}C, only 2 majors products (GlcNAc and chitobiose $(GlcNAc)_2$) could be obtain (Songsiriritthigul et al., 2010). In order to obtain longer chain of COS, one attractive strategy is to use chitosanase to hydrolyze soluble chitosan in by an endo-type fasion. This strategy has been used to produce oligosaccharides ranging from dimer to octamer of GlcN (Choi et al., 2004) because chitosanase have difference hydrolytic specificities for acetylate and non-acetylated units near the cleavage point (Uragami & Tokura, 2006). An efficience method for the synthesis of longer chain COS is attractive because it has been suggest that these oligomers may be more advantageous than chitin and chitosan in field of food additive and nutraceuticals (Shahidi et al., 1999). Chitooligosaccharides that are longer than hexamer have been reported to have more potent antimicrobial, antitumor, and immunopotentiating activities, when compared to shorter oligosaccharides (Choi et al., 2004). This research focus on using enzyme technology to convert low-value chitin wastes from shrimp and crab shells into high-value chitooligosaccharide.

1.2 Research objective

1. To clone chitosanase genes from *Bacillus subtilis* strain 168
2. To over-express recombinant chitosanase gene using *E. coli* system.
3. To purify recombinant chitosanase by one-step affinity purification using immobilized metal affinity chromatography (IMAC)
4. To characterize the basic properties of the recombinant chitosanase such as amino acid sequence, effect of pH and temperature on enzyme activity, and kinetic parameters.
5. To analyze COS that will be produced from various bioconversion processes.

CHAPTER II

LITERATURE REVIEW

2.1 Chitin

Chitin is the second most abundant biopolymer on earth after cellulose. It is widely distributed in nature, particularly as a structural polysaccharide in a fungal cell wall in the exoskeleton of arthropods, the outer shell of crustaceans, nematodes, etc (Dahiya et al., 2006). The structure of chitin is a β-(1,4)-linked polymer of *N*-acetyl D-Glucosamine (GlcNAc) chains (Figure1) arranged in microcrystalline structures of antiparallel sheets (α-chitin), parallele sheets (β-chitin) and a combination of both (γ-chitin) (Maria et al., 2008). The α -Chitin is the most abundant; it occurs in fungal and yeast cell walls, in krill, in lobster and crab tendons and shells, and in shrimp shells, as well as in insect cuticle. β–chitin occurs in association with proteins in squid pens (Zhang et al., 2000). Chitin is insoluble in water because of the highly extended hydrogen bonded semi-crystalline structure (Pillai et al., 2009) . The non-solubility of chitin in almost all common solvents has been an obstacle in its appropriate utilization . Molecular structure and hydrogen bonding in α-chitin and β-chitin show in figure 2.

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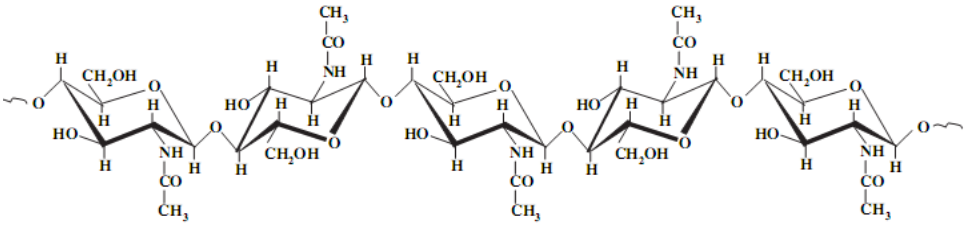

Figure 1. Chemical structure of Chitin (Prashanth & Tharanathan, 2007)

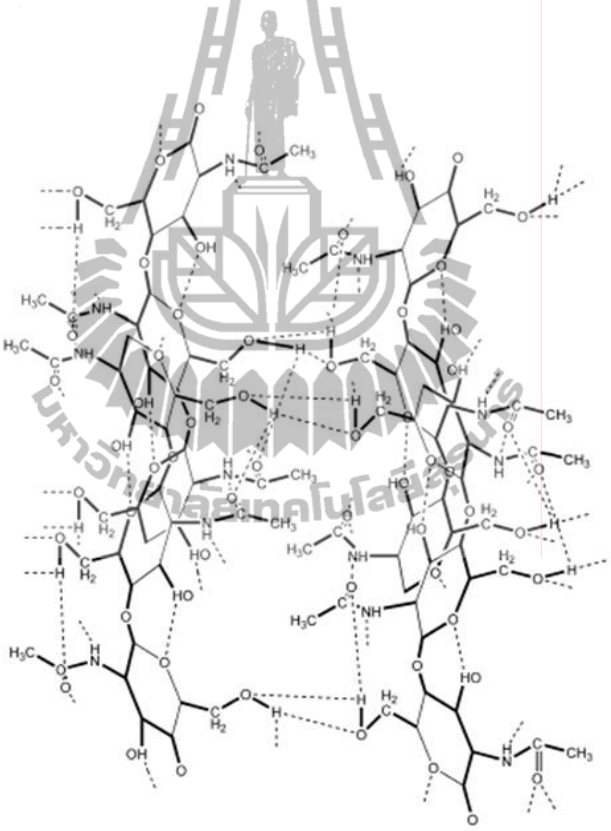

Figure 2. Molecular structure and hydrogen bonding in chitin (Khor, 2001)

2.2 Chitosan

Chitosan is completely or partially deacetylated derivative of chitin by treating with alkali solution , consisting of β-1,4 linkages of GlcNAc and D-glucosamine (GlcN) (Figure 2). It was found in nature in the mycelia and sporangiophore walls of some fungi, mainly in Mucorales (phylum Zigomicota) (Fenton & Eveleigh, 1981). It consists of three types of reactive functional groups, an amino/acetamido group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively. The amino/acetamido contents are the main reason for the differences between their structures and physicochemical properties (Xia et al., 2011). Chitosan becomes water soluble following the formation of salt with organic acids such as formic acid, acetic acid and ascorbic acid (Uragami & Tokura, 2006). It is high molecular weight, high viscosity and poor solubility at pH > 6.5 are serious drawback in many of its potential applications. However, the anvantage of chitosan and its derivatives are biodegradable, non toxic and non-allergenic. Various applications of chitosan derivative include antimicrobial agents, edible films, antioxidants, food additives, etc. (Maria et al., 2008).

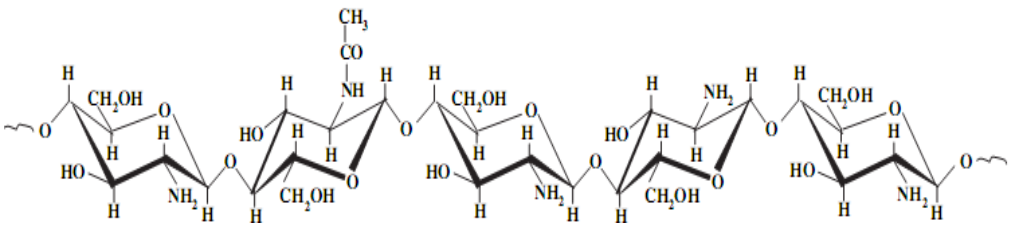

Figure 3. Chemical structure of Chitosan (Prashanth & Tharanathan, 2007)

2.3 Chitinolytic enzyme

2.3.1 Chitinase

The enzymes responsible for chitin degradation are chitinases (EC3.2.1.14), which are found in a variety of organisms such as bacteria, fungi, insects, plants, and animals (Tanaka et al., 2004). Chitinases can be classified into two major categories:,ie., endochitinase and exochitinase. Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites, generating low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose, and diacetylchitobiose. Exochitinases can be divided into two subcategories: chitobiosidases (EC 3.2.1.29), which catalyze the progressive release of diacetylchitobiose starting at the nonreducing end of chitin microfibrilles, and β-(1,4) N-acetyl glucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc.

Enzymatic degradation of chitin is significant to several biological processes such as cuticle destabilization and partial degradation in crustaceans, morphogenesis and nutrition in fungi and pathogenesis in virus (Maria Hayes, 2008).

2.3.2 Chitosanase

Chitosanase or chitosan *N*-acetylglucosaminohydrolase (EC 3.2.1.132) catalyzes the hydrolysis of glycosidic bond of chitosan into chitooligosaccharide (COS). It is a member of glycosyl hydrolase (GH) families including GH5, GH7, GH8, GH46, GH75, and GH80, according to the CAZy database (http://www.cazy.org/). GH 5, 7 and 8 contain a few cases of chitosanase activity. Families GH75 and GH80 have only a few members and there were no structural and very little functional information available. Among these familes, GH46 have been studied extensively in terms of their catalytic features, enzymatic mechanisms and protein structures (Lacombe-Harvey et al., 2009; Marcotte et al., 1996). Chitosanases can be classified into three classes depending on their cleavage specificity (Figure 4). ClassI cleaves GlcNAc–GlcN and GlcN–GlcN bonds, classII cleaves only the GlcN–GlcN bonds and classIII chitosanases cleaves GlcN–GlcNAc and GlcN–GlcN bonds (Tanabe et al., 2003). Chitosanase have been found in a variety of microorganism such as bacteria, fungi and a few in plant (Jo et al., 2003) . Many of them are derived from the genus *Bacillus* that belong to GH families 8 and 46 (Table1). There have been a number of reports on the characterization of chitosanase from difference bacteria including *Bacillus coagulans* CK108 (Yoon et al., 2002), *Bacillus* sp. Strain CK4 (Yoon et al., 2000) , *Bacillus* sp. MET 1299 (P. I. Kim et al., 2004), *Bacillus* sp. DAU101 (Y. Lee et al., 2006), *Jantinobacterium* sp. Strain 4239 (Johnsen et al., 2010), *Burkholderia gladioli* strain CHB101(Shimosaka et al., 2000) and etc. Their properties are shown in table1.

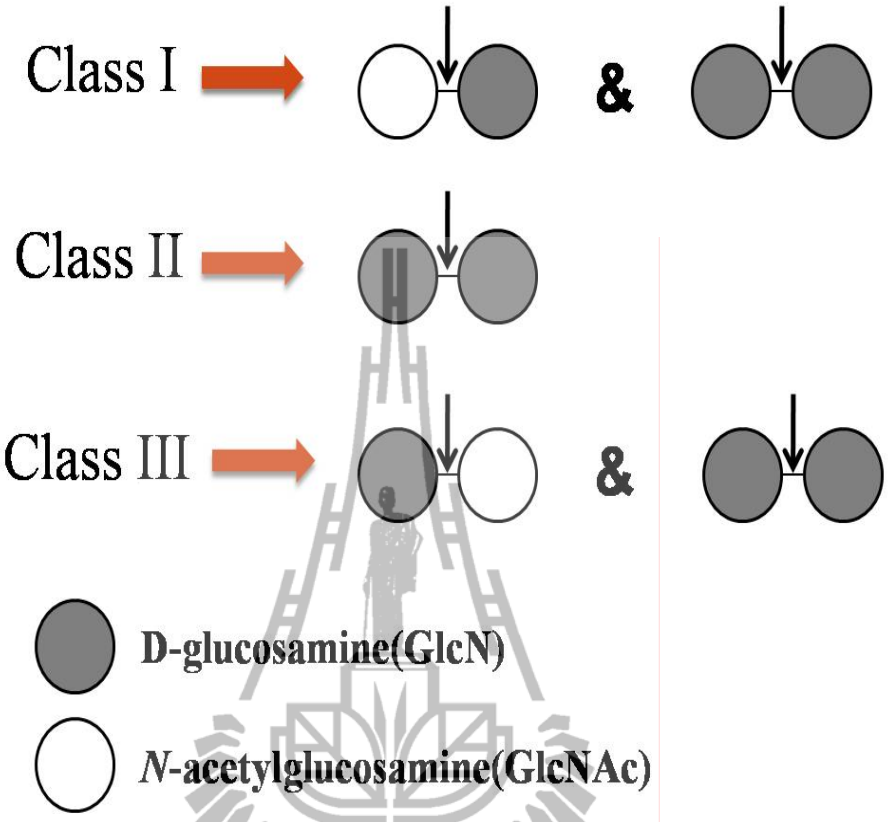

Figure 4. Schematic diagram of three class of chitosanase.

Table 1. Properties of various chitosanases

Organism	Family GH	Native/ Recombinant	Specific activity (Yield)	Kinetic parameter (K_m and V_{max})	Optimal pH/temperature	Stability	Ref
Aeromonas sp. HG08	-	Native	8.5 x 10^2 U/mg [a] (ND)	K_m 7 mg/ml V_{max} 840 U/mg	pH 6.6, 55°C	85% activity at 40°C for 2h	(Yuying et al., 2009)
Aspergillus sp. Cj22-326 ChiA ChiB	ND	Native Native	6.46 U/mg[a] 18.26 U/mg[a] (ND)	ND	pH 4.0, 50°C pH6.0, 65°C	ND	(Xiao'e et al., 2005)
Bacillus cereus D-11	8	Native	347 U/mg[a] (0.55g/l)	K_m 7.5 mg/ml V_{max} 21.5µmol/s/mg	pH 6.0/60°C (chitosan)	Stable below 50°C and from pH5-8	(Gao et al., 2008)
Bacillus cereus S1	-	Native	196 U/mg[a] (ND)	ND	pH 6.0, 60°C	Stable at pH 6-11 at 40°C for 1h and temperature below 60°C	(Kurakake et al., 2000)

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Table 1. (continued)

Organism	Family GH	Native/ Recombinant	Specific activity (Yield)	Kinetic parameter (K_m and V_{max})	Optimal pH/temperature	Stability	Ref
Bacillus circulans MH-K1	46	Native	160 U/mg[d] (ND)	K_m 0.63 mg/ml V_{max} 12.5 µmol/min/ml (colloidal chitosan)	pH 6.5/50°C (colloidal chitosan)	Stable at 0-40°C and pH4.0-9.0	(Yabuki et al., 1988)
Bacillus coagulans CK108	-	Recombinant (*E. coli* BL21(DE3))	ND[a] (ND)	K_m 1.7 mM [a] V_{max} 516 U/mg (soluble chitosan)	pH 6.5/65°C (chitosan)	73% activity at 80°C for 1hr	(Yoon et al., 2002)
Bacillus licheniformis MB-2	-	Native	38.21 units/mg[a] (ND)	K_m 0.23 mg/ml V_{max} 843 U/mg (chitosan 100%DDA)	pH 6-7/70°C (chitosan)	ND	(Ekowati et al., 2006)
Bacillus megaterium P1 (chitosanase A)	-	Native	154.8 U/mg[c] (1.05 mg/l)	K_m 0.8 mg/ml V_{max} 280 U/mg	pH 4.5-6.5/50°C (chitosan)	Stable up to 45°C	(Pelletier & Sygusch, 1990)

Table 1. (continued)

Organism	Family GH	Native/ Recombinant	Specific activity (Yield)	Kinetic parameter (K_m and V_{max})	Optimal pH/temperature	Stability	Ref
Bacillus subtilis 168	46	Native	56.9 U/mg[a] (ND)	K_m 0.1 mg/ml V_{max} 66.3 U/mg	pH 5.7/ 60°C (chitosan)	Stability decrease above 50°C	(Rivas et al., 2000)
Bacillus subtilis 168 (this study)	46	Recombinant	904.7 U/mg[a] (14mg/l)	K_m= 1.57 mg/ml V_{max}= 530.64 µmole min^{-1} mg^{-1} K_{cat} = 2.0x10^5 s^{-1} (Chitosan low MW)	50°C/pH 5-6	Stable at pH 2-10 for 24 h and temperature 4-40 for 30 min (without substrate)	This study
Bacillus subtilis CH2	-	Native	161 U/mg[d] (ND)	ND	pH 5.5/ 60°C	Stable up to 40°C	(Oh et al., 2011)
Bacillus subtilis IMR-NK1	ND	Native (mutant)	2819 units/mg[b] (ND)	ND	pH4.0/45°C	Stable at 30-40°C for 20 min and pH5-9 for 1 hr at 25°C	(Chiang et al, 2003)

Table 1. (continued)

Organism	Family GH	Native/ Recombinant	Specific activity (Yield)	Kinetic parameter (K_m and V_{max})	Optimal pH/temperature	Stability	Ref
Bacillus subtilis KH1	-	Native	1.4 U/mg[d] (ND)	ND	pH7.0/50°C (chitosan)	Stable at pH5.5-6.6 and temperature up to 40°C for 3h	(Omumasaba et al., 2000)
Bacillus subtilis RKY3	-	Native	18.5 U/mg[a] (ND)	ND	pH 6.0/ 60°C (chitosan)	Stable at pH 3-9, and temperture 10-45°C	(Wee et al., 2010)
Bacillus subtilis TKU007	-	Native	0.0603 U/mg[a] (755mg/l)	ND	37°C, pH7	Stable at pH4-9 and 37°C	(San-Lang Wang & Yeh, 2008)
Bacillus thuringiensis Strain JAM-GG01	8	Native	412 units/mg[a] (1mg/l)	K_m 0.23 mg/ml V_{max}7.7µmol/sec/mg (chitosan)	pH 5.6/60°C (chitosan)	pH 2-4 at 30°C for 30 min and inactive at 45°C for 15 min	(Kobayashi et al., 2011)

Table 1. (continued)

Organism	Family GH	Native/ Recombinant	Specific activity (Yield)	Kinetic parameter (K_m and V_{max})	Optimal pH/temperature	Stability	Ref
Bacillus sp. CK4	-	Recombinant (*E. coli* BL21(DE3))	ND[a] (ND)	K_m 1.2 mg/ml V_{max} 151 U/mg (chitosan 83%DDA)	pH 7.7/ 55°C (chitosan)	66% activity at 80°C for 1hr	(Yoon et al., 2000)
Bacillus sp. KCTC 0377BP	46	Native	1700 U/mg[a] (1.55mg/l)	K_m 1.1 mg/ml (chitosan)	pH 4-6/60°C (chitosan)	Stable at pH 4-8 and temperature <50°C	(Choi et al., 2004b)
Bacillus sp. MET 1299	-	Recombinant (*E. coli* TOP10)	476 U/mg[a] (7.8 mg/l)	ND	pH 5.6 / 60°C (colloidal chitosan)	Stable at pH 5.5-9.0 and temperature below 60°C	(Kim et al, 2004)
Bacillus sp. P16	8	Native	47.8 U/mg[b] (4.3mg/l)	K_m 0.52 mg/ml V_{max}7.7µmol/sec/mg	60°C, pH5.5	pH 4.5-10 and under 50°C	(Jo et al., 2003)

Table 1. (continued)

Organism	Family GH	Native/ Recombinant	Specific activity (Yield)	Kinetic parameter (K_m and V_{max})	Optimal pH/temperature	Stability	Ref
Bacillus sp. S65	8	Native	93.6 U/mg[a] (8.87 mg/l)	ND	pH6/65°C (chitosan)	Stable from pH5.5-6.5 at temperature below 40°C	(Su et al., 2006)
Bacillus strain	8	Recombinant	270-290 U/mg [b] (500mg/l)	ND	50-55°C	40-50°C	(Liu et al., 2009)
Burkholderia gladioli strain CHB101	46	Native	160 U/mg[a] (0.015mg/l)	ND	pH5-7.7 / 55°C	ND	(Shimosaka et al., 2000)
Gongronella sp. JG	-	Native	82 U/mg[a] (0.82mg/l)	K_m 4.5 mg/ml	pH 4.6-4.8/50°C	Stable at temperature Below 50°C	(Wang et al., 2008)

Table 1. (continued)

Organism	Family GH	Native/ Recombinant	Specific activity (Yield)	Kinetic parameter (K_m and V_{max})	Optimal pH/temperature	Stability	Ref
Janthinobacterium sp. Strain 4239	46	Recombinant (*E. coli*)	1500 U/mg[b] (60mg/l)	ND	pH5-7/45°C (chitosan80%DDA)	Stable at temperture up to 50°C	(Johnsen et al., 2010)
Matsuebacter chitosanotabidus 3001	-	Native	250.2 U/mg[b] (ND)	ND	pH 4 , 30-40°C	Stable at pH 4.0-9.0 at 37°C for 30 min and 95% activity at 40°C for 1h	(Park et al., 1999)
Nocadioides sp.	-	Native	41.8 U/mg [a] (ND)	ND	pH 5/ 55-60°C (chitosan 95-100%DDA)	Stable at pH 5.0-5.5 50% activity at 60°C for 15 min	(Okajima et al., 1995)
Penicillium islandicum	-	Native	10 U/mg [c] (ND)	K_m 1.4 mg/ml V_{max} 2.7 µmol min^{-1}	pH4-6/ 45°C	Stable at pH 3.5-8.5	(Fenton & Eveleigh, 1981)

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Table 1. (continued)

Organism	Family GH	Native/ Recombinant	Specific activity (Yield)	Kinetic parameter (K_m and V_{max})	Optimal pH/temperature	Stability	Ref
Psudomonas sp. A-01	46	Recombinant	3.9 U/mg [b] (8.6mg/L)	ND	pH5	pH 5-8	(Ando et al., 2008)
Serratia marcescens TKU011	-	Native	0.1 U/mg[b] (76.6mg/l)	ND	pH 5/50°C (chitosan)	Stable at pH 4-8 and temperature below 50°C	(Wang et al., 2008)
Sphingomonas sp. CJ-5	-	Native	ND[a] (ND)	ND	pH6.5/56°C (chitosan)	Stable up to 45°C for 1 h at pH 5-8	(Zhu et al., 2007)
Streptomyces cyaneogriseus	-	Native	120 U/mg[b] (0.175 mg/l)	ND	pH 5.0/50°C	Stable at pH 3.0-8.0 and 40-60°C	(El-Sherbity, 2010)

Table 1. (continued)

Organism	Family GH	Native/ Recombinant	Specific activity (Yield)	Kinetic parameter (K_m and V_{max})	Optimal pH/temperature	Stability	Ref
Streptomyces griseus HUT 6037	5	Native	86.2 U/mg[b] (1.14 mg/l)	ND	pH 6.0/ 60°C (chitosan)	Stable at pH 6.0-9.0 and temperature up to 40°C for 15 min	(Tanabe et al., 2003)
Trichoderma reesei PC-3-7 (Exo-β-D-glucosaminidase)	-	Native	27.3 U/mg[a] (0.06 mg/l)	ND	pH 4.0/ 50°C (chitosan 100%DDA)	Stable at pH6-9 and temperature below 50°C	(Nogawa et al., 1998)

Specific chitosanase activities were determined using various assay methods, i.e., [a]DNS method, [b]Schales' procedure (Jarle Horn & Eijsink, 2004), [c]somogyi-nelson method (Nelson, 1944; Smogyi, 1952) , [d]Morgan method (Good & Bessman, 1964)

2.4 Preparation of COS

Chitosan can be cleaved by hydrolyzing agents due to the presence of rather unstable of glycosidic bonds. There are difference methods for degradation of *O*-glycosidic linkages of chitosan, which leads to the production of COS varying in the degree polymerization (Kim & Rajapakse, 2005). Different physical methods, such as acid hydrolysis, enzymatic hydrolysis, ultrasonic degradation, microwave, gamma-ray, or hydrothermal have been used, but these methods are not as efficient as the enzymatic method. (Aam et al., 2010).

The industrial techniques to extract chitin and and chitosan from crab and shrimp shell wastes mainly employs stepwise chemical method consisting of three steps:demineralization, deproteinization and bleaching. Demineralization can be achieved using diluted HCl (1-8%) at room temperature for 1-3 hr or using other acid such as acetic and sulfuric acid. The industrial methods usually utilize aqueous base solutions such as NaOH or KOH for the deproteinization step. Industrial methods may also include a decolourization step using NaOCl or H_2O_2 solutions as a bleaching agent. Chitin deacetylation forming chitosan may follow chitin extraction by using very strong NaOH solution to deesterify the N-acetyl linkages at room or elevated temperatures (Maria Hayes, 2008). After alkaline deacetylation, some of the amino groups may remain acetylated and distribute randomly along the whole polymer chain.

The industrial production of COS, acid hydrolysis using concentrated hydrochloric acid is commonly used to convert chitosan to COS. However, chemical hydrolysis results in low yield and a large amount of monomeric D-glucosamine units. The main disadvantage of this method is that it generate a large quantities of

hazardous chemical wastes and have a possibility for contamination with the toxic chemical compounds (Kim & Rajapakse, 2005). As a result, enzymatic method has been suggested to be a more efficient method because the yield of COS preparation that are enriched for certain know compounds can be regulated by controlling the enzyme, the source of chitosan starting material and the reaction conditions, which determine the extent to which the hydrolysis is allowed to occur (Aam et al., 2010). The schematic representations of chitin and chitosan are shown in Figure 3.

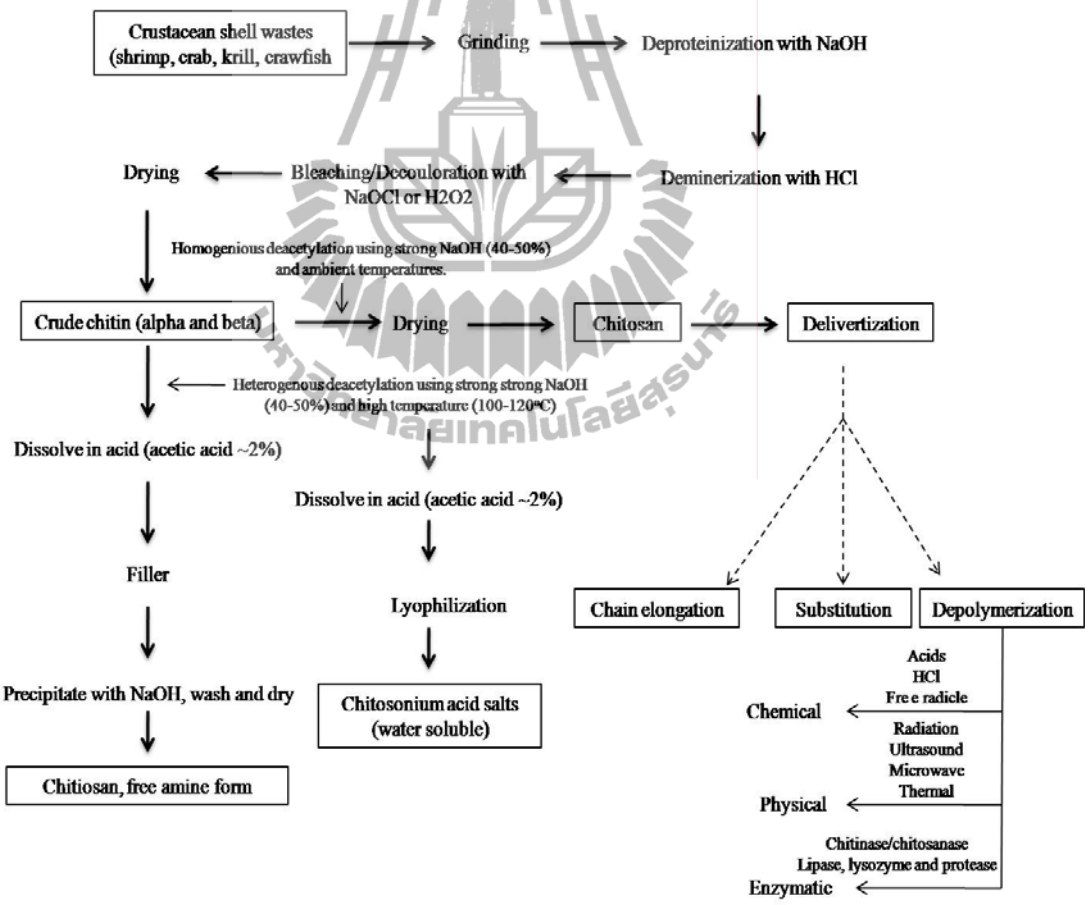

Figure 5. Chitin and chitosan manutacture by industrial methods (Maria Hayes, 2008)

2.5 Applications of chitooligosaccharide (COS)

In recent years, chitooligosaccharides have received growing attention because these oligosaccharides have low viscosity, and water soluble at the neutral pH and possess a numerous of biological properties. Almost of the molecular mechanisms behind these bioactivities are still unknown. The properties of COS such as degree of deacetylation (DDA), degree of polymerization (PA), charge distribution, and nature of chemical modification are important factors influencing the biological activities of COS (Muzzarelli, 1996). COS have the various applications in many fields such as food, agriculture and medical application.

2.5.1 Food application

2.5.1.1 Antimicrobial activity

Antimicrobial activity of COS against several bacterial species has been recognized and is considered as one of the most important properties. The antibacterial activity of these compounds is influenced by a number of factors such as degree of polymerization, level of deacetylation, type of microorganism and some other-chemical properties (Kim & Rajapakse, 2005). It has previously been reported that 85-95% deacetylation of COS showed the highest antibacterial activity (Chung et al., 2004). COS possess primary amino groups in their structures. The number of these amino groups has proven to play a major role in antibacterial activity and several mechanisms have been proposed to describe this activity (Yen-Meng et al., 2002). The mostly accepted mechanism explains that COS can alter permeability characteristics of microbial cell membrane and further prevent the entry of materials or cause leakage of cell constituents that finally leads to death of bacteria (Sudarshan et al., 1992).

2.5.1.2 Nutritional value

Researches on COS in food and nutrition have emphasized their ability to improve food quality and human health promotion. COS are easily absorb through intestine, quickly get into the blood flow and have a systemic biological effects in the organism. In food industry, COS attracts a greater interest as antioxidants and enhancers of nutritional quality of food (Shahidi et al., 1999a). There have previous been reported that the mixture of COS showed a growth stimulatory effect on *Lactobacillus casei*, and *Lactobacillus brevis* at a concentration of 0.1%. These results indicated that COS can be used as a prebiotic ingredient in the food industry, though the mechanism of its growth stimulatory effect is still unknow (Lee et al., 2002).

Recently, a nutritional significance of COS in animals has been demonstrated which indicated that COS are an effective feed additives. The effects of chitooligosaccharide supplementation on the growth performance of pig that fecal *Lactobacillus* was increased while *E. coli* was decreased when COS was used at level of 200 mg/kg (Liu et al., 2008). Similarly, COS supplementation can decrease fecal *E. coli* without impacting the concentration of *Lactobacilli* in the guts of growing broiler chickens (Li et al., 2007).

2.5.2 Agriculture application

2.5.2.1 Inhibitory of plant virus

COS as been reported o suppress viral infections in various biological systems. The mechanism of antiviral activity is not well understood. However, one possible explanation is that cationic charges of amino groups of COS may have additional functions to activate the immune and defense systems in plants. It has been reported that COS could inhibit tobacco mosaic virus (TMV) on tobacco leaves and the best disease control effect was observed when TMV inoculation was conducted at 24 h after 50 g/ml COS application (Yin et al., 2010).

2.5.2.1 plant elicitor

In the recently year, chitooligosaccharide is reported to be effective at eliciting plant innate immunity against plant diseases in lots of plants such as tobacco, rapeseed, rice, grapevine, etc (Yin et al., 2010). For example, it was reported that COS has the ability to stimulate defense responses in leaves of rice. It was found that reactive oxygen species (ROS) accumulated in rice after treatment with 0.1% of COS (Agrawal et al., 2002). Moreover, COS can be used in cooperation with other pesticides and biocontrol agents. COS is effective on several plant–disease interactions. A survey in China suggests that COS can produce resistance to the plant disease (Yin et al., 2010). The mechanism of COS induce plant immunity is still not well understood. Therefore, further research is required on the study mechanisms of COS induction of plant immunity.

2.5.3 Medical application

2.5.3.1 Antitumor activity

There have been reported that COS could inhibit the growth of tumor cells by exerting the immunoenhancing effects. The antitumor activities observed in COS are dependent on their structure characteristics such as DDA and molecular weight. It could be observed that, medium molecular weight of COS ranging from 1.5-5.5 kDa could efficiently inhibit the growth of Sarcoma 180 solid (S180) or Uterine cervix carcinoma No.14 (U14) tumor in BALB/c mice (Jeon & Kim, 2002). Moreover, the water soluble of COS prepare with a mixture of tetramer and pentamer could inhibited the growth of sacroma 180 (S180) tumor cells in the mice. The result showed that the water-soluble chitosan with higher MW than hexamer might have better antitumor activity (Qin et al., 2002).

2.5.3.2 Wound-dressing

The use of chitosan and COS in wound dressings have been explored and positive effects have been documented, which were shown to accelerate the wound healing process (You et al., 2004). Chitosan is converted to COS by naturally occurring enzymes and that the activity observed for chitosan might in fact be caused by COS. Then, COS are thought to accelerate wound healing by enhancing the functions of inflammatory and repairing cells (Usami et al., 1997; Usami et al., 1998). For example, it has been shown that subcutaneous injection of $GlcN_6$ enhanced migration of polymorphonuclear cells in dogs (Usami et al., 1997). It must be noted that most authors ascribe the beneficial effect of longer CHOS and chitosan on wound healing to the ability of these compounds to form biocompatible ordered tissue-like structures (Riccardo A.A, 2009).

Many biological activity of COS have been reported including the potentials to be used as a vector for gene therapy, drugs against asthma, malaria, and antifungal agents, ingredients for bone strengthening, as well as substances for lowering serum glucose in diabetetics. Many biological activity of COS have been reported but the molecular mechanisms are mostly unclear.

CHAPTER III

MATERIAL AND METHOD

3.1 Materials

3.1.1 Bacterial strains and plasmid

Bacillus subtilis strain168 (ATCC23857) was obtained from American Type Culture Collection. The bacteria were grown on NA agar at 30˚C. *E. coli*DH5α (Life Technologies) and TOP10 (Invitrogen) was used as a cloning and expression host, respectively. The plasmid pMY202, which was used for cloning and expression of *B. subtilis* chitosanase gene, was constructed in our laboratory.

3.1.2 Substrate

Chitosan practical grade [product number 417963, ≥75% degree of deacetylation (DDA)], low molecular weight [product number 448869 (75-85% DDA)] and medium molecular weight [product number 448877(75-85% DDA)] were purchased from Sigma-Aldrich. Chitosan middle viscous (28191) and chitosan highly viscous (48165) were purchased from Fluka. Chitosan solution (commercial grade) was purchased from Poodang168.Co, Ltd (Thailand). Chitosan powder (commercial grade) was purchased from NNC products.,Co.,Ltd. (Thailand). Chitosan-oligosaccharides (G2-G6) were purchased from Seikagaku (Tokyo, Japan).

3.1.3 Instruments

Centrifuge:	Sorvall RC 5C plus, USA
	Centrifuge 5810R, Eppendortf, USA
ELISA plate reader:	Sunrise, TECAN, Austria
Heat box:	Thermomixer Comfort, Eppendorf AG, Hamburg, Germany
Incubator shaker:	Innova 2300 platform shaker, New Brunswick, Scientific, USA
	C24 Incubator shaker, New Brunswick Scientific, USA
Electrophoresis:	Mini-PROTEAN tetra cell**,** Bio-Rad, USA
Microcentrifuge:	Mini spin plus, Eppendorf, USA
PCR machine:	DNA Engine PCT 200 peltier Thermo cycler, MJ Research, USA
pH meter:	Ultra basic pH meter UB-10, Denver Instruments, Germany
Rotator:	FINEPCR, Korea

3.2 Methods

3.2.1 Cloning of chitosanase gene from *Bacillus subtilis* strain168

The gene encoding precursor enzyme containing native signal peptide or mature enzyme were clone by PCR-based method according to previously published protocol (Songsiriritthigul et al., 2010, ; Songsiriritthigul et al., 2009). The primers were designed from the published database of the DNA sequence of the chitosanase gene of *B. subtilis* 168 (NCBI accession number: NC_000964 REGION: complement (2747984..2748817). The primers B.subCsnNdeIFw: 5' CTG TGC CAT ATG AAA ATC AGT ATG CAA AAA GCA GAT TTT TGG 3' and B.subCsnBamHIRv: 5' GCA CAG GGA TCC TTT GAT TAC AAA ATT ACC GTA CTC GTT TGA AC 3' were used for PCR amplification of *B. subtilis* chitosanase gene containing native signal peptide (Native-Csn). The PCR products were cut with *Nde*I and *BamH*I and cloned into *Nde*I and *Bgl*II sites on the pMY202 plasmid. For the construction of recombinant chitosanase gene, of which the native signal peptide was replaced with that of *E. coli* OmpA signal peptide (OmpA-Csn), primers B.subCsnOmpAHindIIIFw: 5' CTGTGCAAG CTT CGG CGG GAC TGA ATA AAG ATC AAA AGC3'and B.subCsnBamHIRv: 5' GCA CAG GGA TCC TTT GAT TAC AAA ATT ACC GTA CTC GTT TGA AC 3' were used in the PCR reaction. The PCR products were cut with *Hind*III and *BamH*I and ligated with pMY202 that has been digested with *Nde*I and *Bgl*II restriction enzymes. The recombinant constructs of *B. subtilis* chitosanase containing native or OmpA signal peptide were designated as CsnNativeApMY202and CsnOmpApMY202, respectively. The integrity of the constructs were confirmed by automated DNA sequencing (Macrogen, Korea).

3.2.2 Expression of recombinant chitosanase gene in *E. coli*

The expression of recombinant *B. subtilis* chitosanase was done according to previously published protocol (Juajun et al., 2011). Briefly, a single colony of freshly transformed *E. coli* TOP10 habouring appropriate constructs was grown in Luria Bertani (LB) broth containing 100μg/ml ampicillin (LB-Amp) overnight, at 37°C. Then 2% of the overnight cultures was added into 0.4-1 L LB-Amp broth and grown at 37°C, 250 rpm until an OD_{600} reached 0.6-0.7. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the culture broth to a final concentration of 0.1 - 1 mM, and the incubation was continued at room temperature with vigorous shaking (180rpm) for 4-20 h. The cells were harvested by centrifugation at 4,000 x *g* for 30 min at 4°C. Preparation of periplasmic extract and cell lysate were done as previously described (Songsiriritthigul et al., 2010).

3.2.3 Purification of recombinant chitosanase

An optimized condition for a lab-scale expression of recombinant chitosanase was done by picking 3-5 colonies of *E. coli* Top10, freshly transformed with CsnOmpApMY202 expression vector, and induced with 0.1 M IPTG when an $OD_{600}$of the culture reached 0.6-0.7. The induction was allowed to continue at room temperature (~27 ˚C) for 20 h with vigorous shaking. To facilitate a small-scale preparation in the laboratory, the enzyme was purified from cell lysate instead of from the cuture broth. The recombinant 10x His-tagged chitosanase was purified by immobilized metal affinity chromatography (IMAC), using Ni-NTA agarose (Qiagen), according to the manufacturer's instruction (Qiagen). Twenty ml of the crude enzyme from cell lysate was loaded into an Ni-NTA column that had been pre-equilibrated

with the equilibration buffer (30 mM Tris-HCl, 300 mM NaCl and 10 mM imidazole), and incubated at 4^{o}C with rotation for 2 h. Then, the column was washed with wash buffer (30 mM Tris–HCl buffer, pH 8.0 and 300 mM NaCl and 20 mM Imidazole) three times. The enzyme that bound with Ni-NTA resin was eluted with elution buffer containing 250 mM imidazole (30 mM Tris–HCl buffer, pH 8.0 and 300 mM NaCl and 250mM Imidazole). The eluted enzyme was passed through Vivaspin6 column M_r cut-off 10 kDa (GE Healthcare, Sweden) to remove imidazole and concentrate the protein. The purified enzyme was stored at 4^{o}C until used.

Protein concentration was determined by the method of Bradford (Bradford, 1976) using Bio-Rad protein assay kit, and bovine serum albumin (BSA) as the standard. The standard calibration curve was constructed from 0.05-0.5 mg/ml of BSA.

3.2.4 SDS-PAGE and Zymogram analysis

Denaturing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (Laemmli, 1970). Protein band was stained by Coomassie brilliant blue R-250. Protein ladder (10-200 kDa) was purchased from Fermentas (St. Leon, Germany) and Bio-Rad. The protein samples were briefly heated (3 min) in the loading buffer (Laemmli buffer) at 100˚C, using a heat block.

For the zymogram analysis of chitosanase, the protein samples were mixed in a loading buffer (Laemmli buffer) without reducing agent and heating. The proteins were electrophoresed in a 12% SDS-PAGE that was placed on ice. After electrophoresis, the gel was rinsed with de-ionized water, then soaked in 100 mM sodium acetate

buffer pH5.5, containing 1% Triton X-100 for 3 h with shaking, on ice. After the gel was rinsed with 10 mM sodium acetate buffer pH5.5 several times, the protein gel was laid on top of a polyacrylamide gel, containing 0.1% chitosan. The gel was then incubated in 10 mM sodium acetate buffer, pH5.5, at 50°C for 18 hours. The chitosanase activity on gel was visualized by staining the gel with 0.1% congo red, followed by de-staining with 1N NaCl.

3.2.5 Enzyme activity assay on agar plate

The activity of the recombinant chitosanase was assayed on LB agar plates containing 100 µg/ml ampicillin and 0.1% of chitosan low MW. Freshly transformed cells were spotted onto the plates and incubated at 37 ◦C overnight. *E. coli* colonies expressing recombinant chitosanase were spotted onto LB-Amp agar plates containing 0.1% chitosan and incubated at 37°C. Hydrolytic clear zones were observed after overnight incubation.

3.2.6 Chitosanase activity assay

Standard chitosanase activity was determined using the dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction mixer consists of 40 µl of dilute enzyme (0.4 µg) and 160 µl of 0.5% soluble chitosan in 200 mM sodium acetate buffer, pH5.5, which was pre-incubated at 50˚C for 30 min. The reaction was incubated in a Thermomixer Comfort (Eppendorf AG, Hamburg, Germany) at 50˚C for 5 min, with mixing at 900 rpm**.** The reaction was stopped by adding 200 µl of 3,5 dinitrosalicylic acid (DNS) solution, and centrifuged at 12,000 x g for 5 min to precipitate the remaining chitosan. Then, the colour was developed by heating at 100°C for 20 min and cooled on ice. The reducing sugar in the supernatant was determined by measuring OD

at 540 nm, using D-glucosamine 1-5 µmole/ml as a standard. The reactions were done in duplicate and their mean and standard deviation values were reported. One unit of chitosanase was defined as the amount of enzyme that released 1 µmol of D-glucosamine per minute under the standard assay condition.

3.2.7 Characterization of enzyme

3.2.7.1 Effect of pH on enzyme activity

The optimal pH of *B. subtilis* chitosanase was determined between pH 2.4-6.5 under the standard assay condition, using two buffer system (250 mM each): glycine-HCl (pH 2.4-4.4) and sodium acetate buffer (pH 4-7.0). The reaction mixture consisted of 1.12 µg of purified enzyme and 0.5% low molecular weight chitosan (Sigma, product number 448869) (Notably, it was not possible to determine the enzyme activity at pH > 7.0 because chitosan substrates were insoluble at this condition).

To determine pH stability, 28 µg of purified enzyme in a total volume of 100 µl was pre-incubated in various buffers without the substrate at 30˚C, for 3 or 24 h. The buffers used were 100 mM each of glycine-HCl (pH 2-4), sodium acetate (pH 4-7), Tris-HCl (pH 7-9), and glycine-HCl (pH 9-12). After pre-incubation, the reactions were diluted 10 times in total volume 200 µl, then 40 µl of the diluted samples were taken to determine the remaining activity under the standard assay condition.

3.2.7.2 Effect of temperature on enzymatic activity

The optimal temperature of *B. subtilis* chitosanase was measured by incubating 0.4 µg of the purified enzyme with 0.5% chitosan practical grade (Sigma, product number 417963) at temperatures ranging from 0-80°C, for 5 minutes at pH5.5. Thermal stability of the enzyme without substrate was determined by incubating 15 µg of the purified enzyme in 50 mM sodium acetate buffer, pH 6.0, at various temperatures ranging from 0-80°C for 30 min, then the remaining enzyme activity was measured under the standard assay condition.

To measure the thermal stability of *B. subtilis* chitosanase in the presence of substrates, 18 µg of the purified enzyme in a total volume of 50 µl was pre-incubated with 0.5% chitosan or 10mM of chitosan oligosaccharide (G1-G6) in 50 mM sodium acetate buffer pH5.5, at 50°C for 30 min. After incubation, the reactions were diluted 30 times in a total volume of 300 µl, and then 40 µl of the diluted samples were taken to determine the remaining activity under the standard assay condition. In addition, the thermal inactivation kinetics at 50˚C in the presence of chitosan was measured by incubating 18 µg of the purified enzyme with 0.5% chitosan in 50 mM sodium acetate buffer pH 5.5, at 50°C for 30 min. After incubation, the reactions were diluted 10 times in a total volume of 300 µl, and then 40 µl of the diluted samples was taken to determine the remaining activity under the standard assay condition at various time points (0, 0.5, 1, 6, 12 and 24 h).

3.2.8 Kinetic analysis

The kinetic parameters were analyzed using various concentrations of low molecular weight chitosan, ranging from 0.8-9.6 mg/ml. The reactions were performed at 50°C for 5 minutes. The kinetic parameters were calculated by nonlinear regression, and the observed data were fitted to the Henri–Michaelis–Menten equation using Sigma Plot (SPSS Inc., Chicago, IL, USA).

3.2.9 Analysis of the hydrolytic products by thin layer chromatography (TLC)

Hydrolysis of chito-oligosaccharides (G2-G6) was carried out in a 50 µl of reaction mixture, containing 10mM substrate and 0.16 µg of purified enzyme in 50mM sodium acetate buffer, pH 5.5. The reaction was incubated at 50°C with shaking for 2, 5, 10, 30 and 60 minutes, and then terminated by boiling for 10 min. To analyze the product by TLC, each reaction mixture was applied five times (1 µl each) onto a Silicagel 60 F254 aluminum sheet (6.0x10.0 cm) purchased from Merck (Damsladt, Germany) and chromatographed two times (2 h each) in a mobile phase containing 28-30% ammonium water: water : Isopropanol (2:4:14) (v/v). The products were detected by wiping the TLC plate with a cotton ball soaked with 10% sulphuric acid in ethanol, followed by baking at 180°C for 3 min. The mixture of 10 mM chito-oligosaccharides (G1-G2) and 5 mM chito-oligosaccharides (G3-G6) was used as a standard.

CHAPTER IV

RESULT

4.1 Cloning of chitosanase gene from *Bacillus subtilis* strain168

Chitosanase gene from *B. subtilis* 168 was cloned into pMY202 vector, which is a cloning and expression vector. The *tac* promoter was used to control the expression of recombinant chitosanase, which can be induced for over-expression by IPTG. The vector contains OmpA signal peptide was fused at the N-terminal and a deca-histidine tag was fused at the C-terminal (Figure 6). Two forms of recombinant chitosanase (Csn) were constructed by PCR-based cloning into pMY202 expression vector to compare the secretion efficiency and specific activity. For the first construct (CsnOmpApMY202), the native signal peptide of *B. subtilis* Csn was replaced with that of *E. coli* OmpA signal peptide by cloning the DNA insert into *Hind*III and *Bgl*II sites of the expression vector. The PCR products (~ 726 bp) were digested with *Hind*III and *Bam*HI and cloned into corresponding restriction sites on the pMY202 plasmid. These resulted in the fusion of *E. coli* OmpA signal peptide with the mature enzyme (OmpA-Csn) (Figure 6A). For the second construct (CsnNativepMY202), the native signal peptide was retained by cloning the entire gene of *B. subtilis* Csn into *Nde*I and *Bgl*II sites of the pMY202 vector (Native-Csn). The PCR products (~ 831 bp) were digested with *Hind*III and *Bam*HI and cloned into corresponding restriction sites on the pMY202 plasmid (Figure 6B). The positive clone was check by using restriction enzyme digestion (Figure 7)

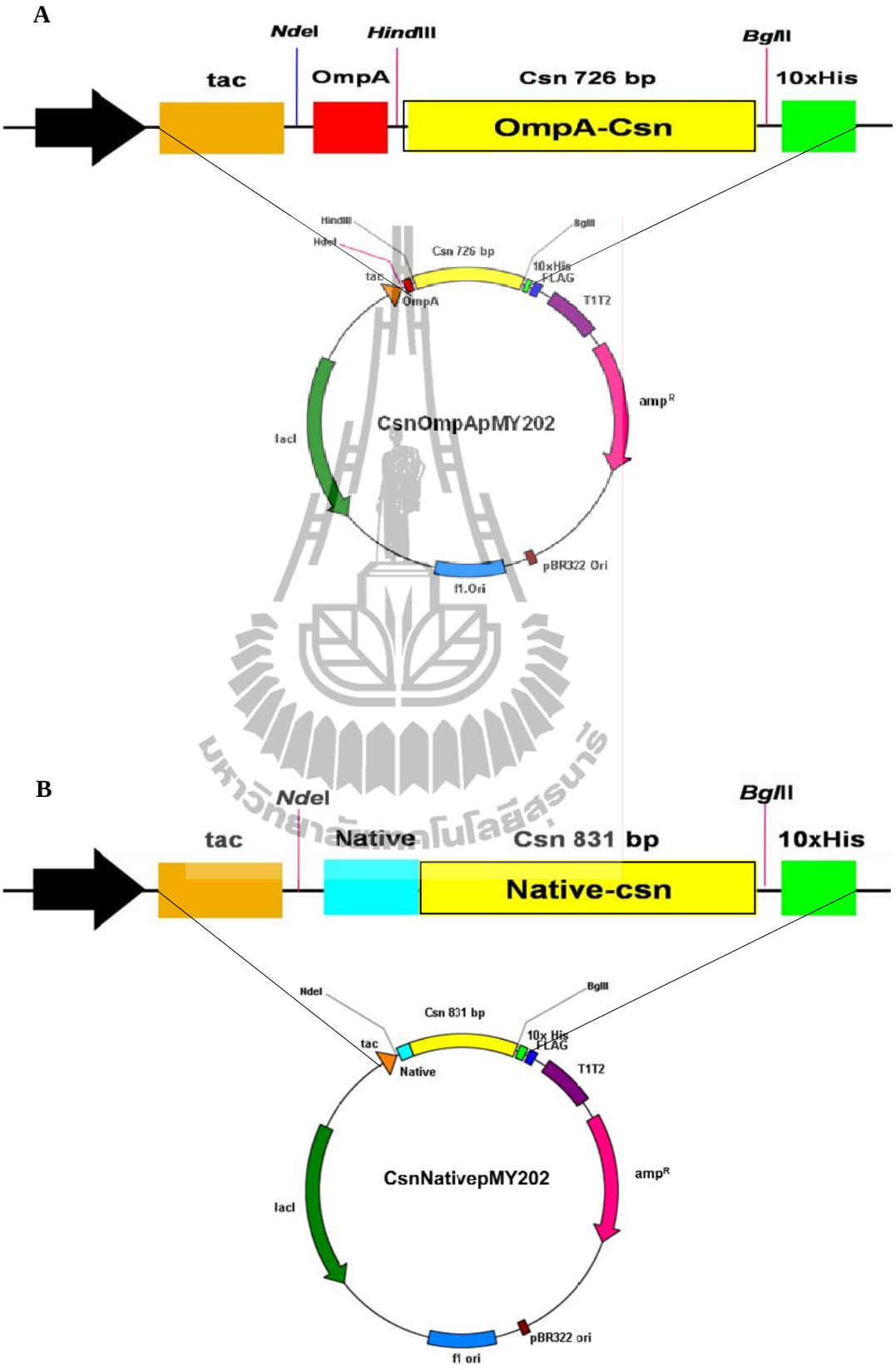

Figure 6. Map of recombinant chitosanase from *Bacillus subtilis* 168

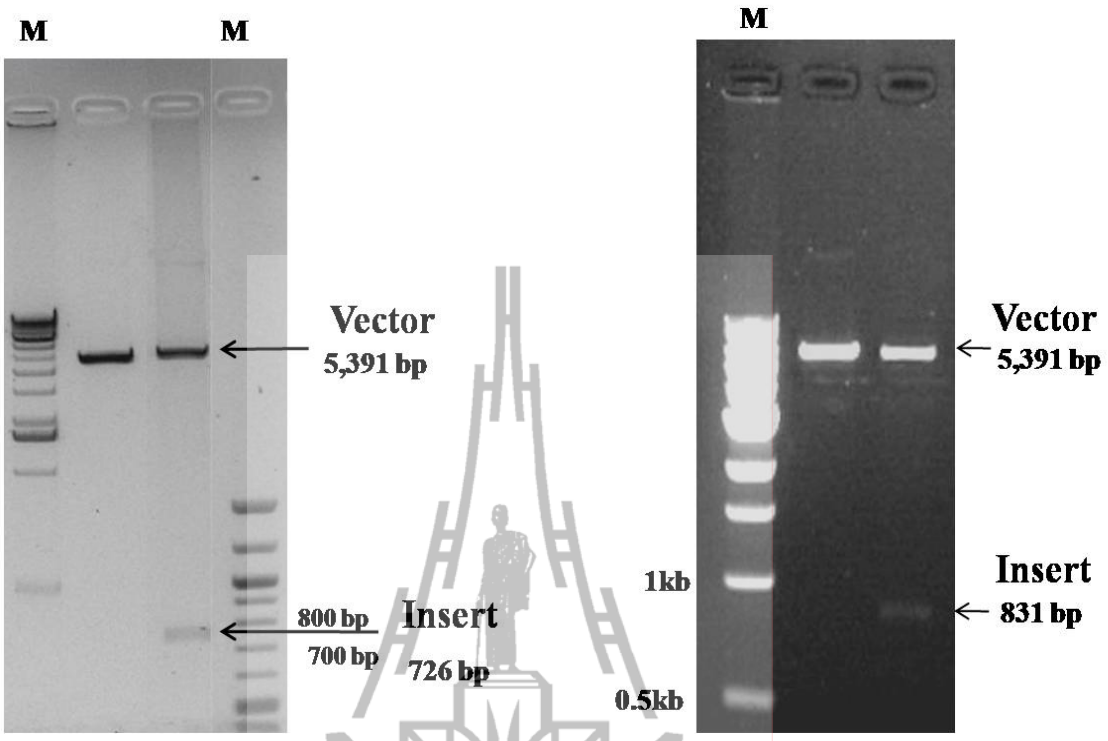

Figure 7. Agarose gel analysis of restriction enzyme digestion to check positive clone was shown. (A) The digestion products of *Hind*III and *Sal*I digestion. M (left); 1kb DNA ladder, lane1; pMY202, lane2; CsnOmpApMY202 and M (right); 1 bp DNA ladder. (B) The digestion products of *Nde*I and *Sal*I digestion. M; 1kb DNA ladder, lane1; pMY202 vector and lane 2; CsnNativepMY202.

The amino acid sequences of CsnNativepMY202 and CsnOmpApMY202 were investigated automated DNA sequencing (Macrogen, Korea). The native signal peptide and OmpA signal peptide were fused at the N-terminal of CsnNativepMY202 and CsnOmpApMY202. Both of the recombinant Csn constructs were fused with C-terminal 10x Histidine tag to facilitate one-step affinity purification using immobilized metal affinity chromatography (IMAC) (Figure 8).

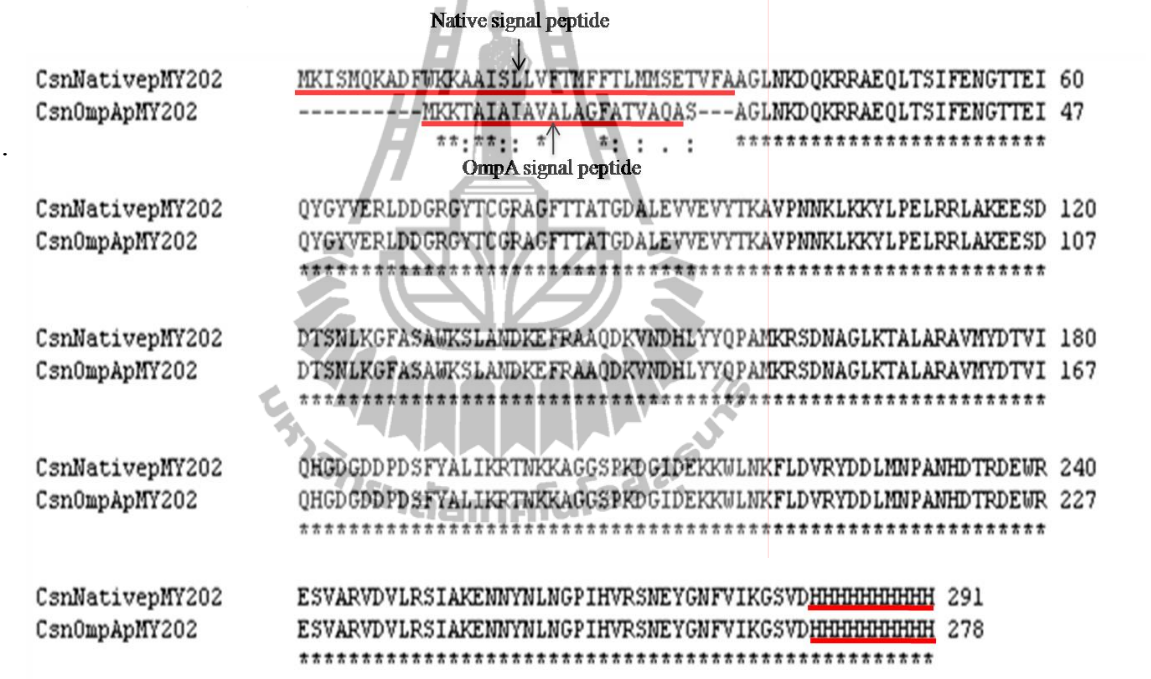

Figure 8. Amino acids sequence alignment of CsnNativepMY202 and CsnOmpApMY202.The alignment was done by using ClustalW program.(http;//www.ebi.ac.uk/Tools/msa/clustalw2/)

4.2 Expression of recombinant chitosanase gene in *E. coli* system

To assay the chitosanase activity on plate, a single colony of *E. coli* TOP10 CsnOmpApMY202 and CsnNativepMY202 was grown on LB-Amp agar containing 0.1% chitosan low MW and incubated at 37°C for overnight. Clearing zones were found from colonies harbouring CsnOmpApMY202 and CsnNativepMY202, but not an empty vector (Figure 9).

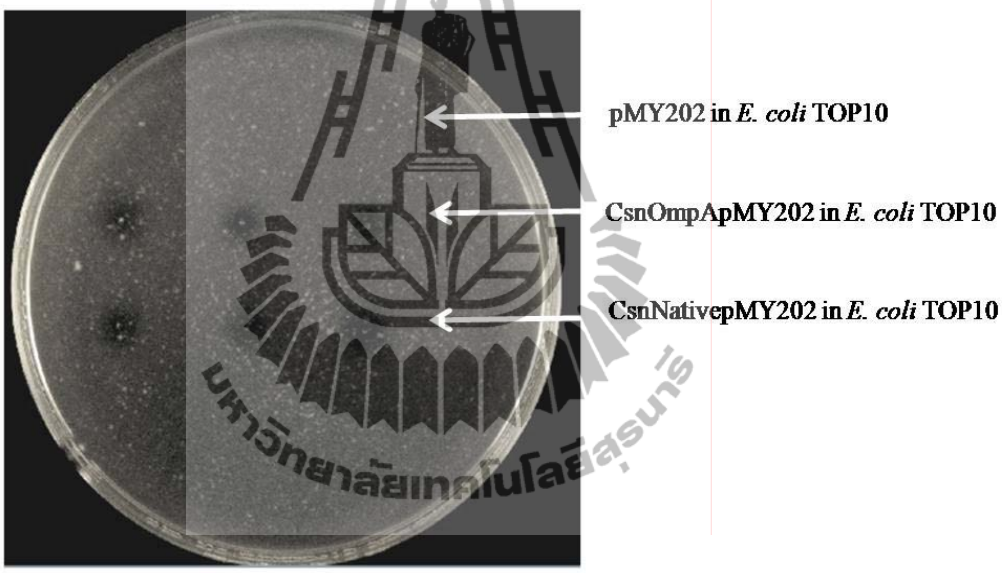

Figure 9. Chitosanase activity assay on plate

The recombinant CsnNativepMY202 and CsnOmpApMY202 were expressed in the *E. coli* strain TOP10 to compare the total yield and specific activity. The conditions of expression were optimized by induction at difference time and extract the enzyme from difference compartments. Two hundred milliliter of two constructs were grown in LB-Amp medium until OD_{600} reach ~0.6-0.7 before IPTG was added to a final concentration of 0.5 mM, and incubation continued at room temperature(~ 27°C). Fifty ml of samples were taken at 0, 4 hr, and overnight (20 h, o/n) after induction with

IPTG and extract the enzyme from culture medium (broth), periplasm and cell lysate. The SDS-PAGE analysis of recombinant *B. subtilis* chitosanase from difference compartments was shown in figure 10. After induce for 20 hr, enzyme from CsnOmpApMY202 was secrete into the culture supernatant. The molecular weight is approximately 33 kDa from SDS-PAGE analysis. The specific activities of the recombinant enzyme in three different compartments, i.e., cytoplasm, periplasmic space, and culture broth were reported in Table 2. The result demonstrated that the yield and specificity of the recombinant OmpA-Csn was higher than those of Native-Csn in all three compartments. The specific activity of OmpA-Csn in cytosol was highest after induction for 20 hr (Table 2). Therefore, the construct CsnOmpApMY202 was used for the subsequent experiments and expressed at the optimal condition.

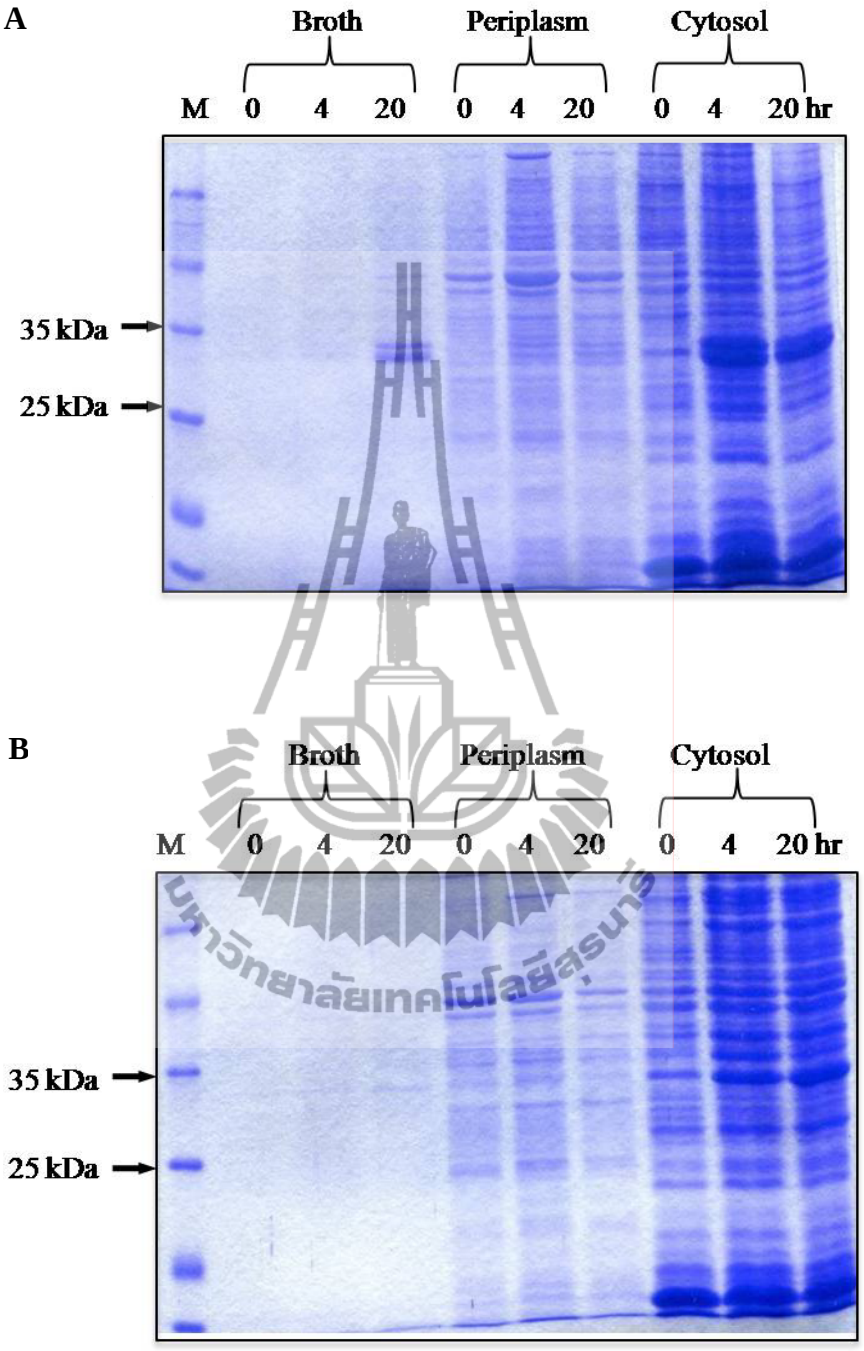

Figure 10. SDS-PAGE analysis of recombinant *B. subtilis* chitosanase from difference compartments of *E. coli* expressing CsnOmpApMY202 and CsnNativepMY202 are shown in panel A and B, respectively. Approximately 10 µl of broth and periplasm and 5 µl of cytosol were loaded onto each lane.

Table 2. Yield of recombinant *B. subtilis* chitosanase in different compartment of *E. coli* TOP10

	Broth			Periplasmic Space			Cytosol		
Induction Time	**0 hr**	**4 hr**	**20 hr**	**0 hr**	**4 hr**	**20 hr**	**0 hr**	**4 hr**	**20 hr**
CsnOmpApMY202									
Total unit (U)	97.95	244.35	1385.55	1.00	22.15	27.62	20.51	561.08	564.33
Total Protein (mg)	2.00	2.50	6.65	0.36	0.68	0.52	2.72	4.89	3.02
Specific activity (U/mg)	**48.97±0.01**	**97.74±0.02**	**208.35±0.00**	**2.76±0.00**	**32.43±0.01**	**53.53±0.02**	**7.53±0.01**	**114.65±0.02**	**186.74±0.00**
CsnNativepMY202									
Total unit (U)	85.45	114.95	265.10	0.93	2.02	4.90	21.32	388.45	528.25
Protein (mg)	2.15	2.65	3.55	0.30	0.35	0.39	3.01	4.92	4.85
Specific activity (U/mg)	**39.74±0.01**	**43.38±0.01**	**74.68±0.01**	**3.13±0.01**	**5.76±0.00**	**12.63±0.02**	**7.09±0.00**	**78.89±0.00**	**108.92±0.01**

The chitosanase activity was measured under the standard assay condition using 0.5% chitosan practical grade as substrate.

4.3 Optimization of enzyme secretion and IPTG concentration

The conditions of expression were optimized by induction at difference time and IPTG concentration. Two hundred milliliter of CsnOmpApMY202 were grown in LB-Amp medium until OD_{600} reach ~ 0.6-0.7 before IPTG was added to a final concentration of 0.1, 0.5 and 1 mM, and incubation continued at room temperature (~ 27°C). Fifty ml of samples were taken at 0, 4 hr, and overnight (20 h, o/n) after induction with IPTG and extract the enzyme from culture supernatant and cell lysate (periplasmic + cytosol). The SDS-PAGE analysis of recombinant *B. subtilis* chitosanase from difference compartments was shown in figure 11. After induce with 0.1-1 mM IPTG for 20 hr, enzyme was secrete into the culture supernatant (Figure11A) and found that IPTG concentration did not have the effect on enzyme expression in the cell lysate (Figure11B). Then, the conditions for enzyme expression were induction with 0.1 mM IPTG at room temperature (~27^{o}C) for 20 hr.

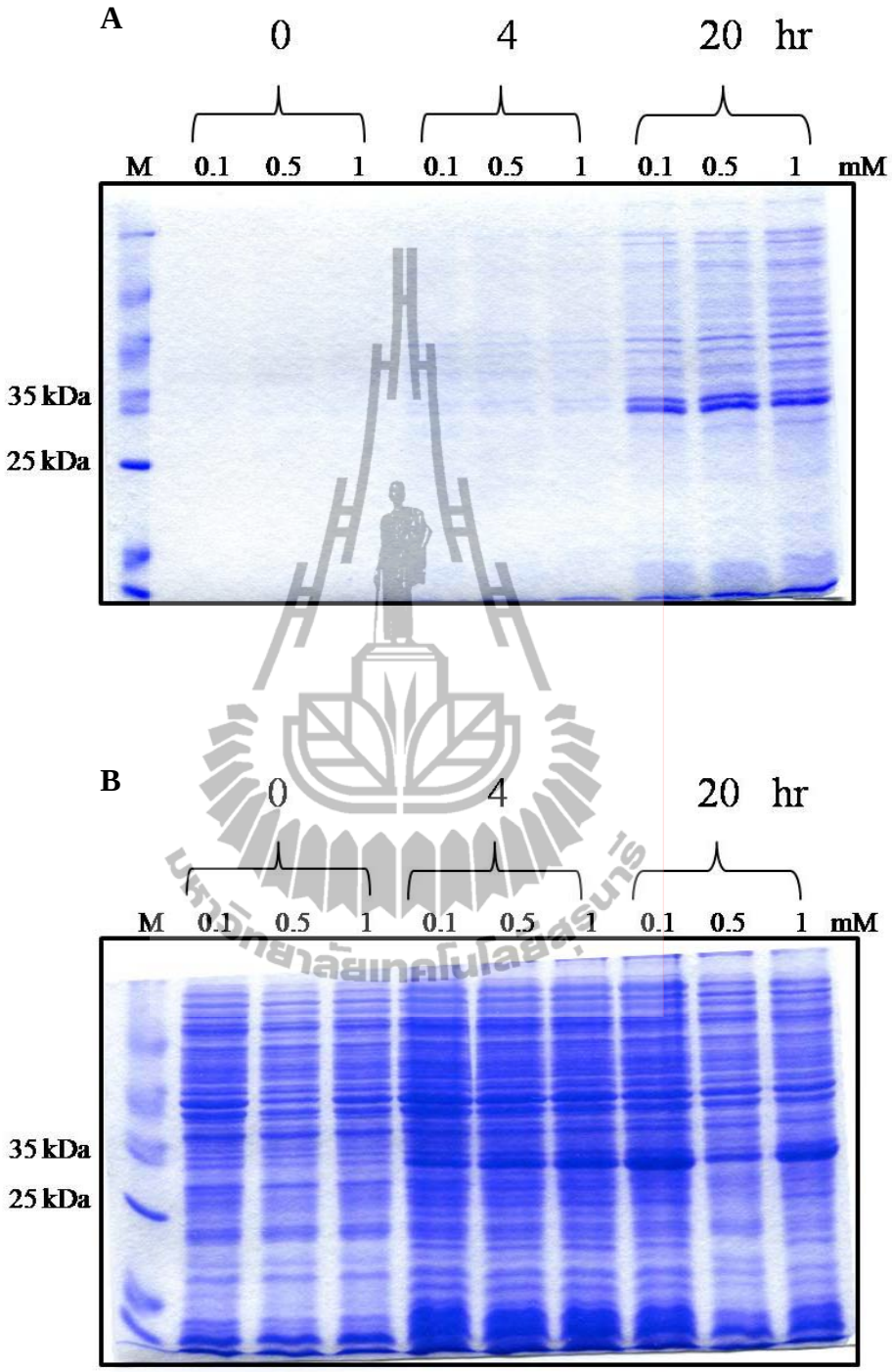

Figure 11. Expression and secretion of recombinant *B. subtilis* chitosanse. The enzyme were taken at 0, 4 hr, and overnight (20 h) after induction with IPTG and extract the enzyme from culture supernatant (A) and cell lysate (B).

4.4 Purification of recombinant *B. subtilis* chitosanase

The recombinant chitosanase CsnOmpApMY202 was purified by only-one step by using Ni-NTA chromatography. We optimized the conditions for enzyme expression, and found that the enzyme induction with 0.1 mM IPTG at room temperature (~27^{o}C) for 20 hr to produce the maximum level of active enzyme. Cells were cultured in 400-ml shaken flasks and the recombinant enzyme was purified from cell lysate (periplasmic space and cytosol) after induction at the optimal condition.The enzyme from was purified to homogeneity by using Ni-NTA affinity column chromatography. The result indicated that the recombinant Csn could be expressed in *E.coli* TOP10 as demonstrated by SDS-PAGE and zymogram analysis The molecular weight (MW) of the purified Csn was approximately 33 kDa on SDS-PAGE (Figure 12A), which corresponded well to the theoretical MW of 31.265 kDa. Chitosanase activity of the purified recombinant enzyme was shown by in-gel activity staining (Figure 12B) as well as by standard chitosanase assay. The purification procedure yielded a 4.43-fold with 35.91% recovery. The specific activity of purified enzyme was 904.8 U/mg. The purification of recombinant Csn was summarized in table 3.

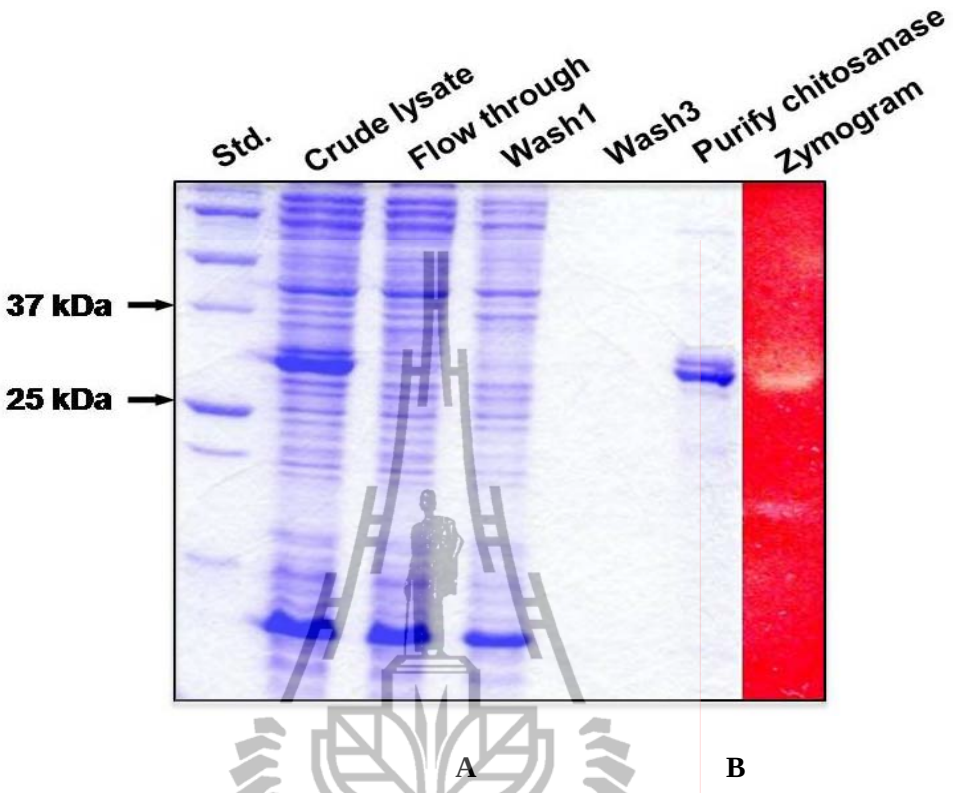

Figure 12. Coomassie staining and Zymogram analysis of purified recombinant chitosanase from *B. subtilis* 168. SDS-PAGE analysis of purified recombinant chitosanase is shown in panel A. Panel B illustrated zymogram analysis of the purified enzyme. White band indicated chitosanase activity.

Table 3. One-step purification of recombinant *B. subtilis* chitosanase

Purification step	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	%Yield
crude extract	69.1	14101.0	204.0	1.0	100.0
Ni-NTA chromatography	5.6	5063.2	904.8	4.4	35.9

The chitosanase was measured under standard assay condition using 0.5% chitosan low molecular weight as substrate.

4.5 Characterization of enzyme

4.5.1 Effect of pH on enzyme activity

4.5.1.1 The Optimal pH

The optimal pH was determined at 50^{o}C for 5 min, using glycine-HCl buffer (pH 2.4-4.4) and sodium acetate buffer (pH 4-7). The optimal pH of the enzyme was between pH 5.0-6.0 in sodium acetate buffer as show in figure 13. But the chitosanase activity was not possible to determine the enzyme activity at pH > 7.0 because chitosan substrates were insoluble at this condition.

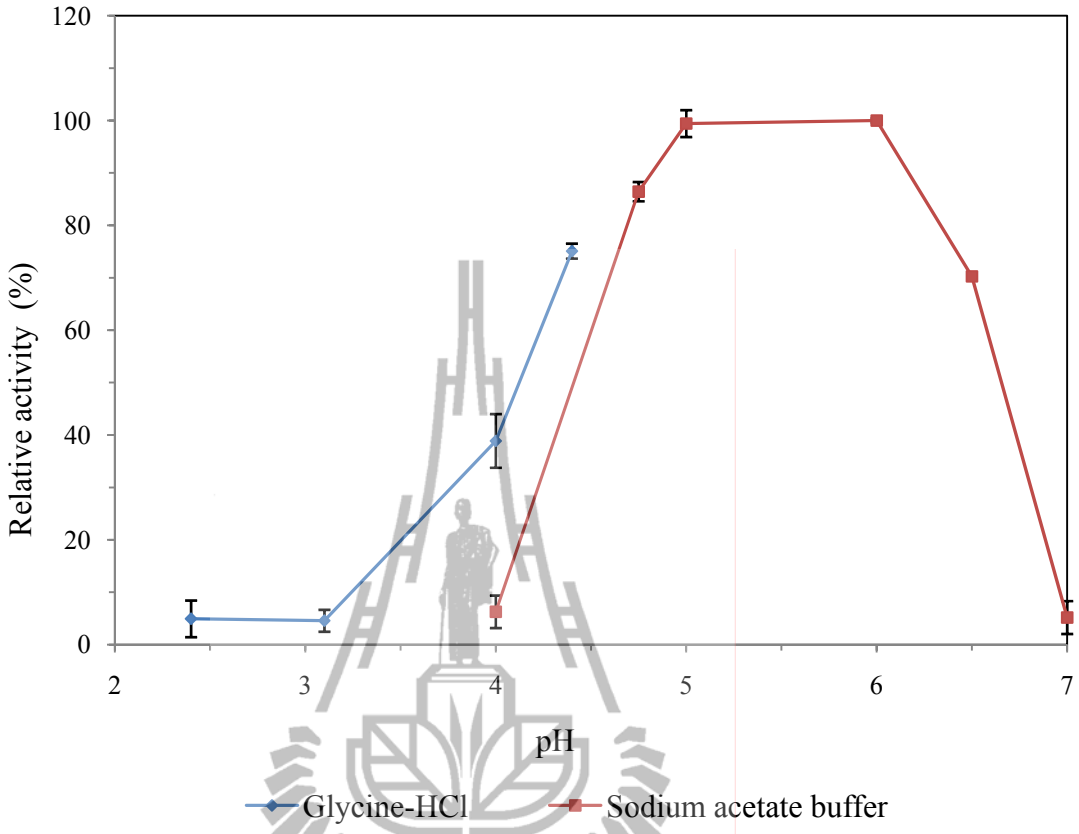

Figure 13. Effect of pH on the activity of *B. subtilis* 168 chitosanase. The optimal pH was determined at 50°C using 0.5% chitosan low MW in 250 mM of different buffers. The buffers used were glycine-HCl buffer (diamond) from pH 2.4-4.4; sodium acetate buffer (square) from pH 4-7.

4.5.1.2 The pH stability

The pH stability was determined by measuring the remaining activity after pre-incubated the enzyme with difference pH buffer range at 30°C for 3 hr and 24 hr. The buffer was 100 mM each of glycine-HCl (pH 2-4), sodium acetate buffer pH (4-7), Tris-HCl, buffer (pH 7-9) and glycine-HCl buffer (pH 9-12). The enzyme is more active in glycine buffer than in sodium acetate buffer. The enzyme also showed stability under a wide range of pH (2-11), after incubation at 30°C for 3 hr without substrate, more than 70% of activity retained (Figure 14A). After incubation for 24 hr, the enzyme was stable at pH 4-9, more than 70% of activity retained when using sodium acetate and Tris-HCl buffer (Figure 14B).

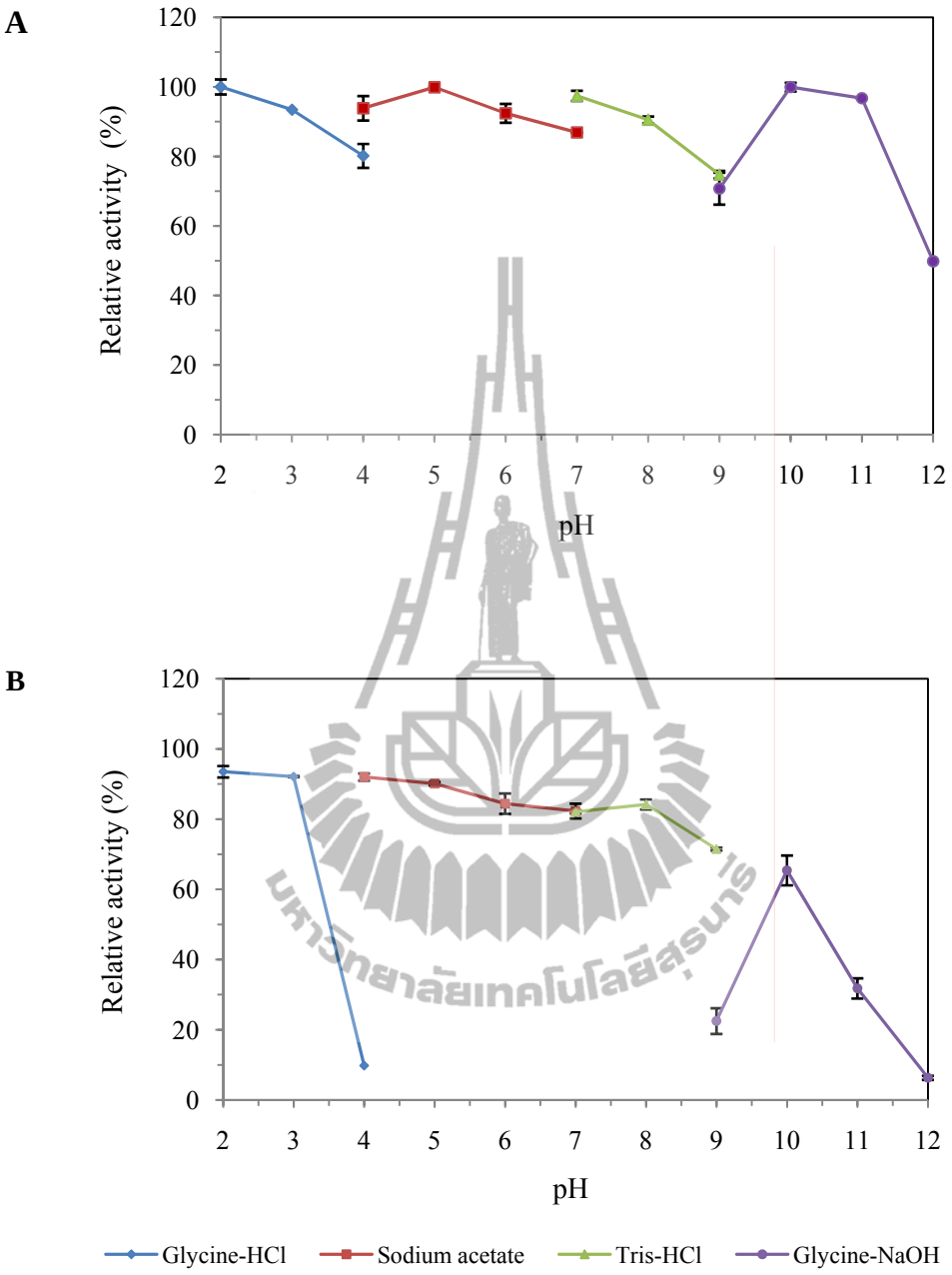

Figure 14. Effect of pH on the stability of *B. subtilis* 168 chitosanase. The pH stability was determined by measuring the remaining activity after incubation at various pH values at 30°C for 3 h (A) and 24 h (B). The buffers (100 mM each) used were glycine-HCl buffer (diamond) from pH 2-4; sodium acetate buffer (square) from pH 4-7;Tris-HCl (triangle) from pH 7-9, and glycine-HCl (circle) from pH 9-12.

4.5.2 Effect of temperature on enzyme activity

4.5.2.1 The optimal temperature

The optimal temperature was obtained by incubation the enzyme with 0.5% chitosan in 200 mM sodium acetate buffer at temperature ranging from 0-80^{o}C for 5 min and determined the chitosanase activity under the standard assay condition. The optimal temperature for *B. subtilis* Csn activity was between 40-50˚C (Figure 15).

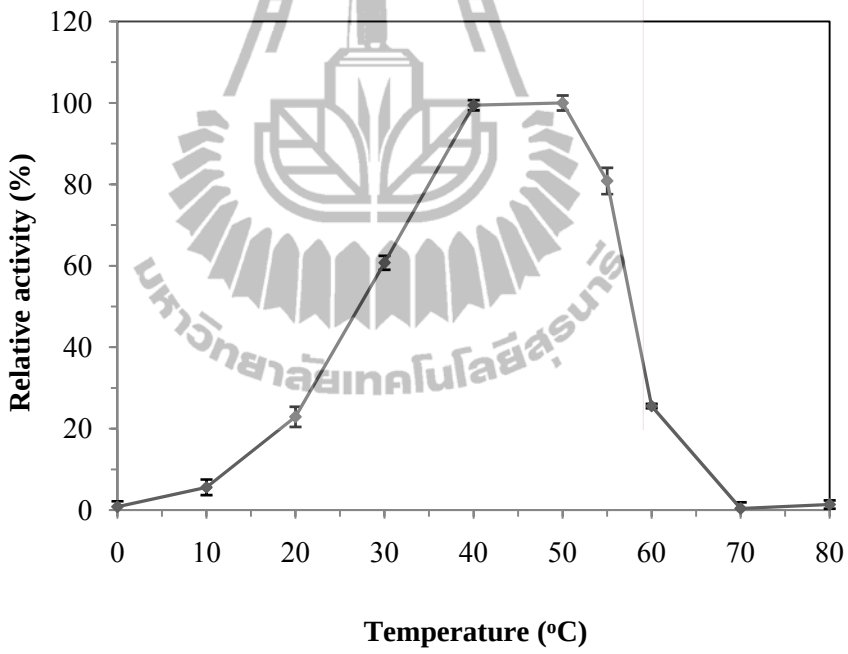

Figure 15. Effect of temperature on activity of *B. subtilis* 168 chitosanase. The optimal temperature of *B. subtilis* chitosanase was measured by incubating 0.4 µg of the purified enzyme with 0.5% chitosan practical grade at temperatures ranging from 0-80^{o}C, for 5 minutes at pH5.5.

4.5.2.2 The thermal stability

To examine the thermal stability of *B. subtilis* Csn, the enzyme was pre-incubated at various temperatures ranging from 4-80°C, pH 5.5 for 30 min without substrate and determined the remaining activity under the standard assay condition. The enzyme was stable up to 40˚C, approximately 80% of the activity was retained. But the enzyme was completely inactivated at 50°C, less than 5% of residual activity could be detected in this condition (Figure 16).

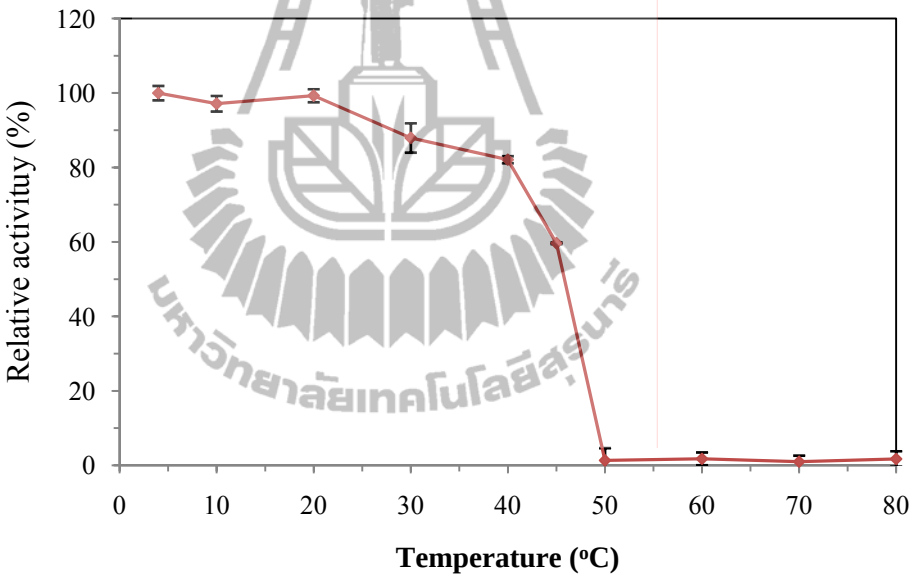

Figure 16. Effect of temperature stability of *B. subtilis* 168 chitosanase. The temperature stability was determined by measuring the remaining activity after incubation without substrate at various temperatures at pH 5.5 for 30 min, and measuring the residual activity was under the standard assay using 0.5% chitosan practical grade as substrate.

4.5.2.3 Thermal stability in the presence of substrate

The thermal stability of purified chitosanase in the presence of substrates was determined by pre-incubation the enzyme with 0.5% chitosan low MW or 10 mM of chitosan oligosaccharide (G1-G6) in 50 mM sodium acetate buffer pH 5.5, at 50^{o}C for 30 min and the remaining activity was determined under the standard assay condition. The result shown that, when enzyme was pre-incubated with 10 mM chitosanbiose (G2), chitosantriose (G3), chitosantetraose (G4), chitosanpentaose (G5), chitosanhexaose (G6) and 0.5% chitosan low MW, the residual activities were 33%, 51%, 78%, 80%, 83% and 85%, respectively (Figure 17A). The result indicated that chitosan oligosaccharide and chitosan low MW could be prevent thermal inactivation of the chitosanase activity. The enhancement of thermal stability by substrate was confirmed by the thermal inactivation results as shown in Figure 11B. The residual activity after incubation at 50^{o}C with 0.5% chitosan for 12 hr was about 70 %. The recombinant chitosanase showed a half-life time of activity, $\tau 1/2$ of approximately 19 hr (Figure 17B).

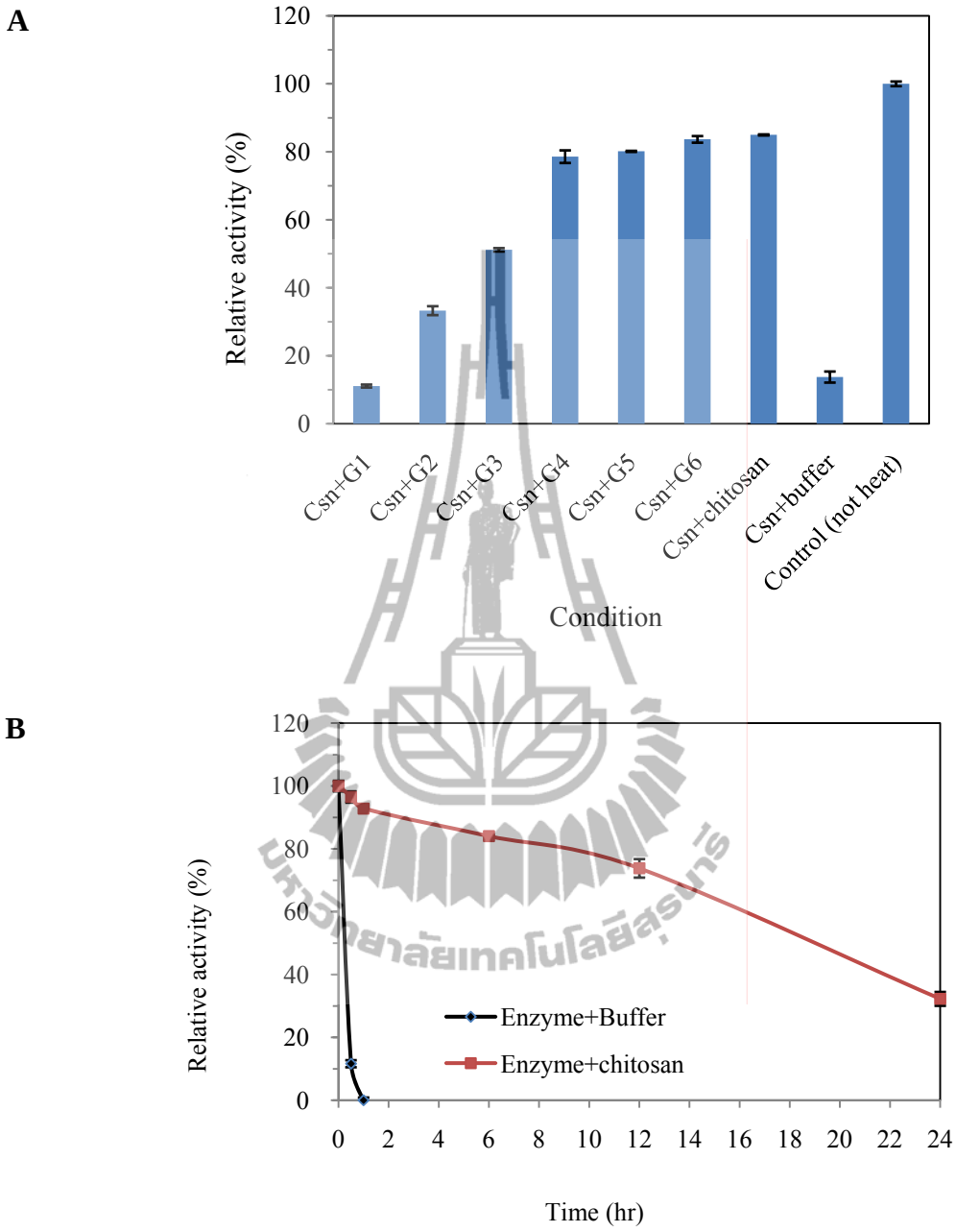

Figure 17. Thermal stability in the presence of substrate. (A) The thermal stability was determined by measuring the remaining activity after incubation with 10 mM chitooligosaccharide (G1-G6) or 0.5% chitosan at 50˚C for 30 min. (B) The remaining activity of *B. subtilis* chitosanase after incubation without (diamond) or with (square) 0.5% chitosan at 50°C, at various time points (0, 0.5, 1, 6, 12 and 24 h).

4.6 Substrate specificity

The activity of the recombinant *B. subtilis* chitosanase was determined under standard assay conditions using each substrate at final concentration of 0.5%, such as chitosan low MW, chitosan practical grade, chitosan medium MW, commercial chitosan, glycol chitosan, and colloidal chitin. The enzyme could not hydrolyze glycol chitosan and colloidal chitin, but it hydrolyzed chitosan. The activity toward low MW chitosan was higher than that of the medium MW chitosan. The relative activities were shown in table 4.

Table 4. Substrate specificity of recombinant *B. subtilis* chitosanase

Substrate	Relative activity (%)
Chitosan low molecular weight (75-85%DDA)	100*
Chitosan Practical grade (≥75%DDA)	112
Chitosan medium molecular weight (75-85%DDA)	49
Commercial chitosan	160
Glycol chitosan	nd**
Colloidal chitin	nd**

* The relative activity with standard low MW chitosan (75 – 85 % DDA) was defined as 100%

** nd: no apparent activity at standard assay condition was detected.

4.7 Kinetic analysis

The Michaeleis-Menten kinetic parameters were determined using 0.8-9.6 mg/ml of low MW chitosan as a substrate. Kinetic analysis revealed that K_m, V_{max}, and k_{cat}values of recombinant *B. subtilis* Csn were shown in table 4.

Table 5. Kinetic parameters of recombinant *B. subtilis* chitosanase

Substrate	**K_m (mg/mL)**	**V_{max} (µmole min^{-1} mg^{-1})**	**K_{cat} (s^{-1})**	**K_{cat}/K_m (s^{-1}/mg/mL)**
Chitosan low MW	1.57 ± 0.09	562.41± 22.65	2.99 x 10^5±1.21 x10^4	1.9x10^5±1.88 x10^4

4.8 Hydrolytic analysis by thin layer chromatography (TLC)

Product analysis by thin layer chromatography (TLC) using various substrates were performed to determine the hydrolytic acitivity of *B. subtilis* chitosanase. The enzyme was incubated with various type of chitosan, various chitooligosaccharide products (G2 - G6) were found after enzymatic hydrolysis (Figure 18). The pattern of products differed depending on the types of the chitosan used, and the time of incubation. Recent work in my laboratory revealed that endo-chitinase (ChiA) from *B. licheniformis* DSM8785 has suitable property for industrial application. However, when the enzyme was incubated with colloidal chitin in difference pH at 50°C, only 2 majors products (GlcNAc and chitobiose $(GlcNAc)_2$) could be obtained (Songsiriritthigul et al., 2010) (Figure 19).

The hydrolytic pattern of *B. subtilis* chitosanase was analyzed by using chitosan oligosaccharide (G2-G6) as substrates (Figure 20). When chitobiose (G2) and chitotriose (G3) were used as substrates, no hydrolytic products could be detected, suggesting that chitosan oligosaccharides shorter than G3 were not the substrate of this enzyme. When G4 was used as substrate, G2 could be observed, suggesting that the enzyme efficiently cleaved the middle glycosidic bond of the tetrameric chain. Using G5, G2, and G3 were released after incubation for 2 min. And when G6 was used as a substrate, G3 and G4 were detected after 2 min of incubation. After 5 min of incubation, G4 was further hydrolyzed to G2, and after 1 h of incubation, all G6 substrate was converted to a mixture of G2, G3, and G4. These results demonstrate that the recombinant *B. subtilis* chitosanase is an endo-chitosanase that prefers substrate longer than G3.

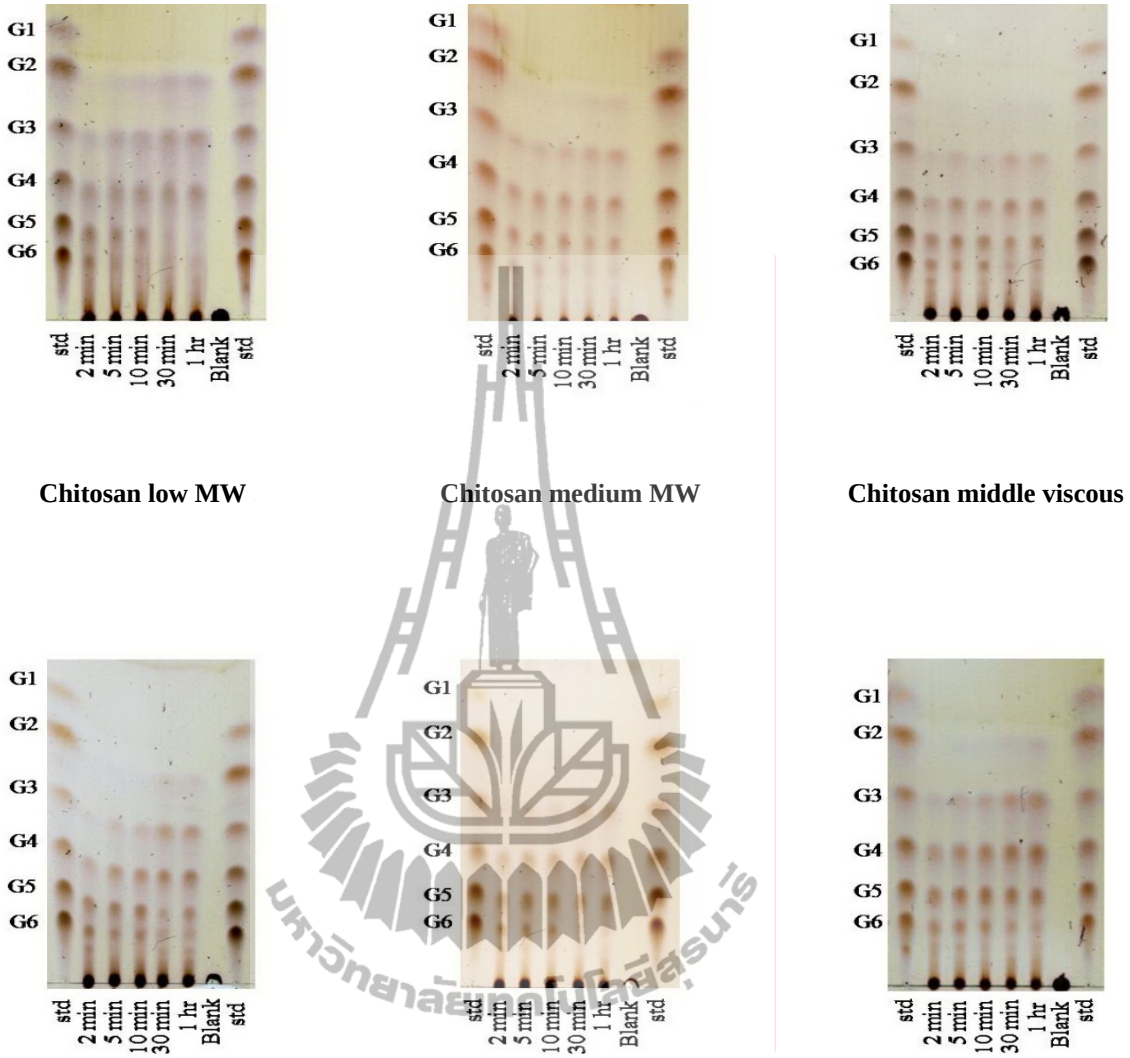

Figure 18. Thin layer chromatography analysis of hydrolysis products using various chitosan as substrates. Products from chitosan hydrolysis at various time points are illustrated. Std: as tandard mixture of G1 - G6; 2 min, 5 min, 10 min, 30 min, and 1hr are the reaction products after incubation at 2, 5, 10, 30, min and 1 hr, respectively; Blank: enzyme blank. Commercial grade chitosan powder was from NNC products., Co., Ltd. (Thailand).

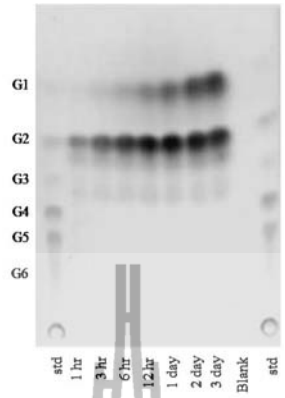

Figure 19. TLC analysis of colloidal chitin hydrolysis by *B. licheniformis* ChiA1(chitinase). The reaction products after incubation for 1 hr, 3 hr, 6 hr, 12 hr, 1 day, 2 day, and 3 day are shown. Std: a standard mixture of G1-G6; SB: Enzyme blank.

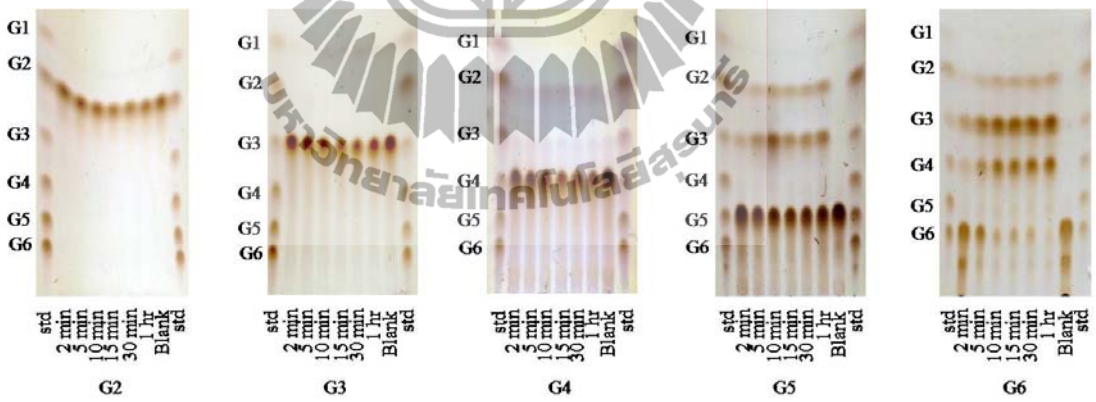

Figure 20. Thin layer chromatography analysis using various chitosan oligosacharide (COS) as substrates. Hydrolysis products when using chitobiose (G2), chitotriose (G3), chitotetraose (G4), chitopentaose (G5) and chitohexaose (G6) as substrates are shown. Std: a standard mixture of G1 - G6; at 2 min, 5 min, 10 min, 15 min, 30 min and 1 hr are the reaction products after incubation at 2, 5, 10, 15, 30 min, and 1 hr, respectively; Blank: enzyme blank.

4.9 Production of chitooligosaccharide using crude secreted enzyme from culture broth

The crude enzyme of the *E. coli* containing CsnOmpAMY202 was secreted into culture supernatant after overnight induction (20 hr). To determine the efficiency of the bioconversion of chitosan into chitosan oligosaccharides, 100 µl of freshly prepared culture broth containing approximately 5 µg of recombinant enzyme was mixed with two types of chitosan, i.e., low MW and commercial grade. The hydrolytic reaction was carried out in sodium acetate buffer, pH 5.5 at 50 °C or 30°C at different time points (2 min, 5min, 10 min, 20 min, 30 min, 1 hr, 2 hr, and 3 hr). The products released from the reactions were subsequently analyzed by TLC. The mixtures chitosan oligosacchatide, ranging from G2 to G6 and longer were released, depending on the temperature and time of incubation and the types of chitosan substrate. The hydrolytic products from commercial chitosan comprised higher amount of G5, G6, and longer chitosan oligosaccharide than those from low MW chitisan. At 30°C, G4 could be observed at every time points; whereas, at 50°C, G4 disappeared after 20 min of incubation, and G5 and G6 were accumulated instead. The hydrolytic products of commercial chitosan at 30^{o}C were longer than those of 50^{o}C (Figure 21). When chitosan low MW was used as substrate, the mixtures of chitooligosaccharide (G2 to G6) were observed after incubation for 2 min at 30^{o}C. At 50^{o}C, G6 could be obtained after 2 min of incubation.

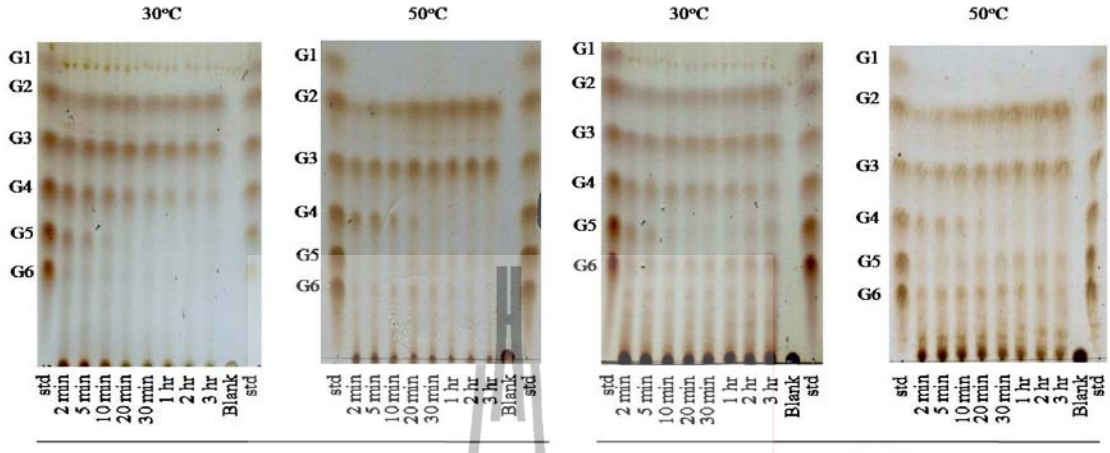

Figure 21. Bioconversion of various chitosans using secreted enzyme from culture broth. Thin layer chromatography analysis of hydrolysis products using chitosan low molecular weight (left panel) and commercial liquid chitosan (Poodang) (right panel) as substrates. The substrates were incubated with crude enzyme from 100 µl of culture supernatant after an overnight cultivation. The reaction products after incubation at pH 5.5, for 2 min, 5 min, 10 min, 20 min, 30 min, 1 hr, 2 hr, and 3 hr at 30°C and 50°C are illustrated.

CHAPTER V

DISCUSSION

B. subtilis 168 was used as a source of chitosanase gene because it is important given the capacity of *Bacillus* strains to secrete large amounts of industrially important enzymes (Kunst et al., 1997) and the complete genome sequence is available on the NCBI DNA database. Many bacillus species are non-pathogenic with GRAS (generally recognized as safe) status (Schallmey et al., 2004). Thus, two forms of recombinant chitosanase was constructed into pMY202 expression vector by PCR base cloning and expressed in *E. coli* TOP10, under the control of tac promoter. The recombinant enzyme tagged with 10x Histidine at the C-terminus, could be purified by one-step immobilized metal affinity chromatography. The first construct (CsnOmpApMY202), the mature enzymes fused with the *E. coli* OmpA signal sequence. For the second construct, the native signal peptide was retained. The amino acid of the signal peptide of *B. subtilis* 168 is 35 amino acid long (MKI SMQ KAD FWK KAA ISL LVF TMF FTL MMS ETV FA), whereas that of *E. coli* OmpA consist of 21 amino acids (MKK TAI AIA VAL AGF ATV AQA). From the result found that *E. coli* outer membrane protein, OmpA, could be used efficiently to direct secretion of the recombinant chitosanase into culture medium after using IPTG induction for 20 hr. The expressed enzymes were observed as clearing zones on chitosan agar plates. However, the mechanism for protein secretion is not known (Mergulhão et al., 2005).

Many chitosanase gene have been cloned from different bacteria including *B. coagulans* CK108 (Yoon et al., 2002), *Bacillus* sp. CK4 (Yoon et al., 2000), *Bacillus* sp. MET1299 (P. I. Kim et al., 2004) , *Burkholderia gladioli* strain CHB101 (Shimosaka et al., 2000), *Janthinobacterium* sp. strain 4239 (Johnsen et al., 2010) and *Pseudomonas* sp. A-01(Ando et al., 2008). Howerver, this is the first report on cloning, expression, and characterization of family GH46 chitosanase from *B. subtilis* in *E. coli*.

The chitosanase gene could be induced for expression with 0.1-1mM IPTG when the OD_{600} reached 0.6-0.7. The recombinant chitosansase could be harvested from three different compartments, i.e., culture supernatant, periplasmic space and cell lysate after induction 4-20 hr. The result revealed that the yield and specific activity of Csn-OmpA was higher than those of Csn-Native in all compartment. The maximum yield of Csn-OmpA was in the cytosol after induction for 20 hr. Therefore, it was more convenient to purify the Csn-OmpA from cell lysate (periplasmic space and cytosol). Most importantly, at 20 hr after induction, the total activity of Csn-OmpA were approximately 5 fold greater when compared with Csn-Native (Table1). Previous studies have reported that *E. coli* OmpA signal sequence could be used efficiently to the direct secretion of the recombinant enzyme into the periplasmic space and culture medium (Yamabhai et al., 2008), ; (Songsiriritthigul et al., 2010). Recent report on the chitosanase gene from *Janthinobacterium* sp. strain 4239 that fused with *E. coli* ompA sequence showed that the enzyme could be secreted into periplasmic space after induction 5 - 5.5 hr (Johnsen et al., 2010).

We routinely expressed the recombinant chitosanase by induction with 0.1 mM IPTG and cultivation for 20 hr at room temperature (~27^{o}C). The recombinant

enzyme from the cell lysate was simply purified in the lab in one-step using Ni-NTA affinity chromatograghy. The total chitosanase activity was about 57,000 U from 1-l shake flask culture. The total activity from culture supernatant was 45,000 U, whereas 12,000 U came from cell lysate (periplasmic space and cytosol). The recombinant chitosanase was secreted into the culture supernatant in amounts about 3.7 fold higher than the cell lysate, which will be potential for large-scale cultivation and downstream processing because cell disruption is not necessary.

It is worthwhile to note that there are many methods to measure chitosanase activities, based on the determination of reducing ends. These various assay procedures must be taken into consideration when comparing different enzymes. Since the most popular method for chitosanase assay is based on the determination of reducing end with 3,5 dinitrosalicylic acid (DNS) ; therefore, this method was used in this study, even if it has been criticized that it is not accurate (Jarle Horn & Eijsink, 2004). In this study, the specific activity of purified enzyme was about 900 U/mg, it was higher than recombinant chitosanase from *Bacillus* sp. MET 1299 (412 U/mg) (Kim et al., 2004) using the same method. For the other recombinant chitosanase such as *Janthinobacterium* sp. (Johnsen et al., 2010) strain 4239 and *Pseudomonas* sp. A-01 (Ando et al., 2008) used Schales's procedure for determination the activity, the specific activity were 1500 U/mg and 3.9 U/mg respectively. It has been noted that this method depends on chitooligosaccharides of different lengths, thus giving different signals per reducing end (Jarle Horn & Eijsink, 2004).

Analysis of biochemical properties of the purified enzyme revealed that the *B. subtilis* chitosanase show the widest pH stability, ranging from pH 2-11, when compare with previous publication (Table2). The optimal pH was between pH 5-6,

which is suitable for chitosan solubility because chitosan is soluble in dilute aqueous acid solution. The optimal temperature of enzyme was at 40-50^{o}C, and stable at 4-40^{o}C, with more than 80% remaining activities after incubation at pH 6.0 for 30 minutes without substrate. But it was completely inactivated at 50^{o}C, which the discrepancy between a high optimal temperature and low thermal stability. There have been the recently report that chitooligosaccharide could prevent the thermal inactivation of chitosanase (Kobayashi et al., 2011). The thermostability of recombinant chitosanase could be increased when incubated enzyme with the substrate (G4, G5, G6 and chitosan) at 50^{o}C for 30 min. And the residual activity after incubation at 50^{o}C with chitosan for 12 hr was about 70%. There result indicated that *B. subtilis* chitosanase is not inhibited by the end product.

The substrate specificity activity against various type of substrate showed that the enzyme exhibited the highest activity on commercial chitosan, followed by chitosan practical grade, chitosan low MW and chitosan medium MW, but no activity against glycol chitosan and colloidal chitin. The substrate specific activity depends on the degree of deacetylation (DDA), the degree of polymerization (DP) or molecular weight (MW), the molecular weight distribution (PD) and the pattern of *N*-acetylation (P_A) (Aam et al., 2010). The hydrolytic products of the purified chitosanase against various type of chitosan, various chitooligosaccharide (G2-G6) were generated. The pattern of hydrolytic products depend on the type of chitosan used as described above and incubation time. The smallest substrate was G4, the hydrolytic products was G2, suggesting that the enzyme efficiently cleaved in the middle of glycosidic bond. There have been report on the type of chitosanase, almost were endo-type chitosanase such as *B. coagulans* CK108 (Yoon et al., 2002), *B. subtilis* IMR-NK1 (Chiang et al., 2003),

Bacillus sp. MET 1299 (P. I. Kim et al., 2004) and etc. Interestingly, the production of chitooligosaccharide could be produced by using crude secreted enzyme from culture supernatant after overnight induction. The mixture of chitooligosaccharides ranging from G2-G6 and longer were released. There have been report that Chitosan oligosaccharides of longer than a hexamer in chain length perform more potent antimicrobial, antitumor, and immunopotentiating activities than shorter oligosaccharides, even though shorter oligosaccharides also perform activities of significance (Choi et al., 2004). The secreted enzyme will be advantageous for large-scale production. The biological effect of chitooligosaccharide will be investigated in the future.

CHAPTER VI

CONCLUSION

1. The recombinant chitosanase from *Bacillus subtilis* 168 was successfully cloned into pMY202 expression vector and efficiently produced and secreted by *E. coli* expression system.

2. The optimal temperature of the recombinant *B. subtilis* chitosanase was 40-50^{o}C and stable up to 40^{o}C in the absence of substrate. However, in the presence of 10 mM substrates longer than G3, the enzyme was thermostable with a half-life time of activity (τ1/2) of approximately 19 hr at 50°C.

3. The optimal pH was between pH 5-6 and stable within pH 2-11 after incubation for 3 hr, and within pH 2-9 after incubation for 24 hr, at 30°C.

4. The recombinant *B. subtilis* chitosanase is an endo-chitosanase that prefers substrate longer than G3. Culture supernatant containing secreted recombinant chitosanase could be used directly for efficient bioconversion of chitosan into different mixtures of COS, depending on the types of chitosan and the reaction conditions used.

5. The secreted enzyme will be advantageous for large-scale production and the biological effect of chitooligosaccharide will be investigated in the future.

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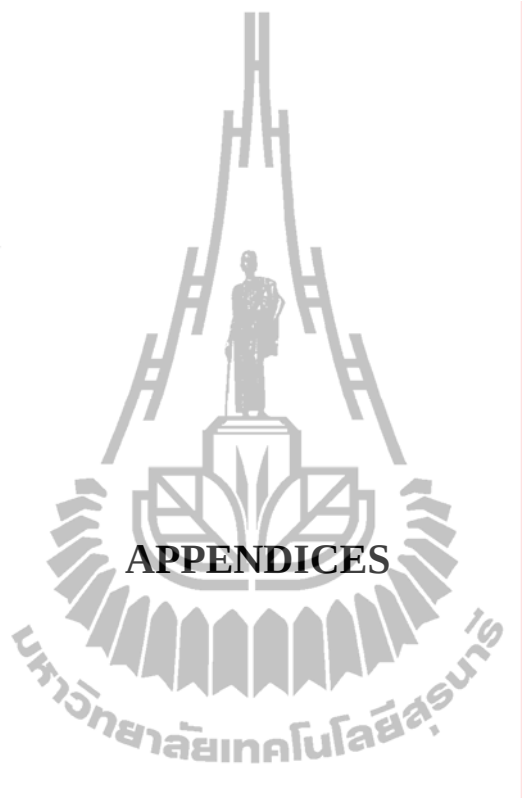


APPENDICES

APPENDIX I

1. REAGENT

1.1 Solution for assay chitosanase activity

1.1.1 Soluble chitosan

Soluble chitosan (10mg/ml) was prepared by dissolving 10 g of chitosan in 400 ml of distilled water, then 90 ml of 1 M acetic acid was added with stirring. The chitosan solution was adjusted to pH 5.5 with 1 M sodium acetate, and to a final volume of 1 L with distilled water.

1.1.2 3,5 dinitrosalicylic acid (DNS solution)

DNS solution was prepare by dissolving 2.5 g of DNS with 2 N NaOH 50 mL. Seventy five g of Potassium sodium tartrate and ddH_2O was added and stirred for overnight. Then adjust the volume to 250 mL and keep in the dark.

1.2.2 4X Stacking gel buffer (0.5 M Tris, pH6.8)

Tris	6 g
DDH_2O	80 mL

Adjust pH to pH 6.8 with HCl and make volume to 100 mL by ddH_2O

1.2.3 Tank buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3)

Tris	30.28 g
Glycine	144.13 g
SDS	10 g
DDH_2O	1 L

1.2.4 Staining solution

Coomassie brilliant blue R-250	1.25 g
Methanol	400 mL
Glacial acetic acid	100 mL

Add distilled water to final volume 1 L

1.2.5 Destaining solution

Methanol	300 mL
Glacial acetic acid	100 mL

Add distilled water to final volume 1 L

12.6 10% (w/v) Ammonium persulfate (APS)

Ammonium persulfate	100 mg
DDH2O	1 mL
Store at -20^{o}C	

1.2.7 12.5% Separating gel SDS-PAGE (15 mL)

40% Acrylamide solution	4.7 mL
4X Running buffer	3.75 mL
10% SDS	0.15 mL
ddH_2O	6.35 mL
10%APS	75 µL
TEMED	7 µL

1.2.8 4% Stacking gel SDS-PAGE

40% Acrylamide solution	0.499 mL
4X Stacking buffer	1.25 mL
10% SDS	0.05 mL
ddH_2O	3.166 mL
10%APS	25 µL
TEMED	12 µL

1.3 Solution for protein purification

1.3.1 Equilibration buffer (30 mM Tris-HCl, 300 mM NaCl and 10 mM Imidazole, pH8)

Tris	3.63 g
NaCl	17.5 g
Imidazole	0.68 g

Adjust pH to 8 with HCl and add distilled water to final volume 1 L.

1.3.2 Washing buffer (30 mM Tris-HCl, 300 mM NaCl and 20 mM Imidazole, pH8)

Tris	3.63 g
NaCl	17.5 g
Imidazole	1.36 g

Adjust pH to 8 with HCl and add distilled water to final volume 1 L.

1.3.3 Elution buffer (30 mM Tris-HCl, 300 mM NaCl and 250 mM Imidazole, pH8)

Tris	3.63 g
NaCl	17.5 g
Imidazole	17.02 g

Adjust pH to 8 with HCl and add distilled water to final volume 1 L.

2. CULTURE MEDIUM

2.1 Nutrient agar (NA)

Peptone	5 g
Yeast extract	3 g
Agar	15 g

Adjust volume to 1L by deionized water (ddH_2O) and autoclave to sterilize.

2.2 Luria bertani (LB)

Bacto-Tryptone	10 g
Bacto-yeast extract	5 g
NaCl	5 g
Agar	15 g (for agar medium)

Adjust volume to 1L by deionized water (ddH_2O) and autoclave to sterilize. After sterilization, add ampicillin final concentration 100 μg/mL.

APPENDIX II

1. STANDARD CURVE

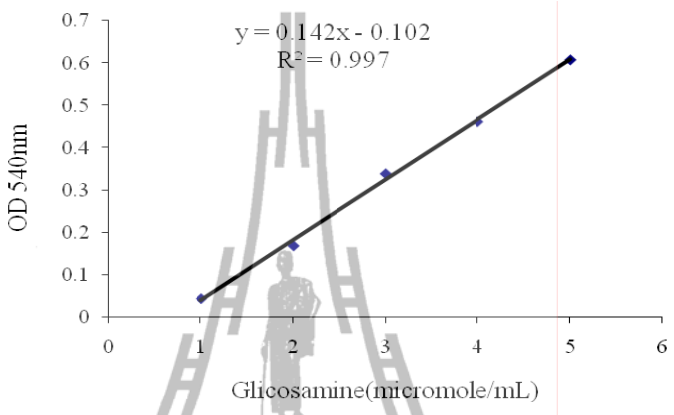


Figure 21. Glucosamine standard curve for determining chitosanase activity using DNS method.

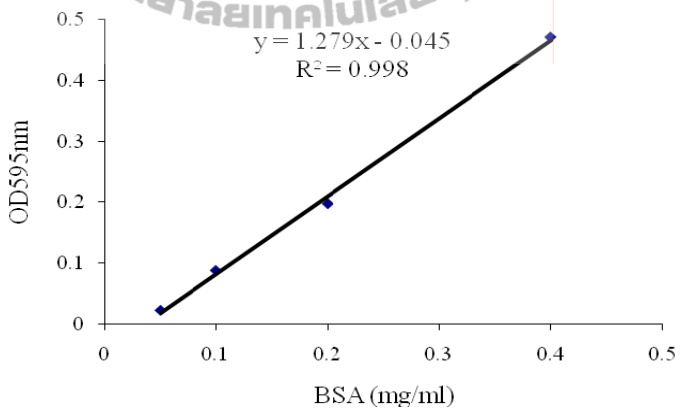


Figure 22. BSA standard curve for determining protein concentration using Bradford method.

2. RESULTS DATA

2.1 Purification of recombinant chitosanase

Table 6. One-step purification of recombinant *B. subtilis* chitosanase.

Purification step	Protein (mg/mL)	Volume (mL)	Total Protein (mg)	Activity (U/mL)	Total activity (U)	Specific activity (U/mg)	Purity (fold)	%Yield
crude extract	4.1	17	69.1	829.5	14101.0	204.0	1.00	100.0
Ni-NTA	1.2	4	4.8	756.1	3024.5	630.1	3.09	21.4
Viva spin	4.7	1.2	5.6	4219.3	5063.2	904.8	4.43	35.9

2.2 Optimal temperature

Table 7. Effect of temperature on activity of *B. subtilis* 168 chitosanase.

Temperature (^{o}C)	Specific activity U/mg	Relative activity (%)
0	2.405	0.811
10	16.582	5.589
20	67.975	22.910
30	180.253	60.751
40	295.063	99.446
50	296.709	100.000
55	239.873	80.845
60	75.823	25.555
70	1.139	0.384
80	4.051	1.365

2.3 Optimal pH

Table 7. Effect of pH on the act ivity of *B. subtilis* 168 chitosanase.

Buffer	pH	Specific activity (U/mg)	Relative act.(%)
glycine-HCl	2.4	20.927	4.919
glycine-HCl	3.1	19.374	4.554
glycine-HCl	4.0	165.337	38.866
glycine-HCl	4.4	319.422	75.087
Sodium Acetate	4.0	26.660	6.267
Sodium Acetate	4.8	367.678	86.431
Sodium Acetate	5.0	422.942	99.422
Sodium Acetate	6.0	425.370	100.000
Sodium Acetate	6.5	298.877	70.258
Sodium Acetate	7.0	22.002	5.172

2.4 pH stability

Table 8. Effect of pH on the stability of *B. subtilis* 168 chitosanase. The pH stability was determined by measuring the remaining activity after incubation at various pH values at 30°C for 3 hr.

Buffer	pH	Specific activity (U/mg)	Relative activity(%)
Glycine-HCl	2	314.697	100.000
Glycine-HCl	3	294.013	93.426
Glycine-HCl	4	252.277	80.164
Sodium acetate	4	295.359	93.854
Sodium acetate	5	314.330	99.882
Sodium acetate	6	290.830	92.415
Sodium acetate	7	273.451	86.892
Tris-HCl	7	306.619	97.432
Tris-HCl	8	284.833	90.509
Tris-HCl	9	235.264	74.758
Glycine-NaOH	9	222.657	70.752
Glycine-NaOH	10	314.575	99.960
Glycine-NaOH	11	304.416	96.732
Glycine-NaOH	12	156.932	49.867

Table 9. Effect of pH on the stability of *B. subtilis* 168 chitosanase. The pH stability was determined by measuring the remaining activity after incubation at various pH values at 30°C for 24 hr.

Buffer	**pH**	**Specific activity (U/mg)**	**Relative activity (%)**
Glycine-HCl	2	294.380	93.543
Glycine-HCl	3	289.974	92.143
Glycine-HCl	4	30.868	9.809
Sodium acetate	4	289.606	92.026
Sodium acetate	5	283.609	90.120
Sodium acetate	6	265.740	84.442
Sodium acetate	7	259.131	82.342
Tris-HCl	7	258.396	82.109
Tris-HCl	8	265.005	84.209
Tris-HCl	9	224.983	71.491
Glycine-NaOH	9	70.768	22.487
Glycine-NaOH	10	205.890	65.424
Glycine-NaOH	11	100.020	31.783
Glycine-NaOH	12	19.852	6.308

2.5 Thermal stability

Figure 10. Thermal stability in the absence of substrate

Temperature	Specific activity (U/mg)	Relative activity (%)
4°C	400.422	100.000
10°C	389.030	97.155
20°C	397.609	99.297
30°C	352.180	87.952
40°C	328.833	82.122
45°C	238.959	59.677
50°C	5.345	1.335
60°C	7.173	1.791
70°C	4.079	1.019
80°C	7.032	1.756

Table 11. Thermal stability in the absence of 10 mM chitosan oligosaccharide or 0.5% chitosan

Condition	Specific activity (U/mg)	Relative activity (%)
Csn+G1	64.252	11.098
Csn+G2	192.638	33.272
Csn+G3	296.290	51.175
Csn+G4	455.006	78.588
Csn+G5	464.134	80.165
Csn+G6	484.452	83.674
Csn+chitosan	492.108	84.996
Csn+buffer	79.564	13.742
Control (not heat)	578.975	100.000

Table 12. Thermal stability in the absence of 0.5% chitosan low MW.

Time(hr)	Enzyme+Buffer		Enzyme+Chitosan	
	Specific activity (U/mg)	Relative activity (%)	Specific activity (U/mg)	Relative activity (%)
0	220.868	100.000	240.400	100.000
0.5	25.736	11.652	232.057	96.530
1	nd	nd	92.814	92.814
6	nd	nd	84.036	84.036
12	nd	nd	73.828	73.828
24	nd	nd	32.280	32.280

2.6 Substrate specificity

Table 12. Substrate specificity of recombinant *B. subtilis* chitosanase

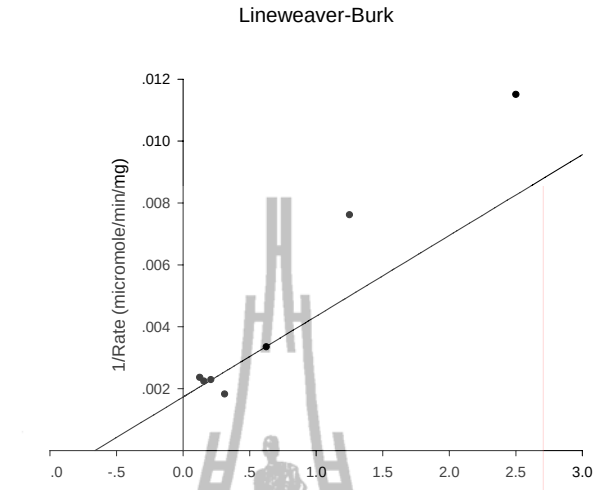
Substrate	Specific activity (U/mg)	Relative activity (%)
Chitosan low MW	280.084	100.000
Chitosan practical grade	312.151	111.483
Chitosan medium MW	137.374	49.062
Chitosan commercial	447.273	159.740
Glycol chitosan	nd	nd
Colloidal chitin	nd	nd

2.7 Kinetic analysis

Table 5. Kinetic parameters of recombinant *B. subtilis* chitosanase

Kinetic parameters	REP1	REP2	Av	RSD	%RSD
K_m	1.51	1.64	1.57	0.09	5.81
V_{max}	578.43	546.40	562.41	22.65	4.03
K_{cat} (min^{-1})	1.85×10^7	1.75×10^7	1.80×10^7	7.24×10^5	4.03
K_{cat} (s^{-1})	3.08×10^5	2.91×10^5	2.99×10^5	1.21×10^4	4.03
K_{cat}/K_m	2.04×10^5	1.77×10^5	1.90×10^5	1.88×10^4	9.85

REP1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.010
.008
.006
.004
.002
.0
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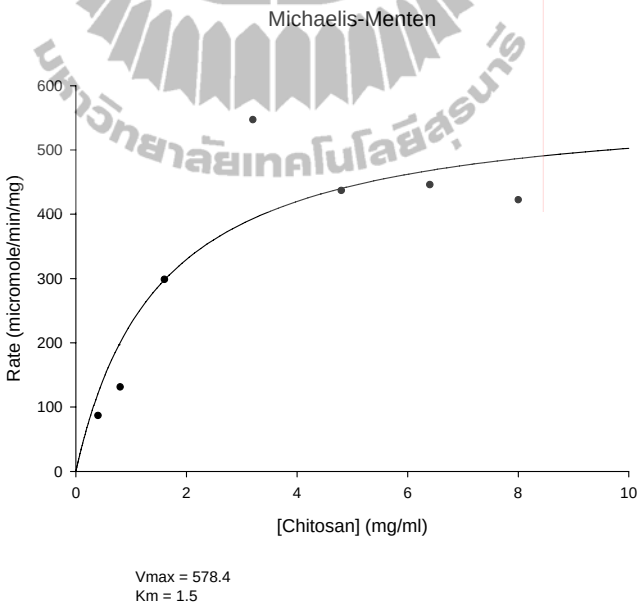
0.0
.5
1.0
1.5
2.0
2.5
3.0
1/[Chitosan] (mg/ml)
Vmax = 578.4
Km = 1.5


Michaelis-Menten
Rate (micromole/min/mg)
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500
400
300
200
100
0
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2
4
6
8
10
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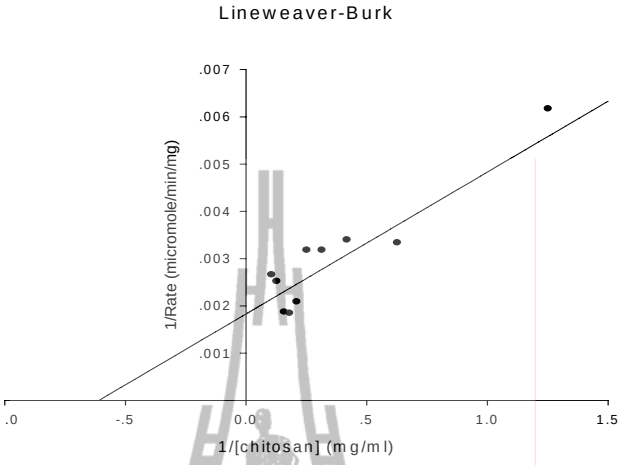
Vmax = 578.4
Km = 1.5

REP2



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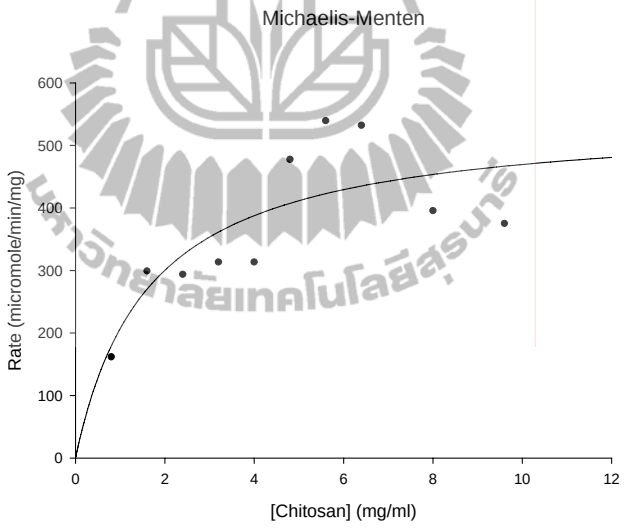

Vmax = 546.4
Km = 1.6

BIOGRAPHY

Miss Phornsiri Pechsrichuang was born on August 24, 1984 in Surin Province, Thailand. She graduated with a Bachelor's Degree from Department of Biotechnology, Facultly of Technology, Khonkaen University in 2007. After graduation, she has been employed under the position of Research and Development manager by Korn Thai Co., Ltd in 2007. She continued with her graduate studies in the School of Biotechnology, Institute of Agricultural at Suranaree University of Technology. She conducted to research in the topic of Cloning, expression and characterization of recombinant *Ba subtilis* chitosanase.