DIREDTED EVOLUTION OF CHITINASE FOR INDUSTRIAL APPLICATIONS

Puntarika Pesutcha

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การใช้เทคโนโลยีการกำกับวิวัฒนาการเพื่อการพัฒนาเอนไซม์ไคติเนสในอุตสาหกรรม

นางสาวปุณฑริกา เภสัชชา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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DIRECTED EVOLUTION OF CHITINASE FOR INDUSTRIAL APPLICATIONS

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

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ปุณฑริกา เภสัชชา : การใช้เทคโนโลยีการกำกับวิวัฒนาการเพื่อการพัฒนาเอนไซม์ไคติเนสในอุตสาหกรรม (DIRECTED EVOLUTION OF CHITINASE FOR INDUSTRIAL APPLICATIONS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. มณฑารพ ยมาภัย, 104 หน้า

ในงานวิจัยนี้ได้คัดเลือกยีนไคติเนสจากเชื้อ *Bacillus licheniformis* และใช้ *Escherichia coli* เป็นระบบในการแสดงออกของเอนไซม์รีคอมบิแนนท์เอนไซม์ไคติเนสจากเชื้อสายพันธุ์ DSM13 และสายพันธุ์ DSM8785 มีค่าอุณหภูมิที่เหมาะสมในการทำงานระหว่าง 50-55°ซ และ 50-60°ซ ตามลำดับ เอนไซม์ทั้งสองสายพันธุ์มีค่า pH ที่เหมาะสมในการทำงานที่ pH 6.0 ยีนไคติเนสถูกทำให้กลายพันธุ์อย่างสุ่มด้วยวิธีการปฏิกิริยาลูกโซ่โพลิ-เมอร์เรส (PCR)ในสภาวะที่มีความแม่นยำต่ำและเอื้อต่อความผิดพลาด (Error-prone PCR) ร่วมกับวิธีการสลับสับเปลี่ยนยีน (DNA Shuffling) คลังของยีนกลายพันธุ์ที่แตกต่างกันของเอนไซม์ไคติเนสได้ถูกคัดเลือกเพื่อหาเอนไซม์ที่สามารถทนต่อสภาวะที่เป็นกรด เอนไซม์ไคติเนสกลายพันธุ์ (128mt) ซึ่งแสดงค่ากิจกรรมที่ดีขึ้นในสภาวะที่เป็นกรดได้ถูกคัดเลือกเพื่อทำการศึกษาในขั้นต่อไป เมื่อใช้ *p*-Nitrophenyl-di-*N*-acetyl-chitobiose เป็นสับสเตรท ค่า k_{cat}/K_m ที่ pH 6.0 ของเอนไซม์ไคติเนสสายพันธุ์ดั้งเดิมจากสายพันธุ์ DSM13, DSM8785 และเอนไซม์ไคติเนสกลายพันธุ์ (128mt) มีค่า 5.17, 6.35 และ 14.73 $s^{-1}mM^{-1}$ ในขณะที่ค่า k_{cat}/K_m ที่ pH 3.0 ของเอนไซม์ไคติเนสเหล่านี้มีค่า 1.69, 1.36 และ 4.10 $s^{-1}mM^{-1}$ ตามลำดับ ค่า specific activity ของเอนไซม์ไคติเนสเหล่านี้มีค่า 290.9, 302.2 และ 351.72 Unit/μg ตามลำดับ เมื่อใช้ colloidal chitin เป็นสับสเตรท เอนไซม์ไคติเนสกลายพันธุ์ (128mt) มีค่าอุณหภูมิที่เหมาะสมในการทำงานที่แปรผันที่อุณหภูมิ 55 ถึง 60ºC และมีค่า pH ที่เหมาะสมในการทำงานที่ pH 6.0 จากการวิเคราะห์ลำดับกรดอะมิโนของเอนไซม์ไคติเนสกลายพันธุ์ (128mt) แสดงให้เห็นว่ามีสองกรดอะมิโนที่ กลายพันธุ์ในส่วนของ catalytic domain ที่ตำแหน่ง 231 จากกรดอะมิโนอะลานีน(Ala) เปลี่ยนไปเป็นกรดอะมิโนวาลีน (Val) และที่ตำแหน่ง 384 จากกรดอะมิโนกลูตามีน (Gln) เปลี่ยนไปเป็นกรดอะมิโนอาจีนีน(Arg) ในส่วนของ fibronectin type III domain ที่ตำแหน่ง 462 จากกรดอะมิโนวาลีน (Val) เปลี่ยนไปเป็นอะลานีน (Ala) และที่ตำแหน่ง 477 จากกรดอะมิโนแอสปาติก (Asp) เปลี่ยนไปเป็นกรดอะโมแอสปาราจีน (Asn) จากผลการทดลองที่ได้ชี้ให้เห็นว่าเทคนิคการกำกับวิวัฒนาการสามารถนำมาใช้พัฒนาเอนไซม์ไคติ-เนสกลายพันธุ์ที่มีกิจกรรมของเอนไซม์ที่ดีขึ้นโดยประมาณสองเท่าทั้งในค่า pH ที่เป็น

กลางและกรดเมื่อเปรียบเทียบกับสายพันธุ์ดั้งเดิมเมื่อใช้ *p*-Nitrophenyl-Di-*N*-Acetyl-chitobiose เป็นสับสเตรท

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

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PUNTARIKA PESATCHA : DIRECTED EVOLUTION OF CHITINASE FOR INDUSTRIAL APPLICATIONS. THESIS ADVISOR : ASST. PROF. MONTAROP YAMABHAI, Ph.D., 104 PP.

CHITINASE/ CHITIN/ DIRECTED EVOLUTION/ DNA SHUFFLING

In this research, the genes encoding chitinase from *Bacillus licheniformis* were isolated and expressed using an *Escherichia coli* expression system. The recombinant chitinase from *Bacillus licheniformis* strains DSM13 and DSM8785 have an optimal temperature between 50-55°C, and 50-60°C, respectively. Both of them have an optimal pH at 6.0. The genes were randomly mutated by a combination of error-prone PCR and DNA shuffling techniques. The library of variants of chitinases was then screened for the acidic tolerant activity. The mutant (128mt), which showed the improved activity, was selected for further study. When using *p*-Nitrophenyl-Di-*N*-Acetyl-chitobiose as a substrate, the k_{cat}/K_m value at pH 6.0 of wild type enzyme from strain DSM13, DSM8785 and mutated chitinase (128mt) were 5.71, 6.35 and 14.73 $s^{-1}mM^{-1}$, respectively, while the k_{cat}/K_m at pH 3.0 of wild type enzyme from strain DSM13, DSM8785 and mutated chitinase (128mt) were 1.69, 1.36 and 4.10 $s^{-1}mM^{-1}$, respectively. The specific activity of mutated chitinase (128mt) at pH 3.0 was 351.72 Unit/µg, while the specific activities of chitinase enzymes from strains DSM13 and DSM8785 were 290.9 and 301.2 Unit/µg, when using colloidal chitin as a substrate. The mutant (128mt) has an optimal temperature ranging from 55 to 60ºC, and an optimal pH at 6.0. Amino acid sequence analysis of this mutant chitinase reveals that two amino acids in catalytic domain (Ala231Val and Gln384Arg), and two amino acids in Fibronectin type III domain (Val426Ala and Asp477Asn) have been mutated.

These results indicated that the directed evolution technique has been used successfully to obtain a mutated chitinase with improved activity at both neutral and low pH, approximately 2-fold better than the wild type, when using *p*-Nitrophenyl-Di-*N*-Aceltyl-chitobiose as a substrate.

School of Biotechnology Student's Signature_____

Academic Year 2007 Advisor's signature_____

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LIST OF ABBREVIATIONS

°C	degree celcius
µg	microgram
µl	microlitre
µmole	micromole
bp	base pair
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide 5’ phosphate
et. al	Et alia (and other)
g	gram
hr	hour
kDa	kilodalton
kbp	Kilo base pair
l	liter
M	molarity
mg	milligram
min	minute
ml	milliliter
mM	millimolar
N	normality
ng	nanogram
PCR	polymerase chain reaction

LIST OF ABBREVIATIONS (Continued)

pmol	picomol
rpm	revolution per minute
v/v	volume per volume
w/v	weight per volume
s^{-1}	per second

CHAPTER I

INTRODUCTION

Chitin is the most abundance biopolymer, which can be found on earth. Chitin can be found in exoskeleton of crustacean (such as shrimp, shell, squid pen, and crab) and cell wall of fungi. Thailand has a large seafood industry which generate a huge amount of chitin waste. Normally, most of the waste is discarded through ocean dumping, incineration and land filling, causing secondary environmental pollution. Chitinase are a member of enzymes that hydrolyze chitin and generate chitooligosacharides (such as chitohexaose and chitoheptaose) and monomers of N-acetylglucosamine (GlcNAc). Chitinase that were produced from soil bacteria have been reported to have anti-fungal activity, which are the new frontiers in biocontrol of plant pathogenic fungi. Furthermore, the products of chitooligosacharides, such as chitohexaose and chitoheptaose, have been reported to have anti-tumor activity. In addition, the GlcNAc itself is also an anti-inflammatory agent useful for the treatment of ulcerative colitis and other gastrointestinal inflammation disorders. Moreover, GlcNAc has been used as a nutritional substrate for pediatric chronic inflammatory bowel disease and pharmaceutical therapy of osteoarthritis. The purpose of this project is to obtain chitinase enzymes that are suitable for industrial application i.e., stable at high temperature and or low pH. This is because during the process of chitin extraction from shrimp and crab shell obtained from food industries, the shell must first be partially digested with hydrochloric acid to extract chitin. In addition the

acidic condition will help to reduce the contamination during the fermentation process. Thus, this research has been proposed to use directed evolution technique to improve the property of *Bacillus* chitinase. The obtained enzymes that are more active whereas acidic condition will be useful for industrial application.

CHAPTER II

LITERATURE REVIEW

2.1 Chitin

Chitin is a polymer of N-acetylglucosamine (GlcNAc) monomers which is linkaged by β-1,4 N-glycosidic bond as shown in Figure1. It is one of the most abundant biopolymers that is found on earth, second to cellulose.

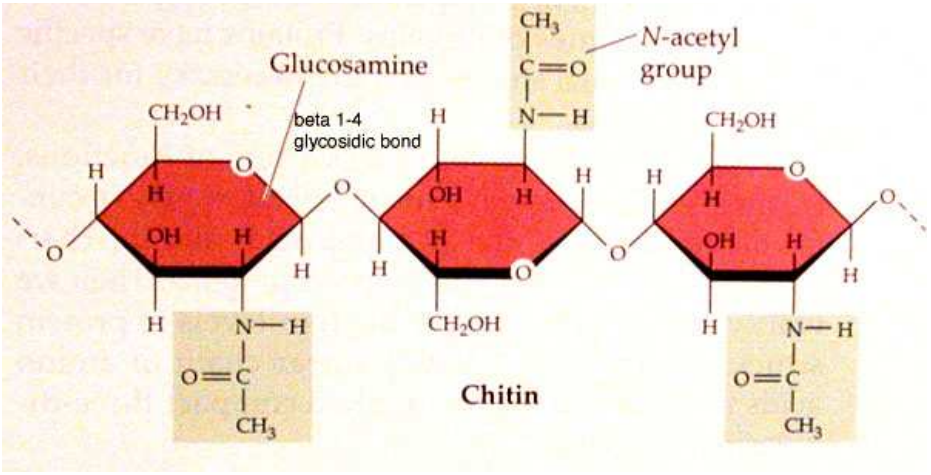

Figure 1. Structure of chitin.

The polymorphic forms of chitin differ in packing and polarities of adjacent chains in successive sheets. In the Beta-form, all chains are aligned in a parallel manner, whereas in alpha-chitin they are antiparallel. For the gamma-form, all chains are aligned randomly. Alpha-chitin is found in the calyces of hydrozoa, the eggshells of nematodes and rotifers, the radulae of mollusks and the cuticles of arthropods.

Beta-chitin is found in shells of brachiopods and mollusks, cuttlefish bone, squid pen, and pogonophora tubes. Gamma-chitin is found in cell wall of fungi and mushroom (Muzzarelli,1998)

2.2 Chitinase

Chitinase (EC.3.2.1.14) are a member of enzymes that hydrolyze chitin by cleaving its β-1,4 N-glycosidic bond. Chohen and Chet classified chitinase into two major categories, i.e. endochitinase and exochitinase (Cohen-Kupiec *et al.*,1998). Endochitinase (EC 3.2.1.14) act randomly on chitin to give chitoligomers, then chitobioses ($(GlcNAc)_2$) as the main products. Chitobiases (EC 3.2.1.52 or formally EC 3.2.1.30) and *N*-acetyl-*β*-hexosaminidases (EC 3.2.1.52) further hydrolyze the dimers and chitoligomers to yield *N*-acetylglucosamine (GlcNAc) as the final product (Sahai *et al.*,1993). Bernard Henrissat (1991) classified chitinase by comparison of their amino acid sequences, which have revealed that their catalytic domain could be grouped into two different families of glycosyl hydrolases family 18 and 19. The catalytic domains in family 18 chitinase have a $(\beta/\alpha)_8$ where as family 19 chitinase have high α-helical contents and share some structural similarity with chitosanases and lysozyme. Family 18 chitinase use substrate assisted double-displacement mechanism whereas family 19 chitinase use a single-displacement mechanism. In addition, chitinase can be divided into different classes. Chitinase from classes I, II and IV are of plant origin and make up the family 19 glycosyl hydrolases (Sahai and Manocha,1993; Hamel *et al.*,1995). Class III chitinase are mainly plant and fungal in origin, and class V is mainly comprised of bacterial chitinase. Both of classes III and V make up the family 18 glycosyl hydrolases. Plant chitinase play a role in defence

mechanism against fungal pathogens such as barley chitinase that inhibited the growth of fungal hyphae (Hejgaard *et al.*,1992; Kirubakaran *et al.*,2007). Fungal chitinase, play a role not only in nutrition, but they are also active in fungal developmental processes and in morphogenesis, because chitin is a major component in the cell wall of fungi (Sahai and Manocha,1993; Rast *et al.*,2003). In addition, chitinase from *Trichoderma* spp. has been shown to have activity against several plant pathogenic fungi (Cohen-Kupiec *et al.*,1998).

2.3 Application of chitinase and the products from chitinase degradation

2.3.1 Chitinase for the production of useful products

Chitinase can degrade chitin to generate chitooligasacharides such as chitohexaose and chitoheptaose, both of them has been reported to have anti-tumor activity (Patil *et al.*,2000). In addition, the GlcNAc itself is also an anti-inflammatory agent useful for the treatment of ulcerative colitis and other (Friedman *et al.*,1980; Russell,1999). Moreover, N-acetylglucosamine has been used as a nutritional substrate for pediatric chronic inflammatory bowel disease (Salvatore *et al.*,2000).

2.3.2 Chitinase in biocontrol of plant pathogenic fungi

A number of soil bacteria produce chitinolytic enzymes that have anti-fungal activity. For example, *Nocardiopsis prasina* OPC-131 soil bacteria have been reported to produce chitinase with anti-fungal activity (Tsujibo *et al.*,2003). Furthermore, rice chitinase (class I) had been cloned into cucumber by

Agrobacterium-mediated transformation, and it was showed that the high expression and intracellular localization of rice chitinase may be involved in enhancing resistance of transgenic plants to gray mold (Kishimoto *et al.*,2002). Recently, yam chitinase E solution (class IV, family 19) had been reported to be effective against the powder mildew (*Sphaerotheca humuli*) when sprayed to strawberry powder mildew infecting the leaves and barriers of strawberry plant (Karasuda *et al.*,2003).

There have been a number of reports on the cloning, expression and characterization of chitinase from varies bacteria as shown in Table 1.

Table 1. Overview of cloning and expression of chitinase genes from bacteria.

Microorganism	Gene	Expression host	Basic Properties	References
Serratia marcescens	Chitinase	*Pseudomonas* spp.	Inhibition growth of *Fusarium oxysporum* f. sp.	(Sundheim *et al.*,1988)
Streptomyces plicatus	Chitinase	*E. coli*	MW. 43.838 kDa.	(Robbins *et al.*,1992)
S. thermoviolaceus OPC-520	Chitinase	*E. coli* JM109	MW. 50 kDa.	(Hiroshi *et al.*,1993)
Aeromonas sp. No. 10S-24	ChitinaseII	*E. coli* DH5	Investigation of DNA sequence.	(Ueda *et al.*,1994)
Bacillus circulans WL-1	Chic	*E. coli*	Investigation of DNA sequence.	(Alam *et al.*,1995)
Janthinobacterium lividum	chi69	*E. coli*	Investigation of DNA sequence.	(Gleave *et al.*,1995)
Aeromonas sp. No. 10S-24	Chitinase	*E. coli* DH5α	Investigation of DNA sequence.	(Shiro *et al.*,1996)
Serratia marcescens KCTC2172	chitinase	*E. coli*	The specific activity of the 54-kDa and 22-kDa chitinase were 300 μM/min/mg and 17 M/min/mg on the natural swollen chitin, respectively.	(Gal *et al.*,1997)
Enterobacter sp. G-1	chiA	*E. coli*	The products of positive clones were found to degrade water insoluble chitin.	(Park *et al.*,1997)
Arthrobacter protophormiae	Endo-β-N-acetylgluco-saminidase	*E. coli*	Endo-A showed broad optima pH and were most active in the range of pH 5.0 to 9.0. Both enzymes were stable up to 60°C.	(Takegawa *et al.*,1997)

Table 1. (Continued)

Microorganism	Gene	Expression host	Basic Properties	References
Serratia marcescens KCTC2172	Chitinase	*E. coli*	The optimal temperature and pH of recombinant of enzyme were 45ºC and 5.5, respectively. The specific activitieson natural swollen chitin is 67 μmol min^{-1} mg^{-1}. The molecular weight is 52 kDa.	(Gal *et al.*,1998)
Xanthomonas sp. strain AK	chiA	*E. coli* DH5α	The opimal temperature and pH at 35°C and 4.5, respectively. The K_m and V_{max} values for colloidal chitin were estimated to be 1.8 mg/ml and 8.7 μmol/min/mg, respectively	(Sakka *et al.*,1998)
Aeromonas sp. no. 10S-24	Cihitinase	*E. coli* DH5α	The molecular weight of the unprocessed protein was calculated to be 51.4 kDa. Investigation of DNA sequence.	(Ueda *et al.*,1998)
Vibrio alginolyticus H-8	chiB	*E. coli* JM109	Investigation of DNA sequence.	(Ohishi *et al.*,2000)
Aeromonas sp.No.10S-24	β-N-acetylgluco-saminidase	*E. coli*	The recombinant chitinase had isoelectric point at 5.0. The Optimal pH and temperature were 7.0and 37°C, respectively. The K_m value for *p*-nitrophenyl-D-N-acetylglucosaminide was 0.24 mM.	(Ueda *et al.*,2000)

Table 1. (Continued).

Microorganism	Gene	Expression host	Basic Properties	References
Bacillus cereus 6E1	chi36	*E. coli* BL2 l-Gold (DE3)	Chi36 belongs to subfamily B of bacterial chitinase in family 18 of glycosyl hydrolases.	(Wang *et al.*,2001)
Salinivibrio costicola strain 5SM-1	chic	*E. coli* DH5α	Specific activity of recombinant chitinase was 21.19 mU/mg when using colloidal chitin as substrate.	(Aunpad *et al.*,2003)
Vibrio carchariae	chiA	*E. coli* M15	Investigation of DNA sequence. Chitinae A acts as an endochitinase. The molecular weight of native chtinase was 62.698 kDa.	(Suginta *et al.*,2004)
Bacillus cereus 28-9	chiCH and chiCW	*E. coli*	chiCH is an endo-chitinase and effectively hydrolyzes chitin and chito-multimers to chito-oligomers and the end product chitobiose, and chiCW is an exo-chitinase and degrades chito-oligomers to produce chitobiose.	(Huang *et al.*,2005)
Bacillus sp. DAU101	Chitinase	*E. coli*	The pH and temperature optima of the enzyme were 7.5 and 60 °C, respectively.	(Lee *et al.*,2007)

2.4 Directed evolution

Recent advances in molecular biology and genetic engineering have led to rapid methods for improving protein and enzyme properties (Patten *et al.*,1997; Arnold *et al.*,1999; Kurtzman *et al.*,2001). This technique, termed directed evolution, mimics the natural selection process, where genes are evolved through recursive rounds of mutation, recombination, and selection (Fisher,1995) One of the most efficient methods that are used to direct the evolution of proteins is DNA shuffling, which was introduced by Stemmer, WPC in 1994 (Stemmer,1994). This method allows a much larger spectrum of diversity to be generated than by natural recombination or mutational mechanisms because two or more homologous from multiple species in different ratios are used to recombine. Successful applications of DNA shuffling and other directed evolution techniques for the improvement or alteration of protein and enzymes for various purposed have been reported. These included the improvement of various enzyme activities(Jestin *et al.*,2004; Valetti *et al.*,2004; Vatetti *et al.*,2004; Williams *et al.*,2004) and a wide variety of pharmaceutical products (Chartrain *et al.*,2000; Ryu *et al.*,2000; Kurtzman, Govindarajan, Vahle, Jones, Heinrichs and Patten,2001; Bornscheuer *et al.*,2002; Jaeger *et al.*,2002; Zhao *et al.*,2002; Vasserot *et al.*,2003; Mijts *et al.*,2005; Raviprakash *et al.*,2006). It has been shown that activity of an enzyme could be greatly improved when a family of genes from different organisms are used to recombined (Patten, Howard and Stemmer,1997; Rosic *et al.*,2006). However, the method for recombination has to be optimized according to the homology between the genes that will be used in the recombination process (Lutz *et al.*,2004). In this thesis, a combination of error-prone PCR and DNA shuffling will be used to generated a library of mutant enzyme. The directed evolution method is

divided into 2 main step. 1) Generation of library of mutant enzyme and 2) Screening or selection of the desired property. The principles of the generation of library of mutant enzyme are described below.

2.4.1 Error-prone PCR method

The error–prone PCR is an in vitro random mutagenesis method, which is mostly an effect of a lacking in DNA polymerase combination with low fidelity buffer conditions. Because *Taq* DNA polymerase has a high intrinsic error rate (between 8 $\times 10^{-6}$ and 2×10^{-4} error/nucleotide synthesis depending on the reaction condition) it is favored for error-prone PCR mutagenesis. The increasing of Mn^{2+} concentration, lower template concentration, the different ratio of dNTPs and higher number of PCR cycles result in the increasing higher mutation frequencies in the amplified DNA. *Taq* DNA polymerase was used in this study. This enzyme has been used to improve the properties of a number of different enzyme (Ruan *et al.*,1997; Ruan *et al.*,1997; Reetz *et al.*,1998; Reetz *et al.*,1998; Kohno *et al.*,2001; Chusacultanachai *et al.*,2002; Komeda *et al.*,2003; Nakaniwa *et al.*,2004; Deng *et al.*,2005; Deng *et al.*,2006; Stephens *et al.*,2007).

2.4.2 DNA shuffling method

DNA shuffling method had been developed by Stemmer and colleague, it is an *in vitro* method that is useful for improvement of enzymes properties. The improved gene can be obtained by a recombinant of homologous genes base methods and the appropriate selection step (Stemmer,2002). DNA shuffling is usually done by *DNase*I treatment and then by PCR. *DNase*I enzyme is employed to generated a small DNA

fragment, which is then subjected to the PCR reaction in two steps (Kikuchi *et al.*,1999), the first being without primer and the second with the specific of primer (Kikuchi, Ohnishi and Harayama,1999). This method has been used to created a Human Theta Class 1-1 Glutathione Transferase that exhibited highly catalytic efficiency and substrate promiscuity (Griswold *et al.*,2006).

2.4.3 Screening and selection

The identification of improved gene products can be accomplished by selection or screening of organism that expressed the improved genes. Selection method is less labor intensive because only organisms that harbor improved gene will be selected. For the screening process, high-throughput method is needed to be developed for each gene products (Arnold *et al.*,2003; Reetz,2006).

CHAPTER III

RESEARCH OBJECTIVES

In this research, the combination of error-prone PCR and DNA shuffling was used to improve the property of enzyme chitinase. The method for high-throughput screening of improved enzyme that is more active at lower pH or thermostable was developed. The characteristic of both wild type and mutant enzymes with improved activity was studied.

The summary of the objectives of this research is listed below.

1. To clone chitinase gene from *Bacillus licheniformis* DSM8785 and *Bacillus licheniformis* DSM13.
2. To express chitinase gene in *E. coli*.
3. To determine basic properties of the recombinant enzymes such as DNA sequence, molecular mass, and chitinolytic activity.
4. To utilize directed evolution technique, using error-prone PCR and DNA shuffling methods to improve the property of chitinase.
5. To determine a kinetic property of both wild type and mutant enzymes with improved activity.

CHAPTER IV

MATERIAL AND METHODS

4.1 Material

4.1.1 Bacterial strains and growth conditions

Bacillus licheniformis DSM13/ATCC 14850 and DSM8785, which were used as the source of chitinase genes, were obtained from American Type Culture Collection and Prof. Dr. Dietmar Haltrich, repectively. The bacteria were grown and kept in M1medium. Both of strain of *Bacillus licheniformis* were cultured in M1 medium at 30°C.

Escherichia coli strain DH5αF' was used as a host for transformation while *E. coli* strains TOP10 and BL21(DE3) were used as a host for protein expression. *E. coli* strains were cultured in Luria-Bertani medium at 37°C.

4.2 Methods

4.2.1 Cloning and construction of recombinant plasmids for expression of chitinase genes

Single colonies of *Bacillus licheniformis* strain DSM13/ATCC14850 and strain DSM8785 were boiled in ultra pure water and used as the templates for amplification of chitinase genes. Chitinase genes were amplified by using a mixture of *Tag* DNA polymerase and *Pfu* DNA polymerase with two sets of oligonucleotide primers containing appropriate restriction site, first set was chiHind3 and XhoI to

amplified and cloned into pFLAG-CTS vector and second set was chiNcoI and chiXhoI to amplified and cloned into pET-21(d+), by polymerase chain reaction (PCR). The PCR thermal profile consist of initial denature 95^{o}C for 2 min followed by 30 cycles of 95^{o}C for 45 sec, 56^{o}C for 55sec, and 72^{o}C for 2 min, followed by final extension step at 72^{o}C for 10 min. The amplified DNA fragment of 1.7 kb was cloned into the corresponding restriction site of pET-21(d+) and pFLAG-CTS. The full length of chitinase gene containing signal peptide was cloned into pET-21(d+) vector (designated pEChi13 and pEChi8785), where as the mature of chitinase lacking signal peptide were fused with OmpA signal peptide of *E. coli* when cloned into the pFLAG-CTS vector (designed pFChi13 and pFChi8785). The construction in pET-21(d+) or pFLAG-CTS expression vector were expressed in *E. coli* BL21(DE3) or TOP10, respectively.

4.2.2 Chitinase activity assay on colloidal chitin containing LB agar plate

E. coli BL21(DE3) carrying recombinant chitinase and *E. coli* TOP10 carrying recombinant chitinase (pFChi13 and pFChi8785) were grown on LB agar plate that containing 1mM isopropyl β-D-thiogalactopyranoside and 0.2% colloidal chitin (colloidal chitin was prepared by chitin from crab (Sigma) at 37°C for 18 hr. After that, colonies showing clear halos of hydrolyzing overlaid chitin were observed by staining with 0.2% Congo Red (Lee *et al.*,2003).

4.2.3 Determination optimal induction time for the production of recombinant enzyme

To determine the optima induction time for the expression of recombinant enzyme**,** *E. coli* carrying appropriate recombinant chitinase was grown in 5 ml LB

medium with 100 μg/ml ampicillin. Then, 2 ml of overnight culture was inoculated into 250 ml LB medium containing 100 μg/ml ampicillin and grown at 37^{O}C until the A_{600} reached 0.6. The expression of recombinant chitinase was induced by 1 mM IPTG at 28^{O}C**.** The sample was collected at different period of time by centrifugation at 4000 rpm for 30 min. Crude enzyme was prepared from bacterial cells by modified osmotic shock method (Chen *et al.*,2004). Culture supernatant and crude enzyme extracts were determined by SDS-PAGE.

4.2.4 SDS-PAGE and Zymogram analysis

SDS-PAGE was performed according Sambrook and Russell (2001) by prepared 4% stacking gel and 15% separating gel. Protein sample was added with protein sample buffer (0.0625 M Tris-HCl pH 6.0.8, 0.01% bromophenol blue, 2% SDS, 20% v/v glycerol, and 0.1 M dithiothreitol (DTT)) and boiled for 3 min before loading into the gel. Electrophoresis was performed using BioRad Mini Protean II Cell (BioRAD), and was run at 120 V for 1.3 hr. After electrophoresis, the gel were stained with Coomassie brilliant blue G-250. Zymogram was performed as follows: protein sample was added into SDS-PAGE sample buffer (0.06 M Tris-HCl, pH 6.0.8, 15% sucrose, 2% SDS and 0.1% bromophenol blue) and boiled for 3 min. The proteins were separated through the 12% polyacrylamide gel containing 0.01% glycol chitin. After electrophoresis, the gel was soaked in 0.1 M sodium acetate buffer (pH 5.0) containing 1% Triton X-100 at 37^{o}C for 2 hr under shaking condition. The gel was rinsed with de-mineralized water, then the gel was stained with 0.01% Calcofluor White M2R in 0.5 M Tris-HCl (pH 8.9). Protein band exhibiting chitinase activity were visualized under UV transilluminator (Trudel *et al.*,1989).

4.2.5 Western Blot analysis

SDS-PAGE was performed according Sambrook and Russell (2001) by prepared 4% stacking gel and 15% separating gel. Protein sample was added with protein sample buffer (0.0625 M Tris-HCl pH 6.0.8, 0.01% bromophenol blue, 2% SDS, 20% v/v glycerol, and 0.1 dithiothreitol (DTT)) and boiled for 3 min before loading into the gel. Electrophoresis was performed using BioRad Mini Protean II Cell (BioRAD) and was run at 120 V for 1.5 hr. After electrophoresis, the gel were transferred onto PVDF membrane using Semi-dry blotting according to the manufacture's protocol. After blotting, membrane was incubated with blocking buffer (25 mg/ml BSA in Tris-buffer Saline Tween (TBST: 100 mM Tris-HCl, 0.9% NaCl, and 0.1% Tween-20) for 1 hr and then wash three times with TBST. Then, the membrane was incubated with HisProbe-HRP in blocking buffer for 1 hr under rotating condition. After that, the membrane was washed four times with TBST. The membrane was incubated with ECL solution (Solution 1:9 ml of ddH_2O, 1 ml of 1M Tris-HCl (pH 8.5), 45 µl of 250 mM Coumaric acid (Sigma), and 100 µl of 250 mM Luminol (Fluka); Solution 2: 9 ml of ddH_2O, 1 ml of 1M Tris-HCl (pH 8.5), and 6 µl of 30% Hydrogen Peroxide).Then, the membrane was exposed on X-ray film (Kodax). Protein sample that contain histidine tag was exhibited as dark band on X-ray film.

4.2.6 Construction of enzyme library

4.2.6.1 Error-Prone PCR

Error-Prone PCR was preformed according to Arnold and Georgiou (2003). Chitinase genes (pFChi13 and pFChi8785) were used as the templates to amplified the error-prone products. The error-prone reactions consisted of 10 ng of DNA templates, dNTP (New England BioLabs) at the concentrations of 0.2 mM dATP, 0.2

mM dGTP, 1.0 mM dCTP, 1.0 mM dTTP, and 2.9 mM $MgCl_2$, 0.15 mM $MnCl_2$, 5 U *Taq* DNA polymerase (New England BioLabs), 1X *Taq* DNA polymerase buffer, and 0.3 μM of each primers chiHind3 and chiXhoI. The reaction was placed in the PCT-200 Peltier Thermol cycler (MJ Research) that has been preheated to 95°C for 2 min; followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 50 sec, and extension at 72°C for 2 min; with final extension at 72°C for 10 min. The error-prone products (designated Er13 and Er8785) were verified for correct size on an agarose gel, and purified using QIA PCR purification Kit (Qiagen).

4.2.6.2 DNA shuffling

The second part of evolution involved the shuffling of the mutant genes from the first step. The shuffling was essentially performed as described by Arnold and Georgiou (2003) but with the following modifications: approximately 5 μg of each error-prone products (Er13 and Er8785) were digested with 0.1 U *DNase*I (Fermentas) in 50 mM Tris-HCl pH 7.4, 10 mM $MnCl_2$ at 15 °C for 7 min. The reaction was terminated by addition of 7 μl ice-cooled buffer consisting of 0.5 M EDTA pH 8.0. The *DNase*1 digested product was separated on a 15% polyacrylamide gel. Fragments with sizes between 25 and 250 bp were cut from polyacrylamide gel. The DNA fragments (designated DNaseEr13 and DNaseEr8785) were isolated from polyacrylamide gel by following the modified Crurh and SoaK method (Sambrook *et al.*,2001). The polyacrylamide gels were incubated with 0.5 M ammonium acetate at 37°C for 18 hr., then filtrated with siliconized glass wool. The eluted DNA fragments were purified with phenol:chloroform and chloroform; followed by ethanol precipitation (Sambrook and Russell,2001). To precipitate the DNA fragment solution, 3 M sodium acetate was added to a final concentration of 0.3 M and then 2

volumes of ice-cold absolute ethanol was added and the solution was mixed well. The solution was stored at -20°C for 30 minutes. The DNA fragment was recovered by centrifugation at 14,000 rpm for 10 min at 4°C and then the supernatant was added with 750 µl of 70% ethanol and centrifuged at 14,000 rpm for 2 min at 4°C; then the supernatant was removed and the open tube was stored at room temperature until the pellet was dry. The DNA pellet was dissolved with 50 µl DNase/RNase free water. To reassemble the digested products, the purified fragments of DNaseEr13 and DNaseEr8785 were equally mixed and reassembled using 3U *Pfu* DNA polymerase (Promaga), 1X *Pfu* DNA polymerase buffer, and 0.2 mM dNTP. This reaction was placed in the PCT-200 Peltier Thermol cycler (MJ Research) that has been preheated to 96°C for 2 min; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min + 5 sec/ cycle; with final extension at 72°C for 10 min. The amplification of the shuffled genes were performed by using a mixture of 2.5 U of *Taq* DNA polymerase (New England BioLabs) and 1.5 U of *Pfu* DNA polymerase, and 1 µl of the reassembled products was used as template. This reaction consisted of 0.2 mM dNTP (New England BioLabs), 1X *Pfu* DNA polymerase, and 0.3 µM of each primers (chiHind3 and chiXhoI). The reactions was placed in the PCT-200 PeltierThermol cycler (MJ Research) that has been preheated to 96°C for 2 min; followed by 10 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 90 sec; followed by 14 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 90 sec + 20 sec/cycle; with final extension at 72°C for 10 min. The shuffled products (designated SH13/8785) were verified for correct size on an agarose gel, and purified using QIA PCR purification Kit (Qiagen). The SH13/8785 products were

cloned into pFLAG-CTS vector and transformed into *E. coli* TOP10 by electroporation as described by Sambrook and Russell (2001).

4.2.7 High-throughput screening

4.2.7.1 First round screening for chitinase activity

E. coli carrying the library of SH13/8785 genes were grown on LB agar plate containing 100 μg/ml of ampicillin, 1mM IPTG and 0.2% colloidal chitin (Screening plate) or an LB agar plate containing 100 μg/ml of ampicillin(Master plate) at 37°C for 18 h. After that, 0.2% Congo Red (Sigma) was poured onto the screening plate. The colonies that produced clear zone (positive clone) were selected for 2nd round screening. The wild type (pFChi13 and pFChi8785) and pFLAG-CTS were used as positive and negative controls, respectively. The corresponding positive clone from the master plate, were collected in 15% glycerol stocks and kept at -20°C.

4.2.7.2 Second round screening for pH tolerant

The positive colonies from the first round, wild type (pFChi13 and pFChi8785) and pFLAG-CTS were picked (triplicate) into 0.5 ml of LB medium containing 100 μg/ml of ampicillin and incubated in microtiter chamber at 37°C for 6 hr. After that the enzymes was induced with 0.5 ml LB medium containing 100 μg/ml of ampicillin and 2 mM IPTG (final concentration is 1 mM) and incubated at 37°C for 16 hr. Then, the culture was chilled in an icebox for 5 min and then centrifuged at 4000 rpm for 20 min at 4°C to collect the cells. The cells were resuspended in 50 μl cold (4°C) spheroplast buffer (100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 20 μg/ml phenylmethylsulfonyl fluoride (PMSF)). After incubation for 5 min on ice, bacterial cells were collected by centrifugation at 4000 rpm at 4°C for 20 min and re-

suspended with 25 µl of cold sterile water supplemented with a proteinase inhibitor (Sigma). This bacterial suspension was incubated for 45s on ice and subsequently mixed with 1.67 µl of 20 mM $MgCl_2$ (a final concentration of 1mM). The supernatant of nearly 60 µl (triplicate) was collected by centrifugation at 4000 rpm at 4°C for 20 min as the periplasmic fraction. Ten µl of periplasmic fraction was added into 100 µl of 0.18 mM p-NP-$(GlcNAc)_2$ in 50 mM buffer (pH 3.0, pH 6.0, and pH10) and incubated at 37°C for 1 hr. The reaction was terminated by adding 10 µl of 1 N NaOH, and the amount of p-nitrophenyl released from p-NP-$(GlcNAc)_2$ was measured by recording the absorbance at 405 nm.

4.2.7.3 Confirmation of acidic tolerant activity

4.2.7.3.1 Using p-Nitrophenyl-chitobiose as substrate

The positive clone from the 2nd round screening**,** wild type (pFChi8785) and pFLAG-CTS were picked (triplicate) into 5 ml of LB medium containing 100 µg/ml of ampicillin and incubated in microtiter chamber at 37°C for 6hr. After that the enzymes was induced with 5ml of LB medium containing 100 µg/ml of ampicillin and 2 mM IPTG (final concentration is 1 mM) and incubated at 37°C for 16 hr. Then, the culture was chilled in an icebox for 5 min and then centrifuged at 4000 rpm for 20 min at 4°C to collect the cells. The cells were resuspended in 500 µl cold (4°C) spheroplast buffer (100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 20 µg/ml phenylmethylsulfonyl fluoride(PMSF)). After incubation for 5 min on ice, bacterial cells were collected by centrifugation at 4000 rpm at 4°C for 20 min and re-suspended with 250 µl of cold sterile water supplemented with a protease inhibitor (Sigma). This bacterial suspension was incubated for 45 sec on ice and subsequently mixed with 16.7 µl of 20 mM $MgCl_2$ (a final concentration of 1 mM). The supernatant

of nearly 260 µl (triplicate) was collected by centrifugation at 4000 rpm at 4^{o}C for 20 min as the periplasmic fraction. Ten µl of periplasmic fraction was added into 100 µl of 0.18 mM p-NP-$(GlcNAc)_2$ in 100 mM buffer (pH 3.0, pH 6.0, and pH10) and incubated at 37°C by using Thermomixer (Eppendorf) for 1 hr. The reaction was terminated by adding 10 µl of 1 N NaOH, and the amount of p-nitrophenol released from p-NP-$(GlcNAc)_2$ was measured by recording absorbance at 405 nm.

4.2.7.3.2 Using colloidal chitin as substrate

Two hundreds µl of enzymes sample was added into 800 µl of 1% colloidal chitin in 100 mM buffer (pH 3.0, pH 6.0, and pH10) and incubated at 37°C by using Thermomixer (Eppendorf) for 1 hr. The reaction was terminated by adding 200 µl of hot 0.8 M Sodiumtrataborate. After that this reaction was centrifuged at 14,000 rpm for 1 min. Then 600 µl of supernatant was boiled for 3 min and then cold down. This reaction was then added with 3 ml of 1X DMAB and incubated at 37°C with shaking. The amount of GlcNAc released from colloidal chitin was measured by recording absorbance at 585 nm.

4.2.8 Purification of recombinant wild type and improved chitinase

E. coli that carry pFChi13, pFChi8785 and 128mt were cultured in LB medium for overnight and then transferred into a 500 ml LB medium containing 100µg/ml ampicillin and cultured at 37°C until OD_{600} reach to 0.6-1.0. Cells were then induced by using IPTG at a final concentration of 1mM and then culture at 28°C for 4 hr. The cells were then harvested by centrifugation at 8000 rpm at 4°C for 20 min. Crude enzyme was prepared from bacterial cells by modified cells lysis method (Suginta, Vongsuwan, Songsiriritthigul, Prinz, Estibeiro, Duncan, Svasti and Fothergill-

Gilmore,2004). The supernatant containing soluble chitinase were purified using Ni-NTA resins according to Qiagen's protocol. Three ml of 50% slurry of Ni-NTA super flow resin is put into a 15 ml FALCON tube. Then it was spin down at short spin for 10 sec and the supernatant is removed and then the resins were equilibrated with lysis buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 10 mM imidazole, pH 8.0). After that, it was spin down at short spin for 10 sec and the supernatant was removed. The cells lysis was added into the tube containing Ni-NTA resins and rotated for 2 hr at 4 ºC and then the tube was spined down and the supernatant was removed. Fifteen ml of washing buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 40 mM imidazole, pH 8.0) was added and the tube was rotated for 15 minutes at 4 ºC. After that, the tube was spin down and the supernatant was removed. The washing step was repeated further 2 times. The samples of the washes were kept for SDS-PAGE analysis later. After tree round of washing the tube was spin down at short spin for 10 sec and the supernatant was removed. Five hundred µl of elution buffer (containing 250 mM imidazole) was added and the tube was rotated at 4 ºC for 10 minutes, spin down, and the supernatant, which contained soluble chitinase, were pooled and concentrated using Vivaspin membrane concentrators (MW cut-off 30,000). Fifty mM sodium phosphate buffer, pH 6.0 was used as washing buffer and the fraction of 1 ml were collected and glycerol was added to a final concentration of 15%, and stored at -20°C. Pure enzymes and crude enzyme was subjected to SDS-PAGE analysis.

4.2.9 Enzyme characterization

4.2.9.1 Optimal temperature for activity of chitinase

Optimal temperature was measured in a reaction mixture containing 0.4 µg of pure enzyme (pFChi13 and pFChi8785) plus 0.18 mM *p*-NP-$(GlcNAc)_2$ in 100 mM

sodium phosphate buffer (pH 6.0). Enzyme activity was assayed at temperature between 0 and 90°C with difference temperature changes, and incubation period of 20 min with shaking for 750 rpm in Thermomixer. The reaction was terminated by adding 10 µl of 1 N NaOH, and the amount of *p*-nitrophenol released from *p*-NP-$(GlcNAc)_2$ was measured by recording absorbance at 405 nm. One unit of chitinase activity was defined as the amount of enzyme required to release of 1 µmole of *p*-nitrophenol from substrate per min (Wang *et al.*,2001).

4.2.9.2 Optimal pH for activity of chitinase

The optimal pH for chitinase activity was determined by incubating 0.4 µg of pure enzyme with varying pH values of the same reaction mixture between 2.0 and 10.0, at increment of 1 pH unit. The buffer used were: 0.1 M glycine-HCl buffer (pH values between 2.0 and 3.0); sodium citrate buffer (pH values between 3.0 and 6.0); sodium phosphate buffer (pH values between 6.0 and 8.0); glycine-NaOH buffer (pH values between 8.0 and 10.0). Chitinase activity was assayed at 37°C for 20 min with shaking for 750 rpm in Thermomixer. After 30 min, 10 µl of 1 N NaOH was added. The chitinase activity was measured as described above.

4.2.9.3 Kinetic property characterization

Substrate was prepared in concentration ranging from 0.008 mM to 0.7 mM in order to obtain steady-state kinetic constant. Each substrate solution was mixed with 0.4 µg pure enzyme solution and was incubated at 37°C for 30 min. The initial velocity was determined from increase in *p*-nitrophenol as described above. The values of the kinetic constant were calculated from the Michaelis-Menten plot using a

non-linear curve fitting method (SigmaPlot2000), and turnover number (k_{cat}) and catalytic efficiency values (k_{cat}/K_m) were further calculated.

CHAPTER V

RESULTS AND DISCUSSIONS

5.1 Cloning and construction of recombinant plasmids for expression of chitinase genes

Chitinase genes from *Bacillus licheniformis* DSM13/DSM8785 were cloned into pET-21(d+) and pFLAG-CTS as shown in Figure 2. The constructs were designated as pETChi13, pETChi8785, pFChi13, and pFChi8785. T7 and *tac* promoter were used to control the expression of recombinant protein in pET-21(d+) and pFLAG-CTS, respectively.

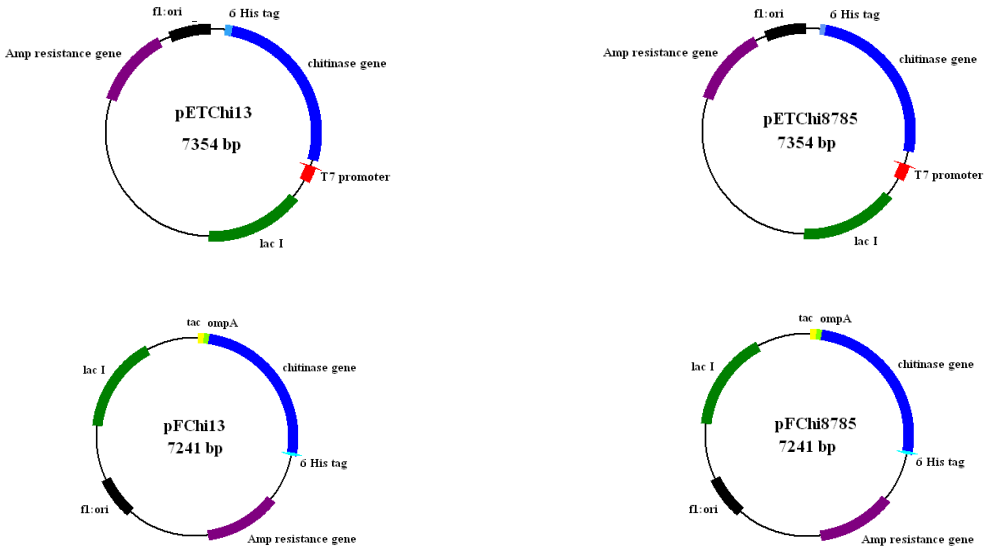

Figure 2. Maps of recombinant chitinase.

The recombinant enzymes were fused 6-his tag at C-terminal by PCR base method. *E. coli* cells that carried recombinant chitinase were grown on LB supplemented with amplicilin and colloidal chitin, the hydrolytic activity of chitinase were demonstrated as clear zone, when induced with IPTG as shown in Figure 3.

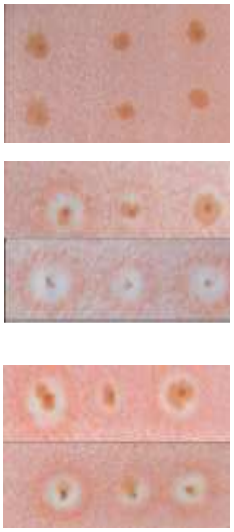

Figure 3. Chitinase activity assay on chitin agar plate.

pFLAG-CTS vector were subsequently selected for expression the recombinant chitinase because of the hydrolytic activity of recombinant chitinase obtained from pFLAG-CTS vector were better than these from pET-21(d+) vector.

The induction time was determined by incubation of the culture medium with 1 mM IPTG at 28^{O}C for 0, 4 hr and overnight. We found that the appropriate induction time was 4 hr as shown in Figure 4.

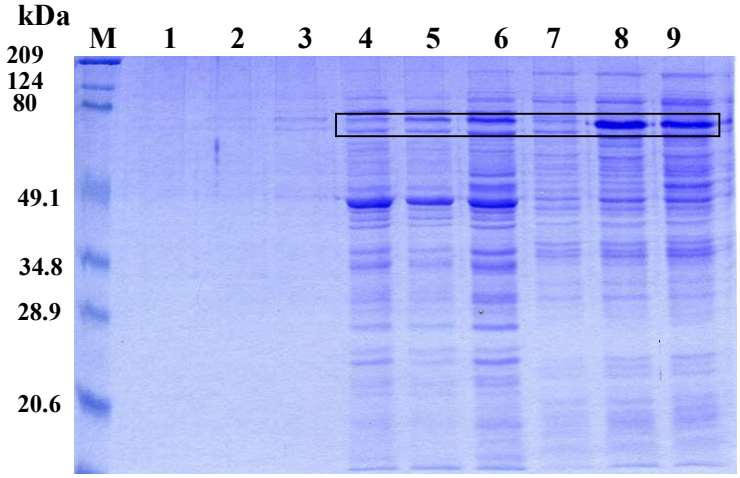

Figure 4. SDS-PAGE analysis for time induction of recombinant chitinase. Crude enzyme preparations from *E. coli* harboring pFChi8785 were load onto each. Lane 1: broth of pFChiG8785 at 0 hr; lane 2: broth of pFChi8785 at 4 hr; lane 3 : broth of pFChi8785 at overnight; lane 4 : periplasmic fraction of pFChi8785 at 0 hr; lane 5 : periplasmic fraction of pFChi8785 at 4 hr; lane 6 : periplasmic fraction of pFChi8785 at overnight; lane 7 : cell lysis of pFChi8785 at 0 hr; lane 8: cell lysis of pFChi8785 at 4 hr; lane 9 : cell lysis of pFChi8785 at overnight. The open box indicate the recombinant chitinase.

When 1 mM IPTG was added into culture medium to induce the recombinant chitinase, the recombinant protein expressions were increased from 0-4 hr while there was secreted to culture medium when the culture medium was incubated at 28^{O}C for overnight. The *tac* promoter has been used to controlled the expression of recombinant cyanase in *E. coli* K-12 by adding 0.5 mM IPTG in the culture medium and incubated at 30°C for 3 hr (Sung *et al.*,1987)

Crude enzyme preparations from *E. coli* harboring different constructs were detected for activity on a zymogram by copolymerization of glycol chitin with SDS-PAGE. The hydrolytic activity was shown as dark band under UV light detection as shown in Figure 5.

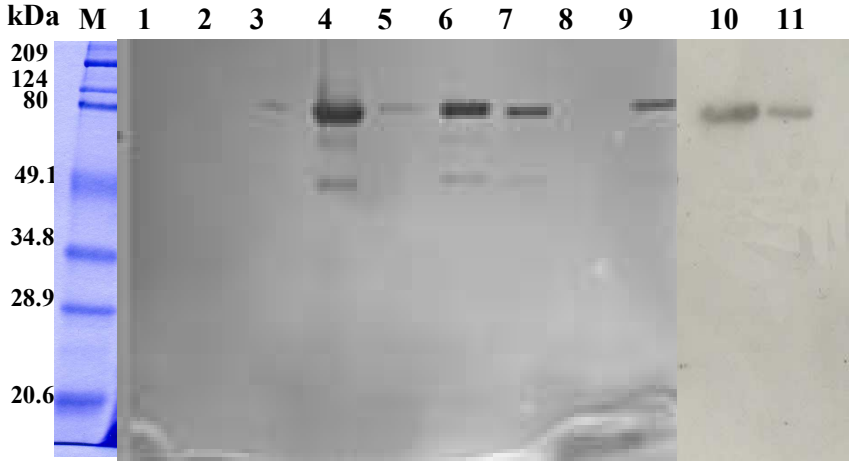

Figure 5. Zymogram analysis and Western blots analysis of recombinant chitinase. Crude enzyme preparations from *E. coli* harboring different constructs were load onto each lane and the activities of the enzymes were detected by in-gel activity staining as described. Lane M; Molecular weight standards; Lane 1: periplasmic fraction of pFLAG-CTS; lane 2: broth of pFLAG-CTS; lane 3: dialysate of pFLAG-CTS; lane 4: periplasmic fraction of pFChi13; lane 5: broth of pFChi13; lane 6: dialysate of pFChi13; lane 7: periplasmic fraction of pFChi8785; lane 8: broth of pFChi8785; lane 9: dialysate of pFChi8785; lane 10: Western blots analysis of cell lysis from pFChi13; lane 11: Western blots analysis of cell lysis from pFChi8785.

The approximate molecular weights of all recombinant enzymes were 64 kDa which correlated with hypothetical MW calculated from amino acid sequence of the enzymes. Other reports from zymogram analysis have shown that purified chitinase from *Bacillus* sp. DAU101 was shown MW were 66 kDa (Lee, Park, Yoo, Chung, Lee, Cho, Ahn, Kim and Choi,2007) while chitinase from *Bacillus* sp.13.26 were 60 kDa (Yuli *et al.*,2004). The recombinant chitinase were subjected to Western blot analysis and the blot was immono stained with rabbit anti-HisProbe conjugated with HRP as shown in Figure 5. The chitinase was detected by exhibition of dark band at 64 kDa, which is similar to the size detected by Zymogram (Land 10-11). This suggested that the 6-his tag was functional for affinity purification with Ni-NTA and that the 6-His tag didn't interfere with the enzyme activity. The amino acid sequences of pFChi13 and pFChi8785 were investigated by automated DNA sequencing (Macrogen, Korea) and revealed highly similarity in 99.8% identity with each other.

The sequence alignment was done by ClustalW program.

(http://www.ebi.ac.uk/Tools/clustalw/).

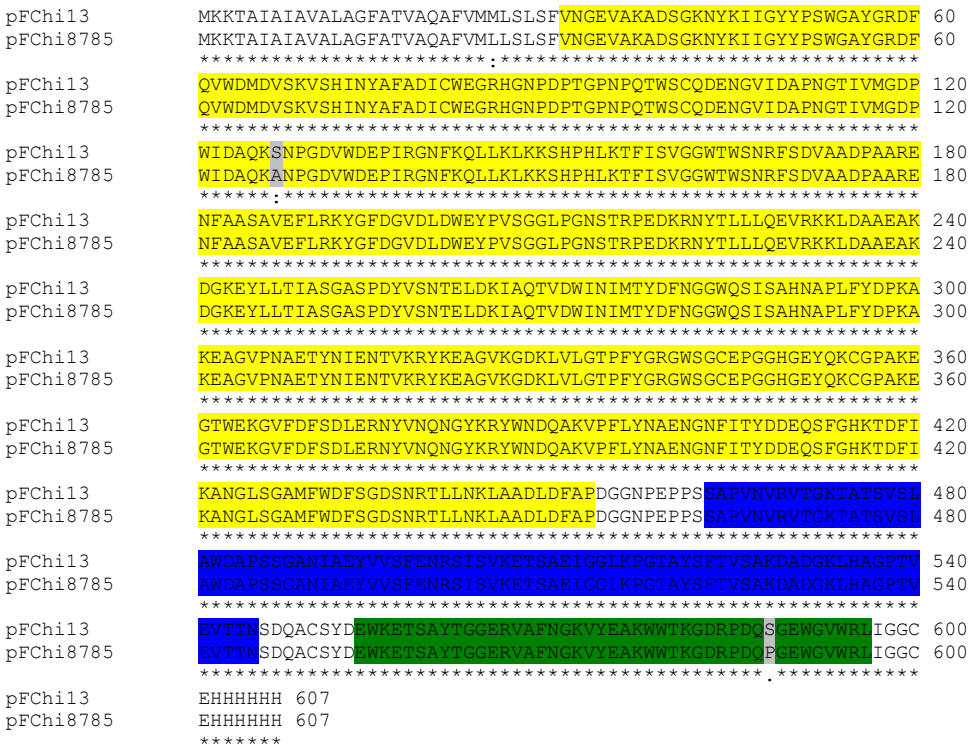

Figure 6. Alignment of recombinant chitinase from *B. licheniformis* strain DSM13 ChitinaseA (ChiA) with *B. licheniformis* strain DSM8785 ChiA sequences. *B. licheniformis* strain DSM13 chitinaseA sequences were identical to the sequence retrieved from the Swiss-Prot/TreEMBL protein databases. The alignment was done using "CLUSTALW". Key: pFChi13: recombinant ChiA from *B. licheniformis* strain DSM13(AAU39296.1); pFChi8785: ChiA from *B. licheniformis* strain DSM8785. Yellow box indicate Catalytic Domain. Blue box indicate Fibronectin type III domain. Green box indicated Chitin binding domain. Gray box indicate amino acid nucleotide differentiation.

From the amino acid sequences alignment, we found that only three amino acids are difference between the two strains, one amino acid in the catalytic domain and one amino acid in the chitin binding domain.

5.2 Directed evolution of chitinase

5.2.1 Construction of cDNA library

5.2.1.1 Error-prone PCR

pFChi13 and pFChi8785 were used as a template to amplified the error-prone PCR products by using a low fidelity buffer conditions, which contained 2.9 mM $MgCl_2$, 0.15 mM $MnCl_2$, and bias dNTA concentration, the PCR cycles were done 40 cycles. To determine the mutation rate, a sample of error-prone products was cloned in to pFLAG-CTS vector (designed pEr13 and pEr8785) and was sequenced. These included both clones that retain activity and lost enzymes activity. Then the DNA and amino acid sequence were shown in Figure 7. and Figure 8.

```
pEr8785_c1      TCGCTACCGTTGCGCAAGCTTTTGTCATGTTGCTGAGCTTGTCATTTGTGAATGGGGAAG 180
pEr8785_C2      TCGCTACCGTTGCGCAAGCTTTTGTCATGTTGCTGAGCTTGTCATTTGTGAATGGGGAAG 180
pEr8785_no      TCGCTACCGTTGCGCAAGCTTTTGTCATGTTGCTGAGCTTGTCATTTGTGAATGGGGAAG 180
pFChi8785       TCGCTACCGTTGCGCAAGCTTTTGTCATGTTGCTGAGCTTGTCATTTGTGAATGGGGAAG 180
                ************************************************************

pEr8785_c1      TTGCAAAAGCCGATTCCGGAAAAAACTATAAAATCATCGGCTACTATCCATCATGGGGTG 240
pEr8785_C2      TTGCAAAAGCCGATTCCGGAAAAAACTATAAAATCATCGGCTACTATCCATCATGGGGTG 240
pEr8785_no      TTGCAAAAGCCGATTCCGGAAAAAACTATAAAATCATCGGCTACTATCCATCATGGGGTG 240
pFChi8785       TTGCAAAAGCCGATTCCGGAAAAAACTATAAAATCATCGGCTACTATCCATCATGGGGTG 240
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Figure 7. Results of ClustalW nucleotide alignment from error-prone mutant and wild type (pFChi8785). pEr8785_c1 and pEr8785_c2, error-prone mutant that retain enzyme activity; pEr8785_no, error-prone mutant that lost enzyme activity. The yellow box indicates the deoxyribonucleic acid substitution.

```
pEr8785_c1      CTTATGGAAGGGATTTTCAAGTTTGGGATATGGACGTTTCGAAAGTCAGCCACATTAATT 300
pEr8785_C2      CTTATGGAAGGGATTTTCAAGTTTGGGATATGGACGTTTCGAAAGTCAGCCACATTAATT 300
pEr8785_no      CTTATGGAAGGGATTTTCAAGTTTGGGATATGGACGTTTCGAAAGTCAGCCACATTAATT 300
pFChi8785       CTTATGGAAGGGATTTTCAAGTTTGGGATATGGACGTTTCGAAAGTCAGCCACATTAATT 300
                ************************************************************

pEr8785_c1      ATGCCTTTGCTGATATTTGCTGGGAGGGAAGGCATGGGAACCCTGATCCGACAGGCCCCA 360
pEr8785_C2      ATGCCTTTGCTGATATTTGCTGGGAGGGAAGGCATGGGAACCCTGATCCGACAGGCCCCA 360
pEr8785_no      ATGCCTTTGCTGATATTTGCTGGGAGGGAAGGCATGGGAACCCTGATCCGACAGGCCCCA 360
pFChi8785       ATGCCTTTGCTGATATTTGCTGGGAGGGAAGGCATGGGAACCCTGATCCGACAGGCCCCA 360
                ************************************************************

pEr8785_c1      ATCCTCAAACGTGGTCATGCCAGGATGAAAACGGAGTGATCGACGCGCCAAATGGAACAA 420
pEr8785_C2      ATCCTCAAACGTGGTCATGCCAGGATGAAAACGGAGTGATCGACGCGCCAAATGGAACAA 420
pEr8785_no      ATCCTCAAACGTGGTCATGCCAGGATGAAAACGGAGTGATCGACGCGCCAAATGGAACAA 420
pFChi8785       ATCCTCAAACGTGGTCATGCCAGGATGAAAACGGAGTGATCGACGCGCCAAATGGAACAA 420
                ************************************************************

pEr8785_c1      TCGTGATGGGCGATCCCTGGATTGACGCACAAAAGGCAAATCCCGGGGATGTCTGGGATG 480
pEr8785_C2      TCGTGATGGGCGATCCCTGGATTGACGCACAAAAGGCAAATCCCGGGGATGTCTGGGATG 480
pEr8785_no      TCGTGATGGGCGATCCCTGGATTGACGCACAAAAGGCAAATCCCGGGGATGTCTGGGATG 480
pFChi8785       TCGTGATGGGCGATCCCTGGATTGACGCACAAAAGGCAAATCCCGGGGATGTCTGGGATG 480
                ************************************************************

pEr8785_c1      AACCGATCCGCGGCAACTTTAAACAATTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGA 540
pEr8785_C2      AACCGATCCGCGGCAACTTTAAACAATTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGA 540
pEr8785_no      AACCGATCCGCGGCAACTTTAAACAATTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGA 540
pFChi8785       AACCGATCCGCGGCAACTTTAAACAATTGTTGAAGCTGAAAAAGAGCCACCCTCATTTGA 540
                ************************************************************

pEr8785_c1      AAACGTTCATATCGGTCGGGGGGTGGACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAG 600
pEr8785_C2      AAACGTTCATATCGGTCGGGGGGTGGACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAG 600
pEr8785_no      AAACGTTCATATCGGTCGGGGGGTGGACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAG 600
pFChi8785       AAACGTTCATATCGGTCGGGGGGTGGACTTGGTCTAACCGCTTTTCAGATGTCGCGGCAG 600
                ************************************************************

pEr8785_c1      ATCCTGCGGCAAGGGAGAATTTCGCCGCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGT 660
pEr8785_C2      ATCCTGCGGCAAGGGAGAATTTCGCCGCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGT 660
pEr8785_no      ATCCTGCGGCAAGGGGGAATTTCGCCGCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGT 660
pFChi8785       ATCCTGCGGCAAGGGAGAATTTCGCCGCTTCGGCCGTTGAGTTTTTAAGGAAATACGGGT 660
                *************** ********************************************

pEr8785_c1      TTGACGGGGTCGATCTTGACTGGGAATATCCGGTCAGCGGAGGATTGCCGGGGAACAACA 720
pEr8785_C2      TTGACGGGGTCGATCTTGACTGGGAATATCCGGTCAGCGGAGGATTGCCGGGGAACAACA 720
pEr8785_no      TTGACGGGGTCGATCTTGACTGGGAATATCCGGTCAGCGGAGGATTGCCGGGGAACAACA 720
pFChi8785       TTGACGGGGTCGATCTTGACTGGGAATATCCGGTCAGCGGAGGATTGCCGGGGAACAGCA 720
                ************************************************************ **

pEr8785_c1      CACGTCCGGAAGATAAAAGAAACTACACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTG 780
pEr8785_C2      CACGTCCGGAAGATAAAAGAAACTACACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTG 780
pEr8785_no      CACGTCCGGAAGATAAAAGAAACTACACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTG 780
pFChi8785       CACGTCCGGAAGATAAAAGAAACTACACGCTGCTCCTGCAAGAGGTGCGCAAAAAACTTG 780
                ************************************************************

pEr8785_c1      ACGCTGTAGAAGCAAAAGACGGCAAGGAATACTTGCTGACGATCGCATCCGGCGCAAGTC 840
pEr8785_C2      ACGCTGTAGAAGCAAAAGACGGCAAGGAATACTTGCTGACGATCGCATCCGGCGCAAGTC 840
pEr8785_no      ACGCTGTAGAAGCAAAAGACGGCAAGGAATACTTGCTGACGATCGCATCCGGCGCAAGTC 840
pFChi8785       ACGCTGCAGAAGCAAAAGACGGCAAGGAATACTTGCTGACGATCGCATCCGGCGCAAGTC 840
                ****** ********************************************************

pEr8785_c1      CCGATTATGTAAGCAACACTGAGCTCGATAAAATCGCTCAAACCGTGGATTGGATTAACA 900
pEr8785_C2      CCGATTATGTAAGCAACACTGAGCTCGATAAAATCGCTCAAACCGTGGATTGGATTAACA 900
pEr8785_no      CCGATTATGTAAGCAACACTGAGCTCGATAAAATCGCTCAAACCGTGGATTGGATTAACA 900
pFChi8785       CCGATTATGTAAGCAACACTGAGCTCGATAAAATCGCTCAAACCGTGGATTGGATTAACA 900
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Figure 7. (Continued).

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pEr8785_c1      TTATGACCTATGACTTTAATGGCGGATGGCAAAGCATAAGCGCCCATAATGCACCGCTGT 960
pEr8785_C2      TTATGACCTATGACTTTAATGGCGGATGGCAAAGCATAAGCGCCCATAATGCACCGCTGT 960
pEr8785_no      TTATGACCTATGACTTTAATGGCGGATGGCAAAGCATAAGCGCCCATAATGCACCGCTGT 960
pFChi8785       TTATGACCTATGACTTTAATGGCGGATGGCAAAGCATAAGCGCCCATAATGCACCGCTGT 960
                ************************************************************

pEr8785_c1      TCTATGATCCAAAAGCGAAAGAAGCAGGCGTTCCAAACGCTGAGACCTACAATATTGAAA 1020
pEr8785_C2      TCTATGATCCAAAAGCGAAAGAAGCAGGCGTTCCAAACGCTGAGACCTACAATATTGAAA 1020
pEr8785_no      TCTATGATCCAAAAGCGAAAGAAGCAGGCGTTCCAAACGCTGAGACCTACAATATTGAAA 1020
pFChi8785       TCTATGATCCAAAAGCGAAAGAAGCAGGCGTTCCAAACGCTGAGACCTACAATATTGAAA 1020
                ************************************************************


pEr8785_c1      ACACTGTGAAACGCTACAAGGAAGCCGGTGTCAAGGGTGACAAATTAGTGCTTGGAACAC 1080
pEr8785_C2      ACACTGTGAAACGCTACAAGGAAGCCGGTGTCAAGGGTGACAAATTAGTGCTTGGAACAC 1080
pEr8785_no      ACACTGTGAAACGCTACAAGGAAGCCGGTGTCAAGGGTGACAAATTAGTGCTTGGAACAC 1080
pFChi8785       ACACTGTGAAACGCTACAAGGAAGCCGGTGTCAAGGGTGACAAATTAGTGCTTGGAACAC 1080
                ************************************************************

pEr8785_c1      CGTTCTACGGAAGGGGCTGGAGCGGTTGTGAACCAGGGGGGCACGGAGAATATCAGAAAT 1140
pEr8785_C2      CGTTCTACGGAAGGGGCTGGAGCGGTTGTGAACCAGGGGGGCACGGAGAATATCAGAAAT 1140
pEr8785_no      CGTTCTACGGAAGGGGCTGGAGCGGTTGTGAACCAGGGGGGCACGGAGAATATCAGAAAT 1140
pFChi8785       CGTTCTACGGAAGGGGCTGGAGCGGTTGTGAACCAGGGGGGCACGGAGAATATCAGAAAT 1140
                ************************************************************

pEr8785_c1      GCGGACCGGCTAAAGAAGGGACATGGGAAAAGGGCGTATTCGATTTTTCAGATCTTGAAA 1200
pEr8785_C2      GCGGACCGGCTAAAGAAGGGACATGGGAAAAGGGCGTATTCGATTTTTCAGATCTTGAAA 1200
pEr8785_no      GCGGACCGGCTAAAGAAGGGACATGGGAAAAGGGCGTATTCGATTTTTCAGATCTTGAAA 1200
pFChi8785       GCGGACCGGCTAAAGAAGGGACATGGGAAAAGGGCGTATTCGATTTTTCAGATCTTGAAA 1200
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pEr8785_c1      GGAACTATGTGAATCAAAACGGCTATAAAAGGTATTGGAACGATCAAGCAAAAGTGCCGT 1260
pEr8785_C2      GGAACTATGTGAATCAAAACGGCTATAAAAGGTATTGGAACGATCAAGCAAAAGTGCCGT 1260
pEr8785_no      GGAACTATGTGAATCAAAACGGCTATAAAAGGTATTGGAACGATCAAGCAAAAGTGCCGT 1260
pFChi8785       GGAACTATGTGAATCAAAACGGCTATAAAAGGTATTGGAACGATCAAGCAAAAGTGCCGT 1260
                ************************************************************

pEr8785_c1      TTTTGTATAATGCGGAAAATGGCAATTTCATCACTTATGATGATGAACAATCATTCGGCC 1320
pEr8785_C2      TTTTGTATAATGCGGAAAATGGCAATTTCATCACTTATGATGATGAACAATCATTCGGCC 1320
pEr8785_no      TTTTGTATAATGCGGAAAATGGCAATTTCATCACTTATGATGATGAACAATCATTCGGCC 1320
pFChi8785       TTTTGTATAATGCGGAAAATGGCAATTTCATCACTTATGATGATGAACAATCATTCGGCC 1320
                ************************************************************

pEr8785_c1      ACAAAACGGATTTTATTAAAGCAAACGGATTAAGCGGAGCAATGTTCTGGGATTTCAGCG 1380
pEr8785_C2      ACAAAACGGATTTTATTAAAGCAAACGGATTAAGCGGAGCAATGTTCTGGGATTTCAGCG 1380
pEr8785_no      ACAAAACGGATTTTATTAAAGCAAACGGATTAAGCGGAGCAATGTTCTGGGATTTCAGCG 1380
pFChi8785       ACAAAACGGATTTTATTAAAGCAAACGGATTAAGCGGAGCAATGTTCTGGGATTTCAGCG 1380
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pEr8785_c1      GCGATTCCAATCGGACGCTCCTCAATAAATTGGCAGCCGATTTAGATTTTGCACCGGACG 1440
pEr8785_C2      GCGATTCCAATCGGACGCTCCTCAATAAATTGGCAGCCGATTTAGATTTTGCACCGGACG 1440
pEr8785_no      GCGATTCCAATCGGACGCTCCTCAATAAATTGGCAGCCGATTTAGATTTTGCACCGGACG 1440
pFChi8785       GCGATTCCAATCGGACGCTTCTCAATAAATTGGCAGCCGATTTAGATTTTGCACCGGACG 1440
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pEr8785_c1      GAGGCAATCCGGAGCCGCCTTCATCGGCACCTGTGAATGTGCGTGTAACCGGAAAAACTG 1500
pEr8785_C2      GAGGCAATCCGGAGCCGCCTTCATCGGCACCTGTGAATGTGCGTGTAACCGGAAAAACTG 1500
pEr8785_no      GAGGCAATCCGGAGCCGCCCTCATCGGCACCTGTGAATGTGCGTGTAACCGGAAAAACTG 1500
pFChi8785       GAGGCAATCCGGAGCCGCCTTCATCGGCACCTGTGAATGTGCGTGTAACCGGAAAAACTG 1500
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pEr8785_c1      CTACAAGTGTCAGCCTGGCGTGGGATGCGCCGAGCAGCGGAGCAAACATTGCGGAATATG 1560
pEr8785_C2      CTACAAGTGTCAGCCTGGCGTGGGATGCGCCGAGCAGCGGAGCAAACATTGCGGAATATG 1560
pEr8785_no      CTACAAGTGTCAGCCTGGCGTGGGATGCGCCGAGCAGCGGAGCAAACATTGCGGAATATG 1560
pFChi8785       CTACAAGTGTCAGCCTGGCGTGGGATGCGCCGAGCAGCGGAGCAAACATTGCGGAATATG 1560
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Figure 7. (Continued).

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pEr8785_c1      TCGTGTCATTTGAAAACCGGTCGATATCTGTAAAAGAAACATCAGCGGAAATAGGCGGCT 1620
pEr8785_C2      TCGTGTCATTTGAAAACCGGTCGATATCTGTAAAAGAAACATCAGCGGAAATAGGCGGCT 1620
pEr8785_no      TCGTGTCATTTGAAAACCGGTCGATATCTGTAAAAGAAACATCAGCGGAAATAGGCGGCT 1620
pFChi8785       TCGTGTCATTTGAAAACCGGTCGATATCTGTAAAAGAAACATCAGCGGAAATAGGCGGCT 1620
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pEr8785_c1      TGAAGCCGGGTACGGCCTACTCATTTACTGTTTCAGCAAAGGATGCGGATGGAAAGCTCC 1680
pEr8785_C2      TGAAGCCGGGTACGGCCTACTCATTTACTGTTTCAGCAAAGGATGCGGATGGAAAGCTCC 1680
pEr8785_no      TGAAGCCGGGTACGGCCTACTCATTTACTGTTTCAGCAAAGGATGCGGATGGAAAGCTCC 1680
pFChi8785       TGAAGCCGGGTACGGCCTACTCATTTACTGTTTCAGCAAAGGATGCGGATGGAAAGCTCC 1680
                ************************************************************

pEr8785_c1      ATGCCGGACCAACGGTAGAGGTCACGACGAATTCTGACCAAGCCTGTTCATATGACGAAT 1740
pEr8785_C2      ATGCCGGACCAACGGTAGAGGTCACGACGAATTCTGACCAAGCCTGTTCATATGACGAAT 1740
pEr8785_no      ATGCCGGACCAACGGTAGAGGTCACGACGAATTCTGACCAAGCCTGTTCATATGACGAAT 1740
pFChi8785       ATGCCGGACCAACGGTAGAGGTCACGACGAATTCTGACCAAGCCTGTTCATATGACGAAT 1740
                ************************************************************

pEr8785_c1      GGAAAGAGACGAGCGCATACACAGGCGGAGAGCGGGTTGCATTTAACGGAAAAGTGTATG 1800
pEr8785_C2      GGAAAGAGACGAGCGCATACACAGGCGGAGAGCGGGTTGCATTTAACGGAAAAGTGTATG 1800
pEr8785_no      GGAAAGAGACGAGCGCATACACAGGCGGAGAGCGGGTTGCATTTAACGGAAAAGTGTATG 1800
pFChi8785       GGAAAGAGACGAGCGCATACACAGGCGGAGAGCGGGTTGCATTTAACGGAAAAGTGTATG 1800
                ************************************************************

pEr8785_c1      AAGCGAAATGGTGGACGAAAGGCGACCGGCCTGATCAATCCGGTGAATGGGGCGTATGGC 1860
pEr8785_C2      AAGCGAAATGGTGGACGAAAGGCGACCGGCCTGATCAATCCGGTGAATGGGGCGTATGGC 1860
pEr8785_no      AAGCGAAATGGTGGACGAAAGGCGACCGGCCTGATCAATCCGGTGAATGGGGCGTATGGC 1860
pFChi8785       AAGCGAAATGGTGGACGAAAGGCGACCGGCCTGATCAACCCGGTGAATGGGGCGTATGGC 1860
                ************************************ ********************

pEr8785_c1      GGCTGATCGGAGGCTGCGAACACCACCACCACCACCACTAG 1737
pEr8785_C2      GGCTGATCGGAGGCTGCGAACACCACCACCACCACCACTAG 1737
pEr8785_no      GGCTGATCGGAGGCTGCGAACACCACCACCACCACCACTAG 1737
pFChi8785       GGCTGATCGGAGGCTGCGAACACCACCACCACCACCACTAG 1901
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Figure 7. (Continued).

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pEr8785_C2      MLLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVWDMDVSKVSHINYAFADICWE 60
pEr8785_no      MLLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVWDMDVSKVSHINYAFADICWE 60
pEr8785_C1      MLLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVWDMDVSKVSHINYAFADICWE 60
pFChi8785       MLLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDFQVWDMDVSKVSHINYAFADICWE 60
                ************************************************************

pEr8785_C2      GRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKANPGDVWDEPIRGNFKQ 120
pEr8785_no      GRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKANPGDVWDEPIRGNFKQ 120
pEr8785_C1      GRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKANPGDVWDEPIRGNFKQ 120
pFChi8785       GRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDPWIDAQKANPGDVWDEPIRGNFKQ 120
                ************************************************************

pEr8785_C2      LLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWE 180
pEr8785_no      LLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARGNFAASAVEFLRKYGFDGVDLDWE 180
pEr8785_C1      LLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWE 180
pFChi8785       LLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARENFAASAVEFLRKYGFDGVDLDWE 180
                ********************************** ************************
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Figure 8. Amino acid alignment of error-prone mutant and wild type (pFChi8785). Gray box indicated the amino acid substitution. Yellow box indicated the Catalytic domain. Blue box indicated the Fibronectin type III domain. Green box indicate Chitin binding domain. Red box indicate two completely conserved motifs. Gray box indicate amino acid substitution.

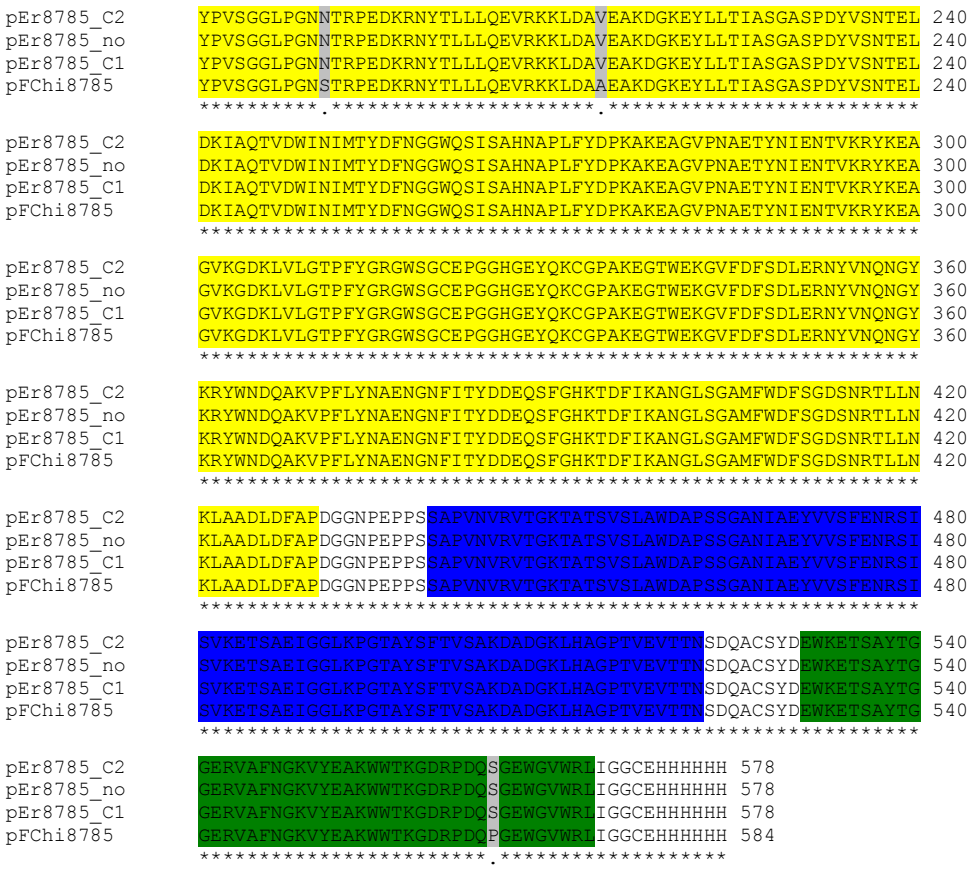


Figure 8. (Continued).

DNA alignment of error-prone PCR products compared with wild type, pFChi8785 demonstrated that the mutations generated by *Taq* DNA polymerase were AT→ GC and GC→ AT substitution. The amino acid alignment revealed that the negative mutant (the mutant that lost chitinase activity) designed as pEr8785_no had four substitution (Glu157Gly, Ser193Asp, Ala216Val, and Pro565Ser) while the positive mutants (the mutant that remain chitinase activity) designed as pEr8785_C1 and pEr8785_C2 had three substitutions (Ser193Asp, Ala216Val, and Pro565Ser), which is the same substitution as the negative mutation except the Glu157Gly substitution. Thus Glu157 seem to be an important amino acid in catalytic activity.

5.2.1.2 DNA shuffling.

The error prone PCR products were digested with *DNase*I to generated a DNaseI fragments. The appropriate unit of *DNase*I was determined by using 1 µg of error prone PCR products and digested with variant unit of *DNase*I. The result was shown that the appropriate unit of *DNase*I is 0.02 unit/µg as shown in Figure 9.

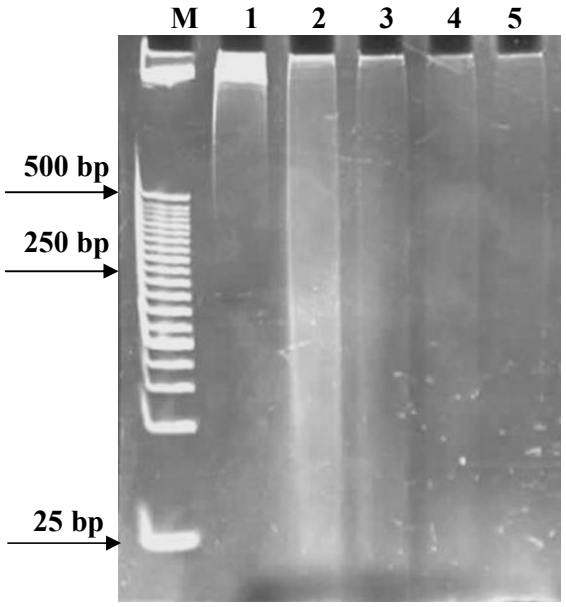


Figure 9. *DNase*I digestion of error prone PCR products. M; 25 bp DNA Ladder, 1; *DNase*I 0.0U/µg, 2; *DNase*I 0.02 U/µg, 3; *DNase*I 0.04 U/µg, 4; *DNase*I 0.07 U/µg, and 5; *DNase*I 0.5 U/µg.

After the appropriate concentration of *DNase*I has been determined, Five µg of error-prone PCR products were digested with 0.1 units of *DNase*I. The *DNase*I fragments in the size 25-250 bases were isolated from polyacrylamide gel then subjected to PCR reassemble step. The reassemble products were analyzed on 1 % agarose gel as shown in Figure 10.

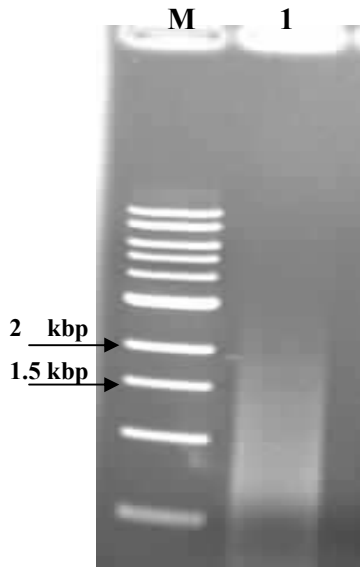


Figure 10. Reassembly by thermocyclig in the presence of *Pfu* DNA polymerase. M: DNA marker; 1: reassemble products.

Then, the shuffled genes were amplified by PCR using the reassemble products with specific primers, the shuffled genes were then subjected to agarose gel electrophorisis. The result was shown in Figure 11.

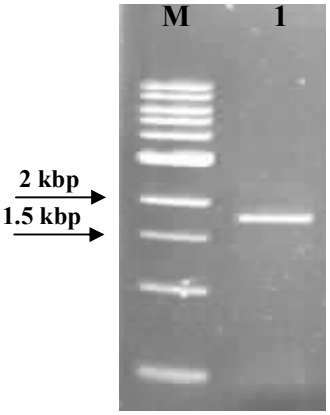


Figure 11. Amplification of shuffled products by a conventional PCR. M: 1 kb DNA marker; 1: shuffled products.

The shuffled products band was 1.7 K bp that related to the chitinase genes. The shuffled products were purified by using QAIGEN PCR purification kits were then cloned into pFLAG-CTS for expression the library of mutant enzyme.

5.2.2 High-throughput screening.

5.2.2.1 First round screening for chitinase activity.

The shuffled products were cloned into pFLAG-CTS vector and transformed into *E. coli* TOP10 by electroporation and then the library of mutant enzymes were screened on LB agar plate containing 0.2% colloidal chitin. The positive mutants could hydrolyzed colloidal chitin as indicated by clear zone when the plates were stained with Congo Red as shown in Figure 12.

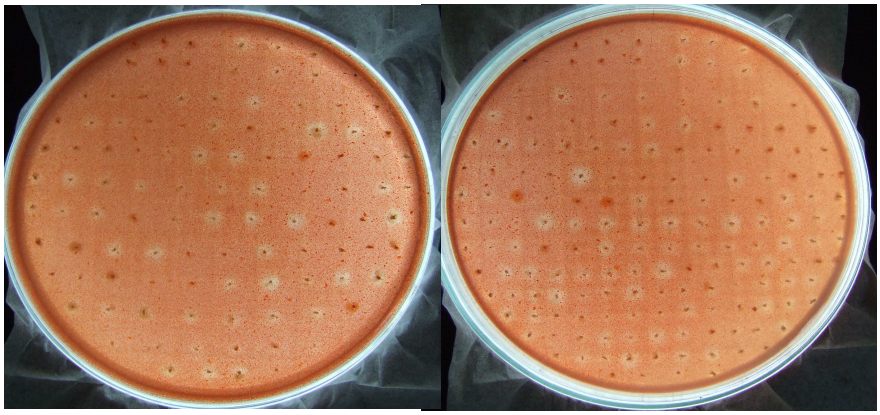


Figure 12. First round screening of mutated library. Each colony of *E. coli* from a mutated library was grown on LB/Ampicillin plate containing 0.2% colloidal chitin. The positive colonies demonstrated clear zone on plate when stained with 0.2 % Congo Red. The total clones from SH13/8785 library were 517 colonies and there were 293 positive clones. Positive and negative control of *E. coli* expressing pFLAG-CTS and wild type enzyme, pFChi8785 were present in every assay plates.

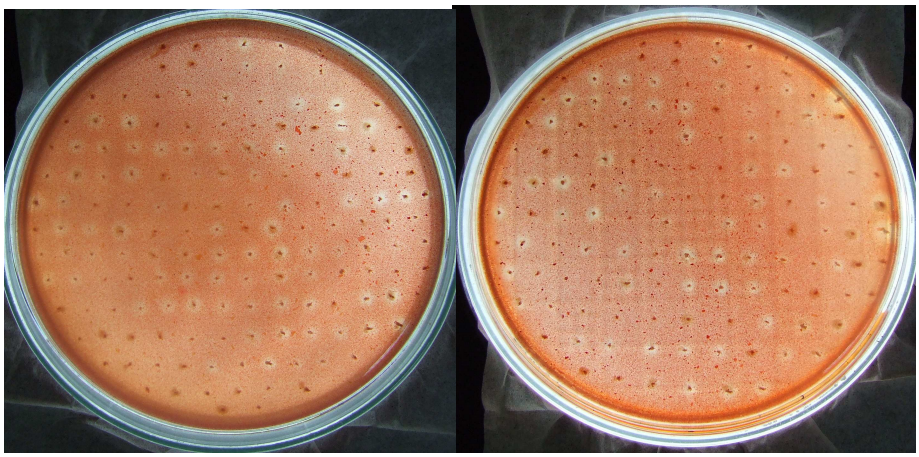


Figure 12. (Continued).

5.2.2.2 Screening for pH tolerant activity.

The positive clones were subjected to secondary screening in microtiter plates by using glycine-HCl pH 3.0, sodium phosphate pH 6.0 and glycine-NaOH pH10.0, as shown in Figure 13.

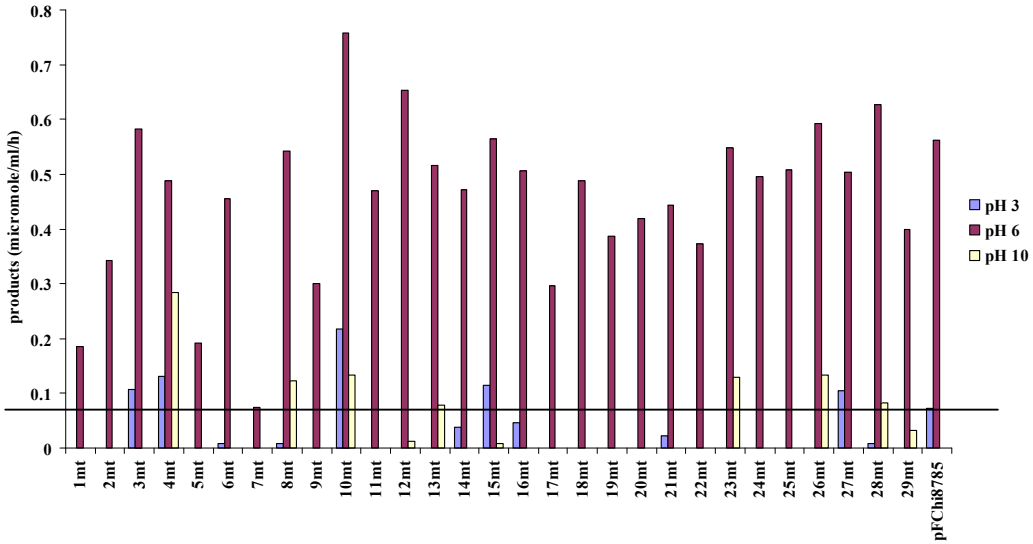


Figure 13. Secondary screening for acidic tolerant chitinase when using *p*- nitrophenyl-chitobiose as a substrate.

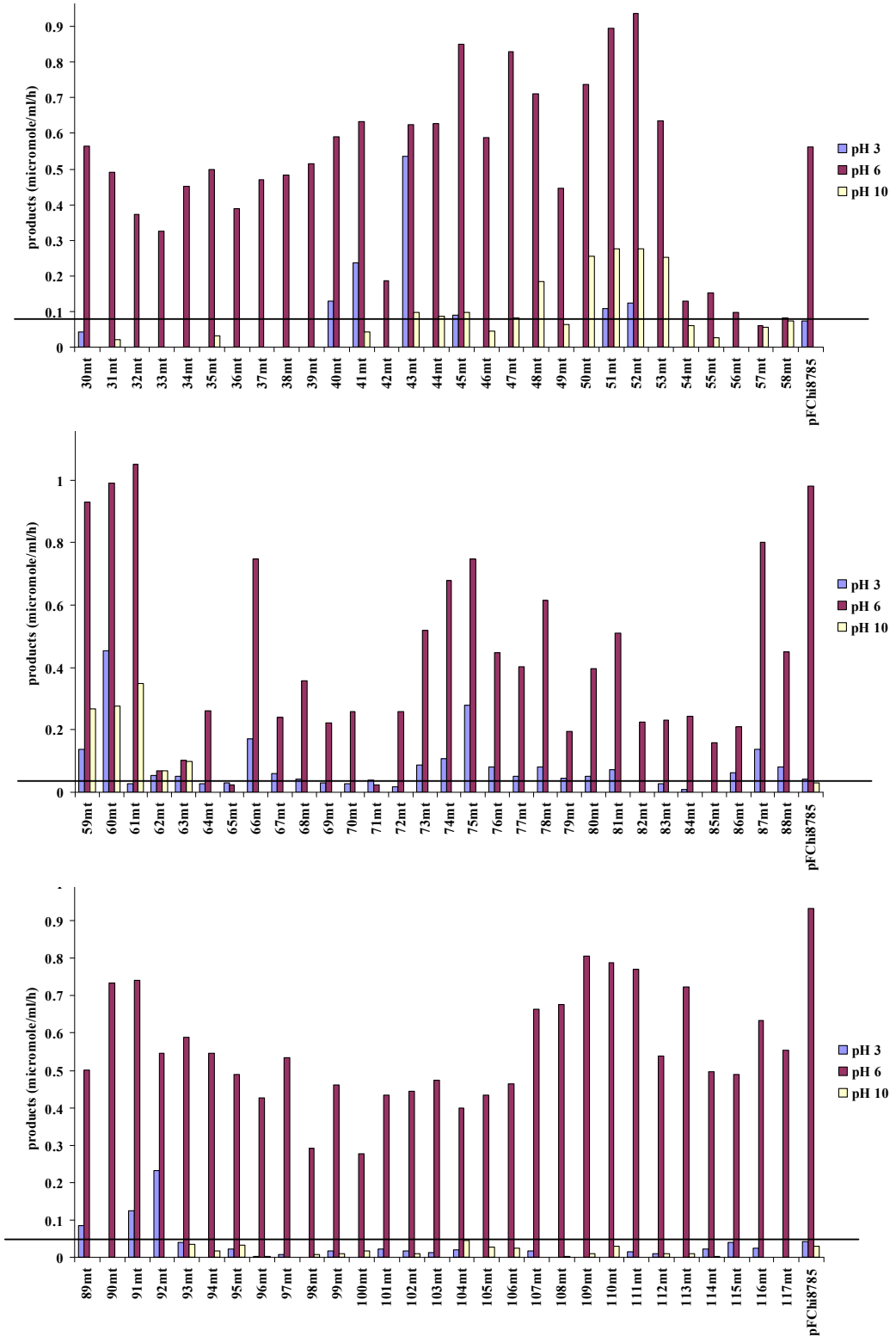


Figure 13. (Continued).

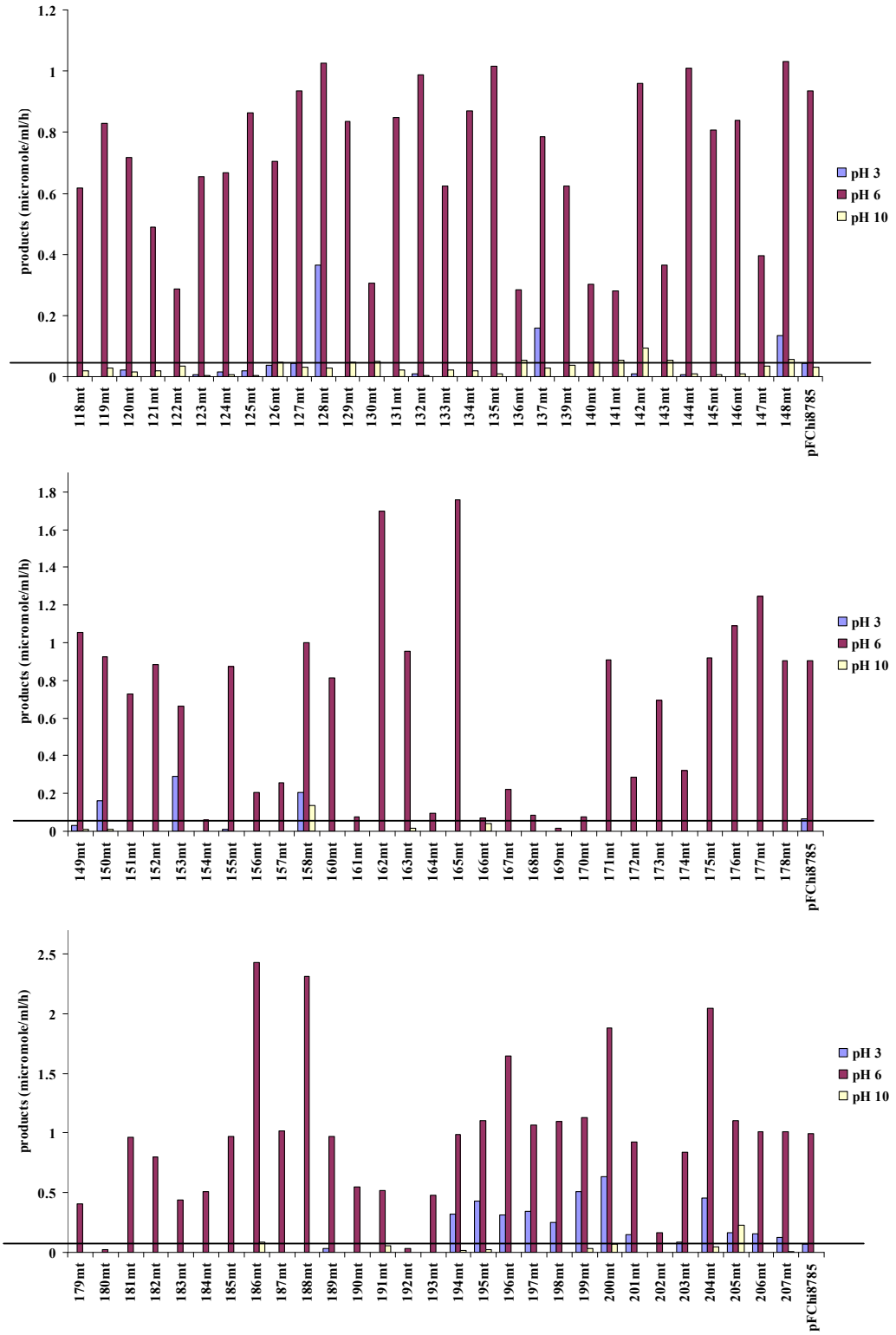


Figure 13. (Continued).

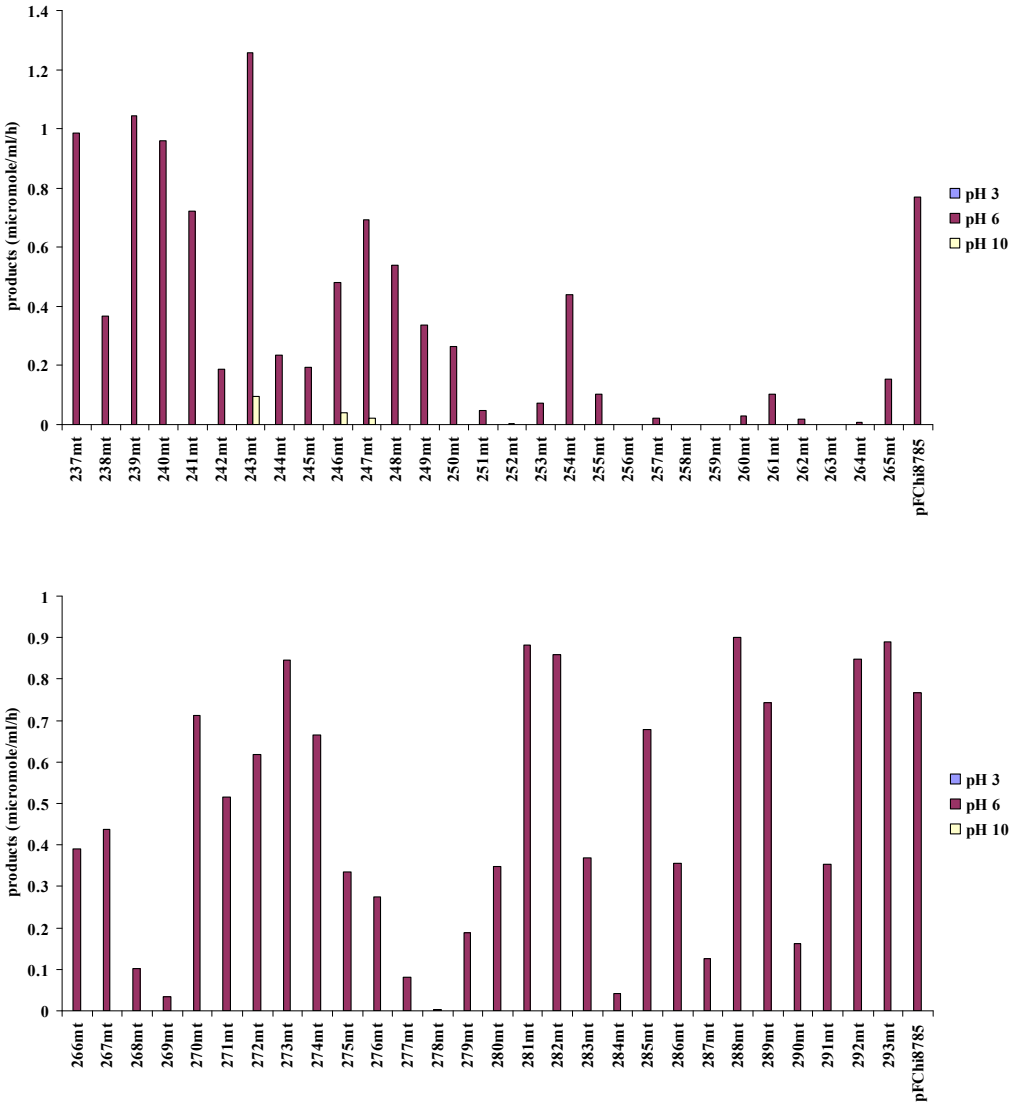


Figure 13. (Continued).

From secondary screening some clone seemed to be active in pH 3.0 such as 43mt, 128mt, 197mt, 199mt, and 204mt. These clones were picked and cultured in 10 ml LB supplement with ampicillin. The crude enzymes were confirmed with colloidal chitin in pH 3.0 and pH 6.0.

5.2.2.3 Confirmation of acidic tolerant activity

After confirmation, we found that only 128mt could hydrolyze colloidal chitin better than wild type at pH 3.0, as shown in Figure 14.

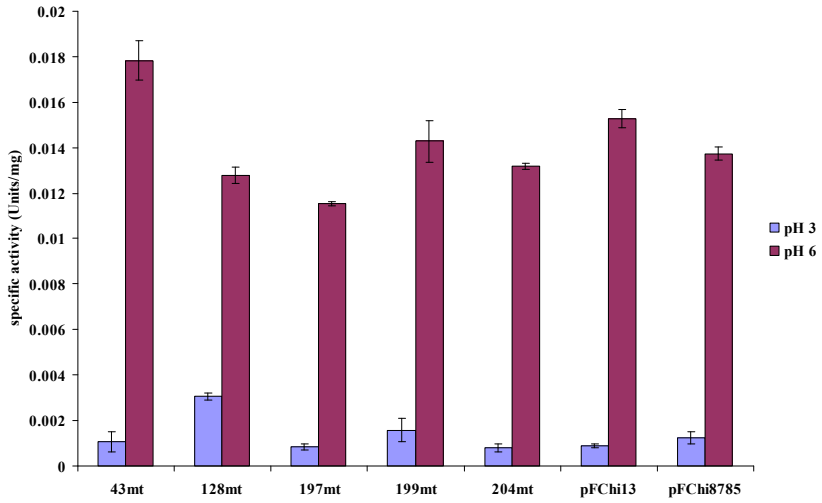


Figure 14. Secondary screening for acidic tolerant chitinase by using colloidal chitin as a substrate.

At pH 3.0, colloidal chitin can be hydrolyzed by all of mutant chitinase when compared with wild type. However clone 128mt demonstrated greatest activity in acidic condition. The affinity purified enzyme was then confirmed for acidic tolerance property by using *p*-nitrophenyl-chitobiose and colloidal chitin as a substrate. The results were shown that purified 128mt can hydrolyze both substrates greater than pFChi13 and pFChi8785 at acidic condition (pH 3.0).

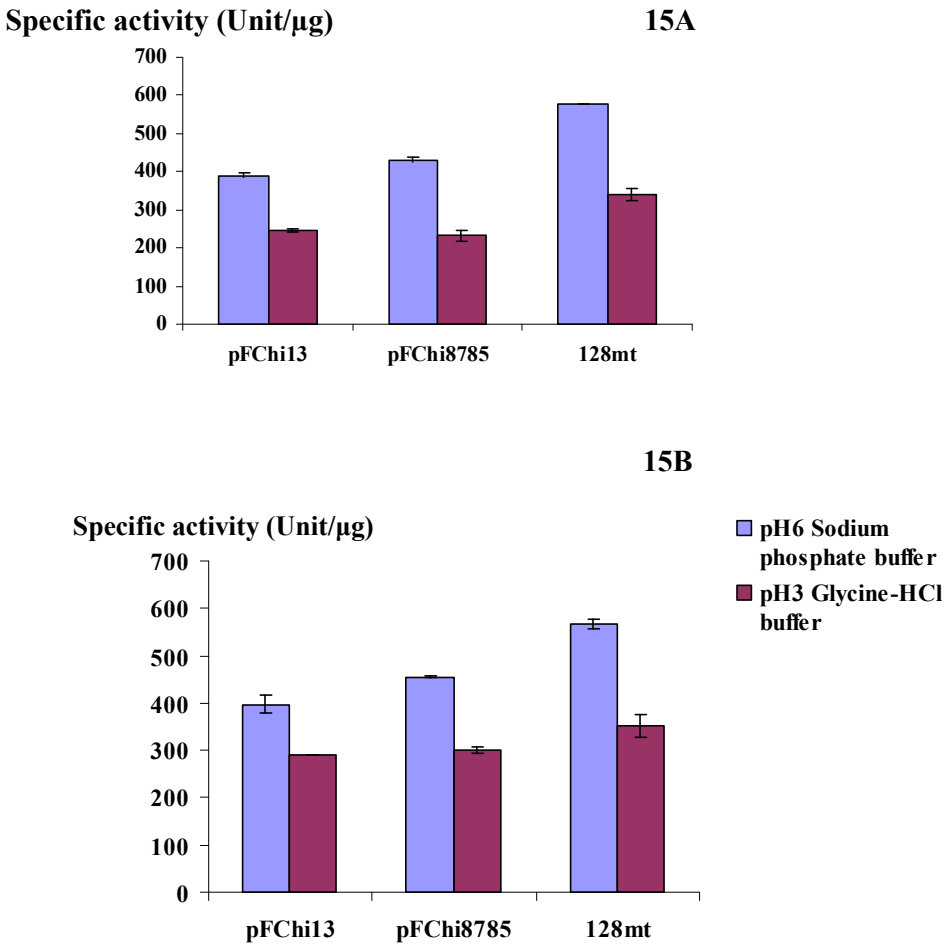


Figure 15. Activities of partial enzymes when using *p*-nitrophenyl-chitobiose (15A) and colloidal chitin (15B) as substrates.

From these results it could be concluded that the combination method of error-prone PCR and DNA shuffling can be used to create an acidic tolerant enzyme. There have been many publications reported the successful of combination method of error-prone PCR and DNA shuffling to improve the enzymes properties. For example serine hydroxymethyltransferase was approximately increased 8-fold of enzyme activity and 41-fold of the enzyme productivity compared with its wild-type parent (Zuo *et al.*,2007). Furthermore, this methods have been used to provide a mutant

tobacco etch virus (TEV) protease in which three amino acid substitutions result in a five-fold increase in the yield of purified protease with retained activity (van den Berg *et al.*,2006). A lipase from *Rhizopus arrhizus* (RAL) had been used to create RAL mutants by combination method, and it was show that the improved thermostability and the optima temperature were higher by 10°C than wild-type RAL (Niu *et al.*,2006). All of this report suggested that the combination of error-prone PCR and DNA shuffling is the efficient enzymatic engineering technique to provide improved enzymes and it can be a basic information to find out the relationship between the function and structure of the enzyme (Zuo, Zheng, Liu, Yi and Zou,2007). This is the first report of improving chitinase activity with the directed evolution method.

The amino acid sequence of 128mt was analyzed then subjected to amino acid alignment by using ClustalW program as shown below.

```
pFChi8785       MKKTAIAIAVALAGFATVAQAFVMLLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDF 60
128mt           MKKTAIAIAVALAGFATVAQAFVMLLSLSFVSGEVAKADSGKNYKIIGYYPSWGAYGRDF 60
pFChi13         MKKTAIAIAVALAGFATVAQAFVMMLSLSFVNGEVAKADSGKNYKIIGYYPSWGAYGRDF 54
                ************************:******.**************************

pFChi8785       QVWDMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDP 120
128mt           QVWDMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDP 120
pFChi13         QVWDMDVSKVSHINYAFADICWEGRHGNPDPTGPNPQTWSCQDENGVIDAPNGTIVMGDP 114
                ************************************************************

pFChi8785       WIDAQKANPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARE 180
128mt           WIDAQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARE 180
pFChi13         WIDAQKSNPGDVWDEPIRGNFKQLLKLKKSHPHLKTFISVGGWTWSNRFSDVAADPAARE 174
                ******:*****************************************************

pFChi8785       NFAASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAK 240
128mt           NFAASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAVEAK 240
pFChi13         NFAASAVEFLRKYGFDGVDLDWEYPVSGGLPGNSTRPEDKRNYTLLLQEVRKKLDAAEAK 234
                ************************************************************.***
```

Figure 16. Amino acid alignment sequence of pFChi13, pFChi8785 and 128mt. Yellow box indicates Catalytic Domain. Blue box indicated Fibronectin type III domain. Green box indicated Chitin binding domain. Grey box indicated amino acid substitution.

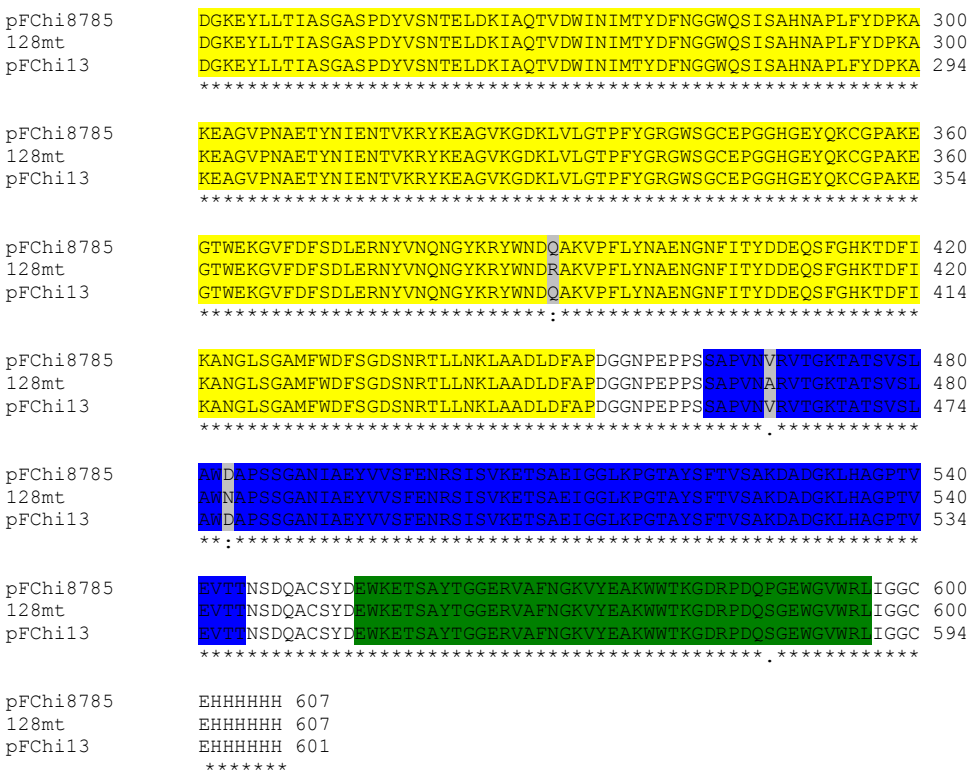


Figure 16. (Continued).

The amino acid sequence analysis demonstrated that 2 amino acids were changed in catalytic domain (Ala231Val and Gln384Arg) and fibronectin type III domain (FnIIID (Val462Ala and Asp477Asn). Modular structure of chitinase with chitin binding domain and catalytic domain separated by distinct functional domain such as FnIIIDs are quite abundant in chitinolytic bacteria. On the other hand, it has been early reported that a deletion of FnIIIDs does not affect the binding activity but causes a significant decrease in the hydrolyzing activity on insoluble chitin (Toratani *et al.*,2006).

5.3 Enzyme characterizations

5.3.1 Purification of chitinase

Chitinase was purified from the soluble protein extract (cell lysis) using Ni-NTA resins followed the modified method. SDS-PAGE analysis (Figure 17.) showed that after the third wash, the chitinase was efficiently eluted from Ni-NTA resin with 250 mM imidazole. This give a partial protein of 64 kDa by SDS-PAGE analysis (Figure 17.) and closely corresponded to values calculated from the amino acid sequences of pFChi13 (64.4244 kDa), pFChi8785 (64.5316 kDa) and 128mt (64.5376 kDa)

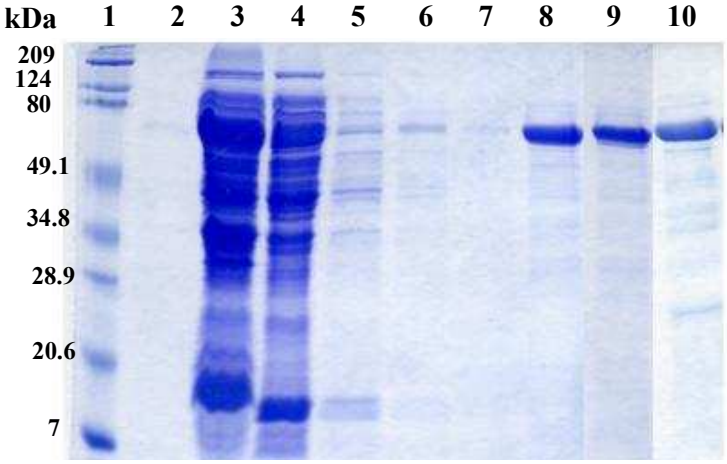


Figure 17. Purification under native conditions. Chitinase was expressed from a pFLAG-CTS vector in *E. coli* TOP10 and purified using Ni-NTA column. Proteins were visualized by Coomassie staining. 2: broth of cells culture; 3: cell lysate of pFChi13 or pFChi8785 or 128mt; 4: flow-through; 5-7: 40 mM imidazole wash; 8-10: 250 mM imidazole elution of pFChi13 or 250 mM imidazole elution of pFChi8785 or 250 mM elution of 128mt. Only one gel shown for sympathy.

There have been previous reported on the functional chitinase after fusion with poly histidine tag. The poly histidine tags have been fused with chitinase from *Thermococcus chitonophagus,* which was purified under native condition by eluting with 300 mM imidazole (Andronopoulou *et al.*,2004). On the other hand, Thermostable Chitinase (Chi40) from *Streptomyces thermoviolaceus* OPC-520 was fused with histidine tags, which was purified under native condition, was eluted with 100 mM imidazole (Christodoulou *et al.*,2001). The pure enzymes were subjected to analysis optimal temperature, optimal pH and kinetic parameter.

5.3.2 The optimal temperature

Activity of the partial enzyme from recombinant chitinase (pFChi13 and pFChi8785) and mutated chitinase (128mt) increased with increasing temperature from 40 to 60°C. The optimal temperature of pFChi13 and pFChi8785 were ranging from 50-55°C and 50-60°C, while 128mt have an optimal temperature fluctuating from 55 to 60°C. The activities of all enzymes were decreased after increasing temperature from 65 to 80°C. They were completely in activated at 90°C as shown in Figure 18.

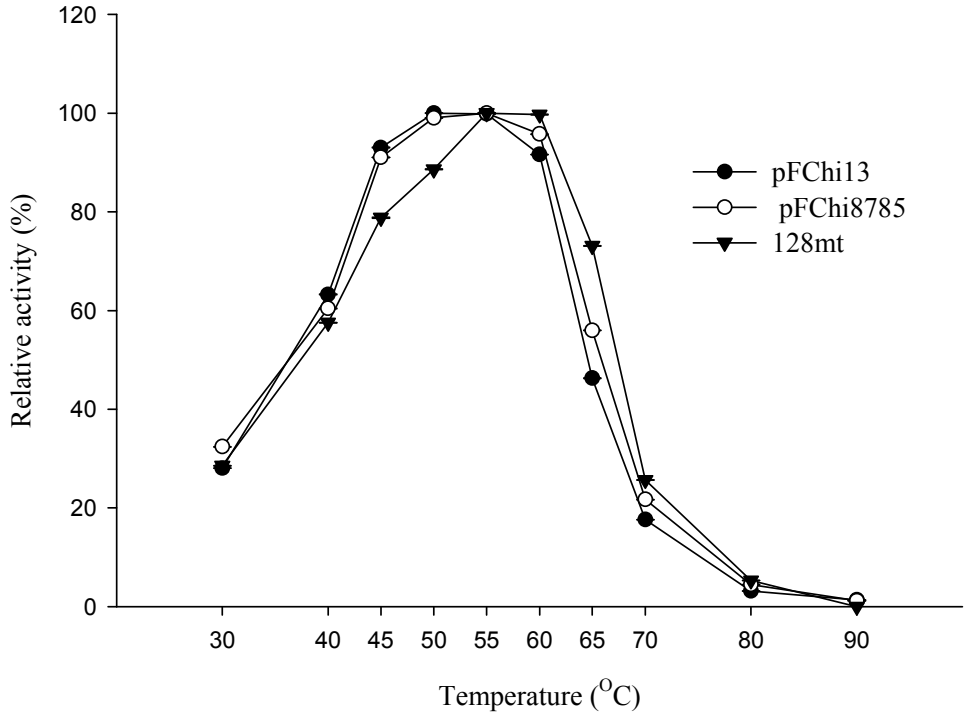


Figure 18. Effects of temperatures on the chitinase activity of recombinant enzyme. Chitinase activity was measured by the standard chitinase assay using *p*-NP-$(GlcNAc)_2$ as substrate for 20 min at designated temperatures, respectively. Symbols: circles (●), pFChi13; open circles (○), pFChi8785; Triangle (▼), 128mt

The differentiation of two amino acids between wild type enzymes did not have an effected on optimal temperature.

5.3.3 The optimal pH

The activities of all partial chitinase have an optimal pH at pH 6.0 as shown below.

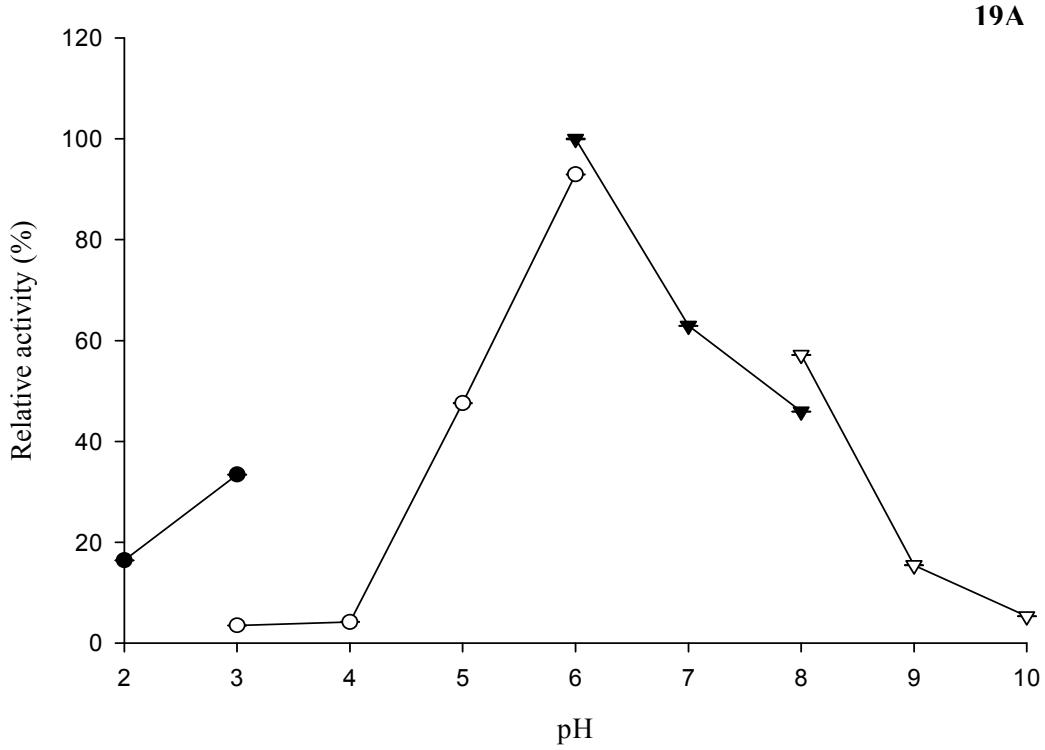
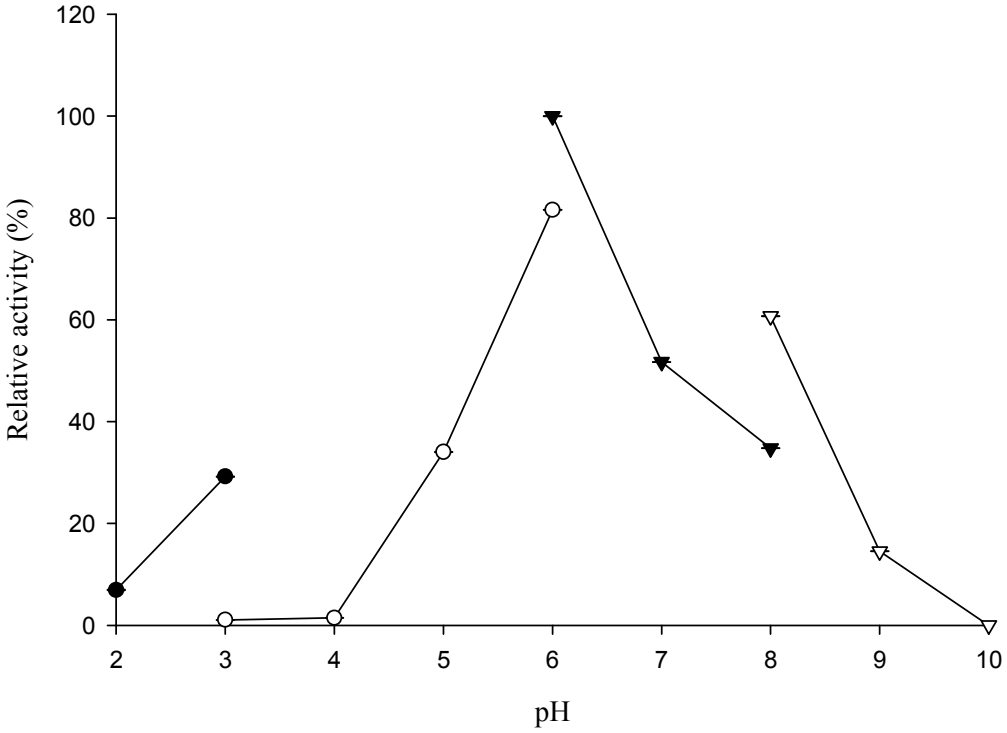


Figure 19. Effects of pH on the chitinase activity of recombinant enzymes. Chitinase activity was measured by the standard chitinase assay using *p*-NP-$(GlcNAc)_2$ as a substrate in the 100 mM buffer for 20 min at 37°C. Symbols: circles (●), glycine-HCl buffer (pH 2−3);open circles (○), sodium citric buffer (pH 4−6); triangles (▼), sodium phosphate buffer (pH 6.0−8); open triangles (▽), glycine-NaOH buffer (pH 9−10); 19A: pFChi13 ; 19B: pFChi8785 and 19C: 128mt.



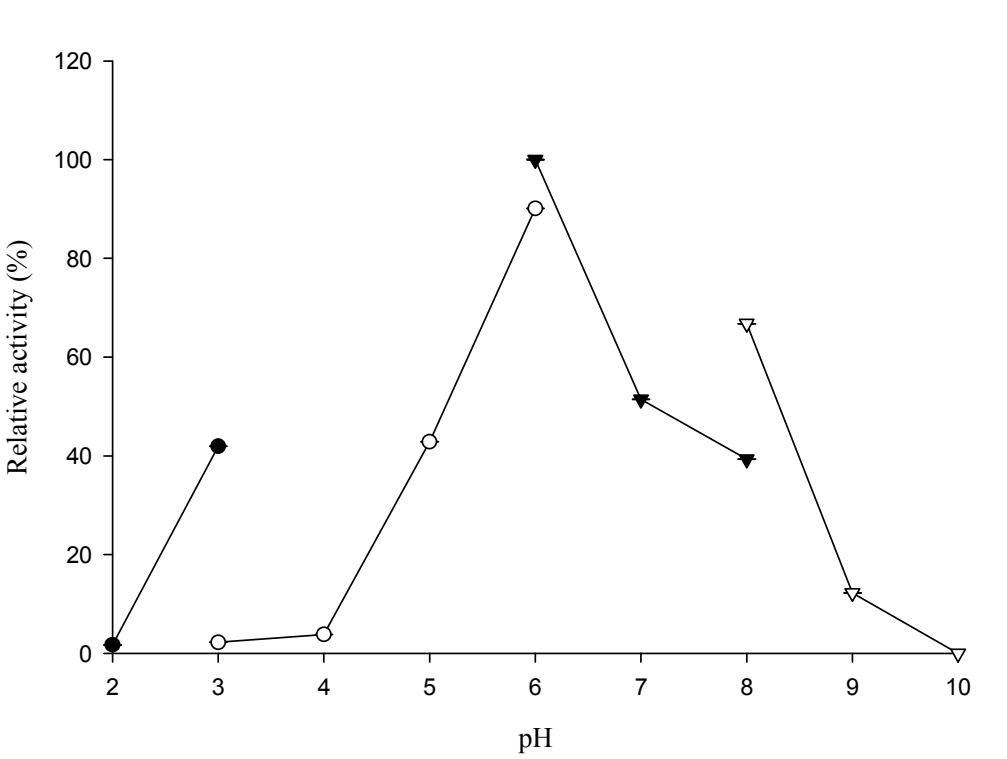


Figure 19. (Continued).

The activity of recombinant chitinase was higher in glycine buffer system than in sodium citric and sodium phosphate buffer system. There have been previously reported that glycine can enhanced xylanase activity. Xylanase activity is increased seven-fold at alkaline pH in the presence of glycine and its pH optimal is shifted from pH 7 to 8 without using any protein engineering techniques (Vinod Vathipadiekal *et al.*,2007). Moreover, glycine have been reported to stabilize the enzyme activity such as after supplementation of Ca_2Cl (10 mM) and glycine (1 mM), the enzyme in Tide detergent retained more than 85% of protease activity after 1h (Nascimento *et al.*,2006). There have been many reports on the optimal pH and optimal temperature of chitinase. Such as optimal pH and optimal temperature of chitinase from *B. cereus* were pH 5.8 and 35°C while it retains some enzymatic activity between pH 2.5-8 (Wang, Moyne, Thottappilly, Wu, Locy and Singh,2001). On the other hand, the optimal temperature and pH of chitinase from *Bacillus* sp. DAU101 was 60°C and pH 7.5 , respectively (Lee, Park, Yoo, Chung, Lee, Cho, Ahn, Kim and Choi,2007). An extracellular chitinase secreted by *Bacillus brevis* was purified, it was shown optimal temperature of 60°C, and was most active at pH 8.0. The enzymatic activity was stable at pH 6.0-10(Li *et al.*,2002). Thermostable chitinase were isolated from the cell-free culture broth of *Bacillus licheniformis* X-7u by successive column chromatographies on Butyl-Toyopearl, Q-Sepharose, and Sephacryl S-200. The enzymes were designated as chitinase I (89 kDa), II(76 kDa), III(66 kDa) and IV(59 kDa). Chitinase II, III and IV possessed extremely high optimal temperatures 70-80°C (Takayanagi *et al.*,1991). Three extracellular chitinase (FB1, FB2, and FB3) were purified from the culture supernatants of *Bacillus cereus* E1, *B. alvei* J1, and *B. sphaericus* J1-1, respectively. The optimal pH and optimal temperature of FB1 were

pH 9 and 50°C; those of FB2 were pH 9 and 60°C; and those of FB3 were pH 7 and 50°C (Wang *et al.*,2001). The purified chitinase from *Bacillus thuringiensis* HD-1 was active at acidic pH, had an optimal activity at pH 6.0.5, and showed maximum activity at 65°C(Salvatore, Heuschkel, Tomlin, Davies, Edwards, Walker-Smith, French and Murch,2000; Arora *et al.*,2003). A chitinase producing bacteria was screened and isolated from Tompaso hot spring in North Sulawesi Indonesia, was identified as *Bacillus* sp. 13.26. The optimal temperature and pH of the 60-kDa chitinase were found at 60°C and pH 7-8 (Yuli *et al.*,2004). *Bacillus subtilis* W-118, a strain that produces antifungal materials, excreted a chitinase when cultured in a medium containing shrimp- and crab-shell powder as the major carbon source. This chitinase, purified by sequential chromatography, had a molecular mass of 20.6 kDa. The optimal pH and optimal temperature were pH 6.0 and 37°C, respectively (Wang *et al.*,2006).

5.3.4 Kinetics of purified chitinase and improved chitinase

To elucidate in more detail the kinetic of each enzyme, parameters of Michaelis-Menten type kinetic for pFChi13, pFChi8785 and 128mt were evaluated by using GraphPad Prism 5 program. The kinetic parameter are summarized in Table 2. The results showed that the enzyme activity was inhibited when the concentration of substrate higher than 0.183 mM (see Appendix II).

Table 2. Kinetics parameter of chitinase and improved chitinase when use *p*-nitrophenyl-chitobiose as a substrate.

Enzyme	K_m (mM)	V_{max} (mM/min)	k_{cat} s^{-1}	k_{cat}/K_m s^{-1}/mM
pH 3.0				
pFChi13	0.10±0.004	0.15±0.004	0.17±0.004	1.69±0.024
pFChi8785	0.11±0.025	0.12±0.033	0.14±0.037	1.36±0.667
128mt	0.06±0.001	0.21±0.059	0.23±0.067	4.10±1.240
pH 6.0				
pFChi13	0.06±0.011	0.38±0.113	0.43±0.126	7.55±0.745
pFChi8785	0.03±0.003	0.28±0.063	0.31±0.070	10.30±3.236
128mt	0.03±0.001	0.59±0.172	0.65±0.191	19.40±4.578

Therefore, the parameter of Eadie-Hofetee plot kinetic has been use for evaluation the kinetic parameter by GraphPad Prism 3 program. The kinetic parameters are summarized in Table 3.

Table 3. Kinetics parameter of chitinase and improved chitinase when use *p*-nitrophenyl-chitobiose as a substrate.

Enzyme	K_m (mM)	V_{max} (mM/min)	k_{cat} s^{-1}	k_{cat}/K_m s^{-1}/mM
pH 3.0				
pFChi13	0.14±0.134	0.18±0.179	0.20±0.004	1.47±0.019
pFChi8785	0.07±0.051	0.12±0.085	0.11±0.028	1.93±0.127
128mt	0.13±0.139	0.40±0.264	0.37±0.106	2.73±0.874
pH 6.0				
pFChi13	0.09±0.017	0.48±0.142	0.54±0.158	5.71±0.674
pFChi8785	0.08±0.014	0.45±0.066	0.50±0.073	6.35±2.041
128mt	0.06±0.025	0.72±0.017	0.80±0.019	14.73±6.517

128mt was the best enzyme which can hydrolyze *p*-NP-chitobiose and shown a highest catalytic constant k_{cat} both at pH 6.0 and pH 3.0. The k_{cat} at pH 6.0 for pFChi13, pFChi8785 and 128mt were shown 0.54, 0.50 and 0.80 s^{-1} respectively. While the k_{cat} at pH 3.0 for pFChi13, pFChi8785 and 128mt were shown 0.20, 0.11 and 0.37 s^{-1} respectively.While the k_{cat} at pH 3.0 were shown 0.595 and 1.023 s^{-1} respectively, this value is higher than recombinant chitinase from *B. cereus* 28-9 (4.6×10^{-2} s^{-1}) (Huang and Chen 2005). The $k_{ca}t/K_m$ of 128mt at pH 3.0 was 2.73 s^{-1} mM^{-1}, which is 1.9 and 1.4 fold of pFChi13 and pFChi8785 respectively.

CHAPTER VI

CONCLUSIONS

1. Recombinant chitinase were highly expressed and secreted out when induced with IPTG and recombinant chitinase could hydrolyze colloidal chitin and p-nitrophenyl-Di-*N*-chitobiose.
2. Recombinant chitinase from strains DSM13 (pFChi13) and DSM8785 (pFChi8785) have an optimal temperature between 50-55°C, and 50-60°C, respectively. While a mutant chitinase (128mt) has optimal temperature ranging from 55 to 60°C. All of them have optimal pH at pH 6.0.
3. The k_{cat}/K_m value of a mutated chitinase (128mt) at pH 6.0 was 14.73 $s^{-1}mM^{-1}$ which was higher than 5.71 $s^{-1}mM^{-1}$ from strains DSM13 and 6.53 $s^{-1}mM^{-1}$ from strain DSM8785. Whereas the k_{cat}/K_m value of mutated chitinase (128mt) at pH 3.0 was 1.47 $s^{-1}mM^{-1}$ which higher than 1.93 $s^{-1}mM^{-1}$ from strains DSM13 and 2.73 $s^{-1}mM^{-1}$, when using *p*-Nitrophenyl-Di-*N*-Acetyl-chitobiose as a substrate.
4. The amino acid sequences of mutated chitinase were substituted in catalytic domain (Ala231Val and Gln384Arg) and Fibronectin type III domain (Val462Ala and Asp477Asn).
5. Directed Evolution method could be successfully applied to improved the activity of chitinase.

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APPENDIX I

1.1 REAGENT

1.1.1 Colloidal chitin

Ten gram of chitin from crab was hydrolyzed with 200 ml of cold conc. HCl acid and stir at 4 °C for overnight. Then, 50% ethyl alcohol was added and filtrated troughs filter paper and then washed by water until pH reach to pH 6.0.

1.1.2 10X p-Dimethylaminobenzaldehyde (DMAB)

Stock Dimthylaminobenzaldehyde (DMAB) (Sigma) reagent: 10g DMAB was dissolved in 12.5% V/V concentrated hydrochloric acid in glacial acetic acid. Stock reagent was diluted 1: 10 with glacial acetic acid before use.

1.1.3 Chemicals

All chemicals were molecular grade or analytic grade.

1. Reagent for PCR amplification.
    - 25 mM dNTP mix (New England BioLabs)
    - 10X *Pfu* buffer (New England BioLabs)
2. Reagent for agarose gel electrophoresis
    - Agarose low EEO. Molecular biology grade (Research organics)
    - 25 bp DNA ladder (Invitrogen)
    - 1X TAE Buffer
    - 1 Kb Ladder marker DNA (Bio Lab)
    - 6X Loading dye

3. Reagent for transformation
    - Chemical competent cell
    - LB agar plate
4. Reagent for assay chitinase on plate
    - Congo Red (Sigma)
    - Colloidal chitin (see Appendix I)
5. Reagent for SDS-PAGE and Zymogram
    - Protein sample buffer (see Appendix I)
    - 30% polyacylamide (BioRAD)
    - 1.5 M Tris-HCl pH 8.8
    - 0.5 M Tris-HCl pH 6.0.8
    - 10% Amonium persulfate
    - 10% Sodium dodecylsulfate
    - 0.01% glycol chitin
    - PVDF membrane (BioRAD)
6. Reagent for Western Blot
    - HisProbe-HRP (PIERCE)
    - ECL solution
    - 30% Hydrogen Peroxide (Sigma)
7. Enzymes
    - *Taq DNA polymerase* (New England BioLabs)
    - *Pfu DNA polymerase* (New England BioLabs)
    - *T4 ligase* (New England BioLabs)
    - *HindIII* (New England BioLabs)

- *XhoI* (New England BioLabs)
- *NcoI* (New England BioLabs)
- *DNase*I (Fermentas)

8. Primer
   - chiHind3

(5'-CTGTGCAAGCTTTTGTCATGTTGCTGAGCTTGTCATTTG-3')

   - chitXhoI

(5'-CTGTGCCTCGAGTCCATTTGACTTTCTGTTATTCGCAGCCTC- 3')

   - chiNcoI

(5'-CTGTGCAAGCTTTTGTCATGTTGCTGAGCTTGTCATTTG-3')

9. Reagent for assay chitinase on plate
   - Congo Red (Sigma)
   - Colloidal chitin (see Appendix I)
10. Reagent for SDS-PAGE and Zymogram
   - Protein sample buffer
   - 30% polyacylamide (BioRAD)
   - 1.5 M Tris-HCl pH 8.8
   - 0.5 M Tris-HCl pH 6.0.8
   - 10% Amonium persulfate
   - 10% Sodium dodecylsulfate
11. Reagent for enzymes purification
   - Ni-NTA resins (QIAGEN)
   - Lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole. pH 8.0)

- Wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 40 mM imidazole. pH 8.0)
- Elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole. pH 8.0)

12. Reagent for analysis of enzyme activity
    - 4-nitrophenyl-β-D-*N,N'*-diacetylchitobiose (Sigma)
    - 1 N NaOH (Sigma)
    - Dimethylaminobenzaldehyde (DMAB)

1.2 EQUIPMENTS

1. Thermomixer (Eppendorf)
2. BioRad Mini Protean II Cell (BioRAD)
3. Microtiter plate reader (Sunrise)
4. Electrotoporater (Eppendorf)
5. Semi-dry blotting (Hoefer)
6. BioRAD Mini Protein II Cell (BioRAD)
7. pH meter (Hanna instrument)

APPENDIX II

2.1 STANDARD CURVE

2.1.1 Standard curve for colorimetric method

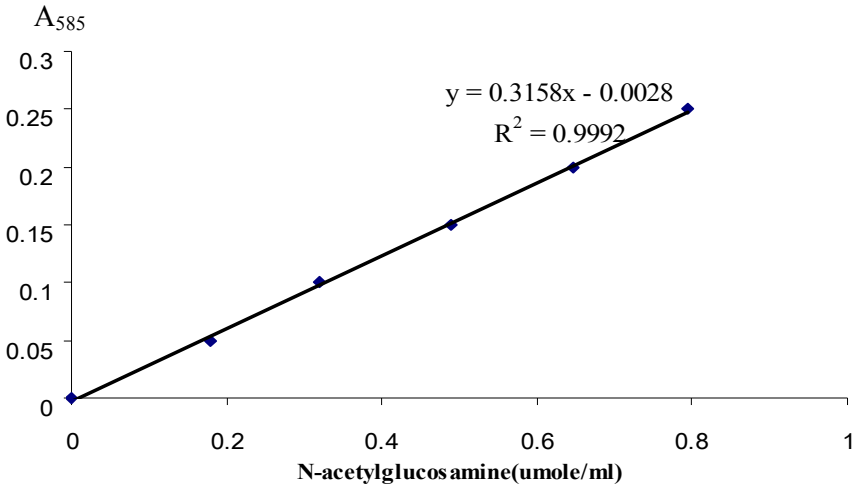

Figure 1A. Standard curve for colorimetric method when using colloidal chitin as a substrate.

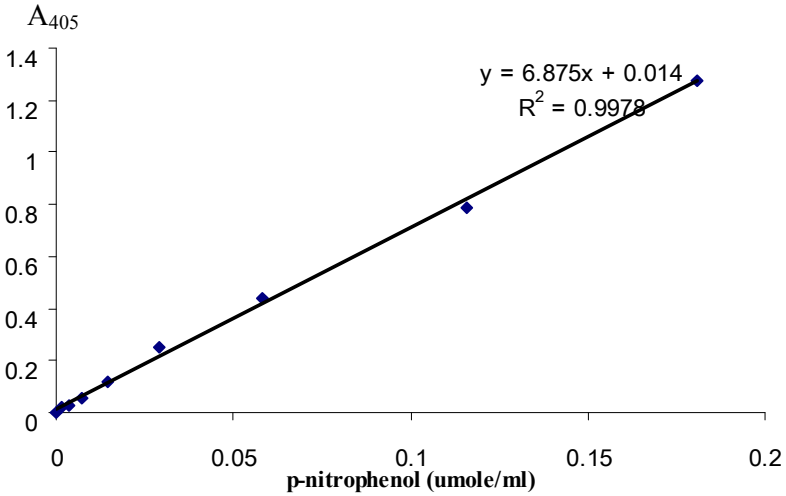

Figure 2A. Standard curve for colorimetric method when using p-nitrophenyl chitobiose as a substrate.

2.2 RESULTS DATA

Table 1A. Chitinase activities of recombinant enzyme on difference temperature.

Temperature (°C)	pFChi13					
	Specific activity (Unit/mg)					Relative activity
	1	2	3	4	5	
30	0.208	0.194	0.222			27.578
40	0.412	0.491	0.503			62.189
45	0.638	0.727	0.704			91.499
50	0.748	0.713	0.763			97.929
55	0.749	0.748	0.723	0.603	0.614	98.029
60	0.690	0.664	0.683	0.575	0.649	90.506
65	0.283	0.339	0.407	0.540	0.602	45.469
70	0.133	0.131	0.128			17.315
80	0.026	0.016	0.028			3.087
90	0	0	0			0

Table 1A. (Continued).

Temperature (°C)	pFChi8785					
	Specific activity (Unit/mg)					Relative activity
	1	2	3	4	5	
30	0.248	0.206	0.215			31.956
40	0.441	0.366	0.443			59.653
45	0.637	0.634	0.611			89.741
50	0.688	0.713	0.647			93.090
55	0.677	0.699	0.692	0.602	0.558	95.182
60	0.644	0.639	0.696	0.591	0.617	96.654
65	0.362	0.379	0.417	0.710	0.637	55.179
70	0.157	0.149	0.142			21.376
80	0.029	0.034	0.028			4.391
90	0	0	0			0

Table 1A. (Continued).

Temperature (°C)	128mt					
	Specific activity (Unit/mg)					Relative activity
	1	2	3	4	5	
30	0.263	0.226	0.240			28.025
40	0.507	0.500	0.465			56.618
45	0.729	0.565	0.721			77.253
50	0.705	0.788	0.774			86.662
55	0.875	0.835	0.847	0.695	0.583	95.366
60	0.860	0.798	0.893	0.712	0.762	98.757
65	0.596	0.642	0.632	0.838	0.786	71.918
70	0.200	0.228	0.228			25.232
80	0.038	0.058	0.039			5.221
90	0	0	0			0

Table 2A. Chitinase activities of recombinant enzymes on difference buffer system.

pH Buffer	pFChi13			
	Specific activity (Unit/mg)			Relative activity
	1	2	3	
Glycine-HCl pH 2.0	0.061	0.091	0.052	16.069
Glycine-HCl pH 3.0	0.148	0.122	0.145	33.341
Sodium citric pH 3.0	0.014	0.028	0.002	3.272
Sodium citric pH 4.0	0.005	0.010	0.037	4.517
Sodium citric pH 5.0	0.206	0.225	0.161	46.710
Sodium citric pH 6.0	0.376	0.416	0.365	92.089
Sodium phophate pH 6.0	0.449	0.449	0.346	98.246
Sodium phophate pH 7.0	0.272	0.245	0.266	62.634
Sodium phophate pH 8.0	0.216	0.187	0.168	45.272
Glycine-NaOH pH 8.0	0.255	0.245	0.211	56.393
Glycine-NaOH pH 9.0	0.038	0.063	0.090	15.816
Glycine-NaOH pH 10.0	0.005	0.028	0.033	5.495

Table 2A. (Continued).

pH Buffer	pFChi8785			
	Specific activity (Unit/mg)			Relative activity
	1	2	3	
Glycine-HCl pH 2.0	0.077	0.006	0.009	7.161
Glycine-HCl pH 3.0	0.164	0.064	0.159	29.028
Sodium citric pH 3.0	0.007	0.003	0.004	1.078
Sodium citric pH 4.0	0.003	0.010	0.007	1.502
Sodium citric pH 5.0	0.146	0.157	0.148	41.816
Sodium citric pH 6.0	0.369	0.345	0.367	81.800
Sodium phophate pH 6.0	0.424	0.421	0.479	100
Sodium phophate pH 7.0	0.237	0.221	0.227	51.905
Sodium phophate pH 8.0	0.152	0.160	0.149	34.966
Glycine-NaOH pH 8.0	0.279	0.257	0.268	60.915
Glycine-NaOH pH 9.0	0.062	0.048	0.082	14.441
Glycine-NaOH pH 10.0	0	0	0	0

Table 2A. (Continued).

pH Buffer	128mt			
	Specific activity (Unit/mg)			Relative activity
	1	2	3	
Glycine-HCl pH 2.0	0.002	0.012	0.009	1.764
Glycine-HCl pH 3.0	0.174	0.202	0.182	41.896
Sodium citric pH 3.0	0.014	0.005	0.011	2.171
Sodium citric pH 4.0	0.012	0.028	0.011	3.939
Sodium citric pH 5.0	0.208	0.195	0.167	42.664
Sodium citric pH 6.0	0.368	0.402	0.428	89.607
Sodium phophate pH 6.0	0.474	0.386	0.470	98.690
Sodium phophate pH 7.0	0.212	0.237	0.235	51.256
Sodium phophate pH 8.0	0.159	0.170	0.193	39.036
Glycine-NaOH pH 8.0	0.285	0.282	0.319	66.195
Glycine-NaOH pH 9.0	0.056	0.056	0.051	12.215
Glycine-NaOH pH 10.0	0	0	0	0

Table 3A. Total activities of the mutant library for acidic tolerant screening.

Clone	Products (μmole/ml/hr)		
	pH 3.0	pH 6.0	pH 10.0
1mt	0	0.185	0
2mt	0	0.342	0
3mt	0.107	0.583	0
4mt	0.132	0.488	0.285
5mt	0	0.192	0
6mt	0.007	0.456	0
7mt	0	0.075	0
8mt	0.009	0.542	0.123
9mt	0	0.301	0
10mt	0.217	0.757	0.134
11mt	0	0.469	0
12mt	0	0.652	0.012
13mt	0	0.517	0.078
14mt	0.039	0.472	0
15mt	0.114	0.565	0.007
16mt	0.046	0.506	0
17mt	0	0.296	0
18mt	0	0.489	0
19mt	0	0.387	0
20mt	0	0.419	0
21mt	0.023	0.444	0
22mt	0	0.372	0
23mt	0	0.549	0.128
24mt	0	0.495	0
25mt	0	0.508	0
26mt	0	0.592	0.134
27mt	0.105	0.504	0
28mt	0.007	0.627	0.082
29mt	0	0.399	0.032
pFCHi8785	0.078	0.553	0.007

Table 3A. (Continued).

Clone	Products (µmole/ml/hr)		
	pH 3.0	pH 6.0	pH 10.0
30mt	0.041	0.564	0
31mt	0	0.489	0.021
32mt	0	0.372	0
33mt	0	0.324	0
34mt	0	0.452	0
35mt	0	0.498	0.032
36mt	0	0.388	0
37mt	0	0.470	0
38mt	0	0.482	0
39mt	0	0.514	0
40mt	0.128	0.589	0
41mt	0.236	0.632	0.040
42mt	0	0.185	0
43mt	0.534	0.623	0.097
44mt	0	0.627	0.085
45mt	0.089	0.849	0.097
46mt	0	0.587	0.044
47mt	0	0.828	0.081
48mt	0	0.710	0.183
49mt	0	0.447	0.062
50mt	0	0.737	0.254
51mt	0.106	0.896	0.274
52mt	0.122	0.937	0.276
53mt	0	0.634	0.253
54mt	0	0.128	0.060
55mt	0	0.151	0.026
56mt	0	0.097	0
57mt	0	0.060	0.055
58mt	0	0.080	0.073
pFChi8785	0.073	0.561	0

Table 3A. (Continued).

Clone	Products (µmole/ml/hr)		
	pH 3.0	pH 6.0	pH 10.0
59mt	0.138	0.930	0.267
60mt	0.452	0.990	0.276
61mt	0.026	1.049	0.347
62mt	0.055	0.069	0.067
63mt	0.049	0.103	0.097
64mt	0.026	0.260	0
65mt	0.028	0.023	0
66mt	0.171	0.748	0
67mt	0.058	0.240	0
68mt	0.042	0.358	0
69mt	0.030	0.220	0
70mt	0.026	0.256	0
71mt	0.039	0.024	0
72mt	0.017	0.256	0
73mt	0.087	0.518	0
74mt	0.106	0.678	0
75mt	0.279	0.748	0
76mt	0.081	0.447	0
77mt	0.049	0.402	0
78mt	0.081	0.616	0
79mt	0.044	0.194	0
80mt	0.051	0.395	0
81mt	0.071	0.509	0
82mt	0	0.224	0
83mt	0.026	0.229	0
84mt	0.008	0.244	0
85mt	0	0.158	0
86mt	0.064	0.210	0
87mt	0.137	0.801	0
pFChi8785	0.042	0.981	0.030

Table 3A. (Continued).

Clone	Products (µmole/ml/hr)		
	pH 3.0	pH 6.0	pH 10.0
88mt	0.080	0.450	0
89mt	0.085	0.502	0
90mt	0	0.732	0
91mt	0.124	0.739	0
92mt	0.231	0.546	0
93mt	0.040	0.587	0.035
94mt	0	0.545	0.017
95mt	0.021	0.488	0.032
96mt	0.002	0.427	0.002
97mt	0.007	0.532	0
98mt	0	0.292	0.007
99mt	0.017	0.461	0.010
100mt	0	0.277	0.017
101mt	0.023	0.432	0
102mt	0.017	0.443	0.010
103mt	0.012	0.473	0
104mt	0.019	0.399	0.044
105mt	0	0.434	0.028
106mt	0	0.465	0.024
107mt	0.017	0.662	0
108mt	0	0.675	0.003
109mt	0	0.805	0.010
110mt	0	0.787	0.030
111mt	0.016	0.769	0
112mt	0.010	0.538	0.010
113mt	0	0.723	0.008
114mt	0.021	0.497	0.001
115mt	0.039	0.488	0
116mt	0.024	0.634	0
pFChi8785	0.042	0.933	0.030

Table 3A. (Continued).

	Products (µmole/ml/hr)		
Clone	**pH 3.0**	**pH 6.0**	**pH 10.0**
118mt	0	0.616	0.017
119mt	0	0.830	0.028
120mt	0.021	0.716	0.016
121mt	0	0.488	0.019
122mt	0	0.286	0.035
123mt	0.007	0.655	0.001
124mt	0.014	0.668	0.007
125mt	0.017	0.864	0.003
126mt	0.037	0.703	0.046
127mt	0.042	0.933	0.030
128mt	0.365	1.024	0.026
129mt	0	0.833	0.046
130mt	0	0.304	0.049
131mt	0	0.846	0.023
132mt	0.010	0.987	0.003
133mt	0	0.623	0.023
134mt	0	0.869	0.019
135mt	0	1.017	0.010
136mt	0	0.285	0.051
137mt	0.160	0.783	0.026
139mt	0	0.623	0.037
140mt	0	0.301	0.048
141mt	0	0.281	0.051
142mt	0.010	0.958	0.094
143mt	0	0.365	0.051
144mt	0.007	1.010	0.008
145mt	0	0.807	0.005
146mt	0	0.837	0.010
147mt	0	0.397	0.033
pFChi8785	0.042	0.933	0.030

Table 3A. (Continued).

Clone	Products (µmole/ml/hr)		
	pH 3.0	pH 6.0	pH 10.0
148mt	0.135	1.031	0.055
149mt	0.032	1.056	0.008
150mt	0.160	0.922	0.008
154mt	0	0.058	0
155mt	0.012	0.874	0
156mt	0	0.208	0
157mt	0	0.258	0
158mt	0.204	0.999	0.135
160mt	0	0.816	0
161mt	0	0.072	0
162mt	0	1.700	0
163mt	0	0.952	0.016
164mt	0	0.097	0
165mt	0	1.757	0
166mt	0	0.069	0.040
167mt	0	0.220	0
168mt	0	0.085	0
169mt	0	0.012	0
170mt	0	0.076	0
171mt	0	0.908	0
172mt	0	0.286	0
173mt	0	0.694	0
174mt	0	0.320	0
175mt	0	0.918	0
176mt	0	1.090	0
177mt	0	1.243	0
178mt	0	0.905	0
179mt	0	0.408	0
180mt	0	0.022	0
pFChi8785	0.064	0.902	0

Table 3A. (Continued).

Clone	Products (µmole/ml/hr)		
	pH 3.0	pH 6.0	pH 10.0
181mt	0	0.966	0
182mt	0	0.798	0
183mt	0	0.438	0
184mt	0	0.508	0
185mt	0	0.968	0
186mt	0	2.426	0.089
187mt	0	1.017	0
188mt	0	2.308	0
189mt	0.030	0.967	0
190mt	0	0.548	0
191mt	0	0.518	0.051
192mt	0	0.033	0
193mt	0	0.475	0
194mt	0.320	0.983	0.012
195mt	0.431	1.107	0.026
196mt	0.317	1.648	0
197mt	0.343	1.063	0.001
198mt	0.249	1.097	0.003
199mt	0.507	1.131	0.033
200mt	0.636	1.876	0.067
201mt	0.149	0.922	0
202mt	0	0.165	0
203mt	0.085	0.839	0
204mt	0.452	2.047	0.044
205mt	0.163	1.101	0.226
206mt	0.158	1.010	0
207mt	0.128	1.010	0.005
208mt	0.329	1.710	0.083
209mt	0.443	1.101	0.032
pFChi8785	0.071	0.992	0

Table 3A. (Continued).

Clone	Products (µmole/ml/hr)		
	pH 3.0	pH 6.0	pH 10.0
210mt	0.128	1.010	0
211mt	0.242	1.110	0.014
212mt	0.253	1.653	0.005
213mt	0	0.707	0.032
214mt	0	1.195	0.021
215mt	0	1.145	0.008
216mt	0	1.045	0.003
217mt	0	1.054	0.007
218mt	0	0.024	0.117
219mt	0.053	1.218	0.060
220mt	0.112	0.037	0.030
221mt	0	1.147	0
222mt	0.057	1.478	0
223mt	0	0.074	0
224mt	0	1.010	0.005
225mt	0	1.040	0.007
226mt	0	0.078	0
227mt	0	1.047	0
228mt	0	1.261	0.195
229mt	0.005	1.175	0.195
230mt	0.012	1.234	0.201
231mt	0	0.027	0
232mt	0	1.248	0.162
233mt	0	1.170	0.172
234mt	0	1.215	0.131
235mt	0	0.876	0
236mt	0	1.231	0.071
237mt	0	0.987	0
238mt	0	0.367	0
pFLAG8785	0.071	0.992	0

Table 3A. (Continued).

Clone	Products (µmole/ml/hr)		
	pH 3.0	pH 6.0	pH 10.0
237mt	0	0.987	0
238mt	0	0.367	0
239mt	0	1.044	0
240mt	0	0.960	0
241mt	0	0.721	0
242mt	0	0.185	0
243mt	0	1.256	0.096
244mt	0	0.235	0
245mt	0	0.194	0
246mt	0	0.481	0.040
247mt	0	0.693	0.021
248mt	0	0.539	0
249mt	0	0.336	0
250mt	0	0.263	0
251mt	0	0.048	0
252mt	0	0.003	0
253mt	0	0.073	0
254mt	0	0.438	0
255mt	0	0.101	0
256mt	0	0	0
257mt	0	0.021	0
258mt	0	0	0
259mt	0	0	0
260mt	0	0.028	0
261mt	0	0.103	0
262mt	0	0.017	0
263mt	0	0	0
264mt	0	0.007	0
265mt	0	0.153	0
pFChi8785	0	0.767	0

Table 3A. (Continued).

Clone	Products (µmole/ml/hr)		
	pH 3.0	pH 6.0	pH 10.0
266mt	0	0.390	0
267mt	0	0.437	0
268mt	0	0.102	0
269mt	0	0.034	0
270mt	0	0.711	0
271mt	0	0.517	0
272mt	0	0.618	0
273mt	0	0.845	0
274mt	0	0.665	0
275mt	0	0.335	0
276mt	0	0.274	0
277mt	0	0.082	0
278mt	0	0.004	0
279mt	0	0.189	0
280mt	0	0.347	0
281mt	0	0.882	0
282mt	0	0.859	0
283mt	0	0.369	0
284mt	0	0.041	0
285mt	0	0.677	0
286mt	0	0.356	0
287mt	0	0.127	0
288mt	0	0.899	0
289mt	0	0.745	0
290mt	0	0.162	0
291mt	0	0.355	0
292mt	0	0.848	0
293mt	0	0.891	0
pFChi8785	0	0.768	0

Table 4A. Confirmation of acidic tolerant activity (crude enzyme) when using colloidal chitin as a substrate.

Clone	Products (Unit/mg)	
	pH 3.0	pH 6.0
43mt	0.001069	0.017821
128mt	0.003056	0.012799
197mt	0.000864	0.011527
199mt	0.001573	0.014281
204mt	0.000789	0.013182
pFChi13	0.000894	0.015293
pFChi8785	0.001229	0.01374

Table 5A. Confirmation of acidic tolerant activity (partial enzyme) when using *p*-nitrophenyl chitobiose as a substrate.

Clone	Specific activity (Unit/µg)	
	pH 6.0	pH 3.0
pFChi13	390.342	247.613
pFChi8785	431.795	231.805
128mt	577.163	339.305

Table 6A. Confirmation of acidic tolerant activity (partial enzyme) when using colloidal chitin as a substrate.

Clone	Specific activity (Unit/µg)	
	pH 6.0	pH 3.0
pFChi13	397.51	290.94
pFChi8785	455.60	301.23
128mt	567.65	351.72

2.3 Kinetic data and Eadie-Hofstee plots

Table 7A. Kinetic data of pFChi13 using *p*-nitrophenyl chitobiose as a substrate in sodium phosphate buffer pH 6.0 and glycine-HCl buffer pH 3.0.

Substrate (mM)	pFChi13 velocity (µmole/min/mg)			
	pH 6.0		pH 3.0	
	V1	V2	V1	V2
0.008	0.034	0.048	0.008	0.01
0.016	0.062	0.064	0.018	0.02
0.023	0.13	0.112	0.028	0.028
0.046	0.162	0.146	0.048	0.046
0.092	0.274	0.272	0.07	0.064
0.138	0.34	0.342	0.09	0.086
0.183	0.412	0.436	0.1	0.102
0.366	0.402	0.378	0.146	0.144
0.7	0.38	0.368	0.116	0.122

Table 8A. Kinetic data of pFChi13 using *p*-nitrophenyl chitobiose as a substrate in sodium phosphate buffer pH 6.0 and glycine-HCl buffer pH 3.0 (duplicate).

Substrate (mM)	pFChi13 velocity (µmole/min/mg)			
	pH 6.0		pH 3.0	
	V1	V2	V1	V2
0.008	0.036	0.04	0.01	0.01
0.016	0.066	0.04	0.018	0.02
0.023	0.092	0.05	0.028	0.026
0.046	0.146	0.146	0.046	0.048
0.092	0.226	0.22	0.07	0.068
0.138	0.238	0.234	0.086	0.084
0.183	0.278	0.284	0.098	0.098
0.366	0.266	0.262	0.144	0.146
0.7	0.246	0.248	0.114	0.112

Table 9A. Kinetic data of pFChi8785 using *p*-nitrophenyl chitobiose as a substrate in sodium phosphate buffer pH 6.0 and glycine-HCl buffer pH 3.0.

Substrate (mM)	pFChi8785 velocity (µmole/min/mg)			
	pH 6.0		pH 3.0	
	V1	V2	V1	V2
0.012	0.07	0.064	0.026	0.032
0.023	0.128	0.132	0.028	0.09
0.046	0.202	0.182	0.08	0.072
0.092	0.284	0.274	0.108	0.082
0.183	0.366	0.348	0.1	0.126
0.366	0.316	0.312	0.13	0.134
0.549	0.228	0.218	0.026	0.032

Table 10A. Kinetic data of p pFChi8785 using *p*-nitrophenyl chitobiose as a substrate in sodium phosphate buffer pH 6.0 and glycine-HCl buffer pH 3.0 (duplicate).

Substrate (mM)	pFChi8785 velocity (µmole/min/mg)			
	pH 6.0		pH 3.0	
	V1	V2	V1	V2
0.008	0.01	0.014	0	0.02
0.016	0.052	0.06	0.026	0.022
0.023	0.086	0.088	0.02	0.018
0.046	0.12	0.136	0.018	0.07
0.092	0.188	0.182	0.06	0.054
0.132	0.272	0.256	0.016	0.008
0.183	0.228	0.24	0.066	0.05
0.366	0.208	0.212	0.068	0.086
0.7	0.152	0.158	0.096	0.09

Table 11A. Kinetic data of 128mt using *p*-nitrophenyl chitobiose as a substrate in sodium phosphate buffer pH 6.0 and glycine-HCl buffer pH 3.0.

Substrate (mM)	128mt velocity (µmole/min/mg)			
	pH 6.0		pH 3.0	
	V1	V2	V1	V2
0.008	0.066	0.086	0.016	0.014
0.016	0.098	0.094	0.04	0.016
0.023	0.152	0.132	0.052	0.016
0.046	0.264	0.254	0.072	0.056
0.092	0.402	0.292	0.118	0.082
0.132	0.462	0.452	0.142	0.102
0.183	0.61	0.626	0.168	0.172
0.366	0.414	0.394	0.154	0.154
0.7	0.248	0.254	0.102	0.132

Table 12A. Kinetic data of 128mt using *p*-nitrophenyl chitobiose as a substrate in sodium phosphate buffer pH 6.0 and glycine-HCl buffer pH 3.0 (duplicate).

Substrate (mM)	128mt velocity (µmole/min/mg)			
	pH 6.0		pH 3.0	
	V1	V2	V1	V2
0.012	0.09	0.094	0.026	0.046
0.023	0.29	0.286	0.078	0.026
0.046	0.41	0.392	0.086	0.112
0.092	0.584	0.584	0.18	0.132
0.183	0.648	0.652	0.254	0.258
0.366	0.586	0.62	0.234	0.23
0.549	0.63	0.62	0.174	0.17

2.3.1 Kinetic parameter graph

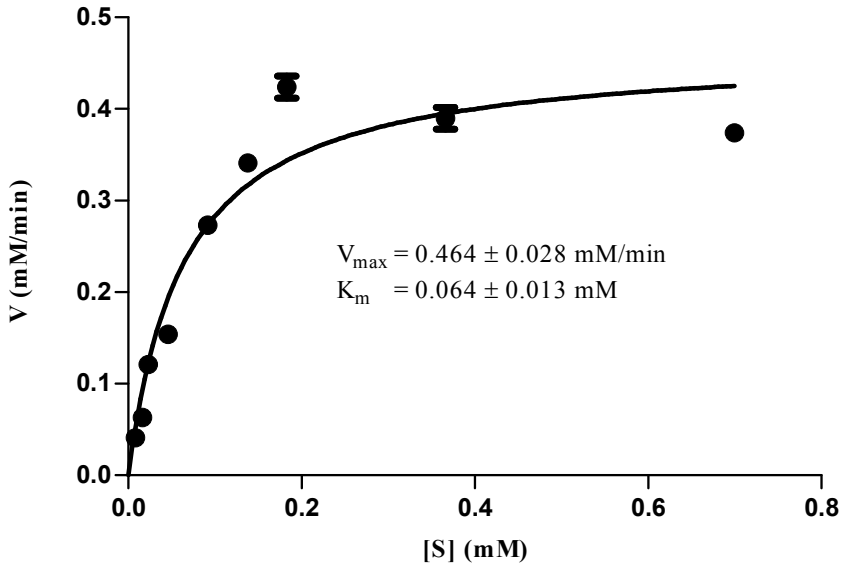

Figure 3A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi13 in sodium phosphate buffer pH 6.0.

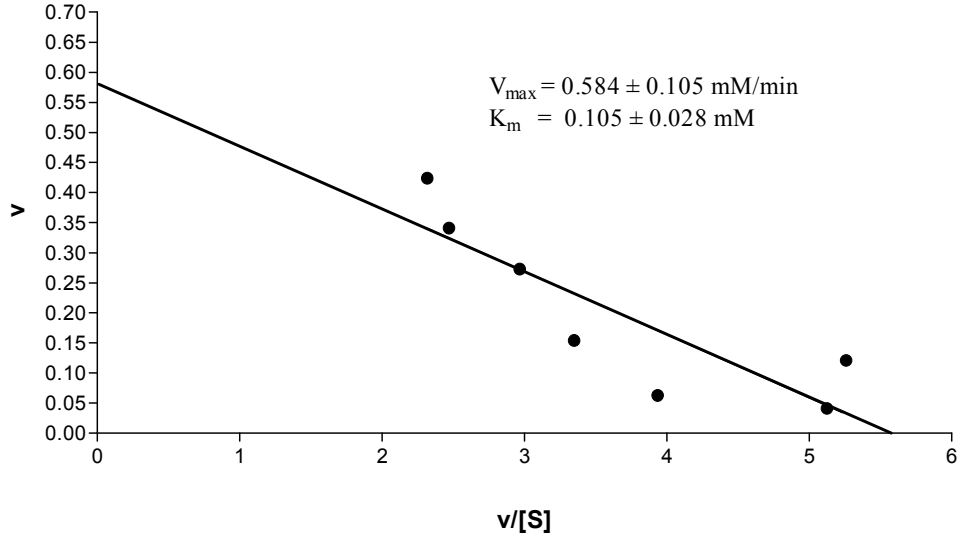

Figure 4A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi13 in sodium phosphate buffer pH 6.0.

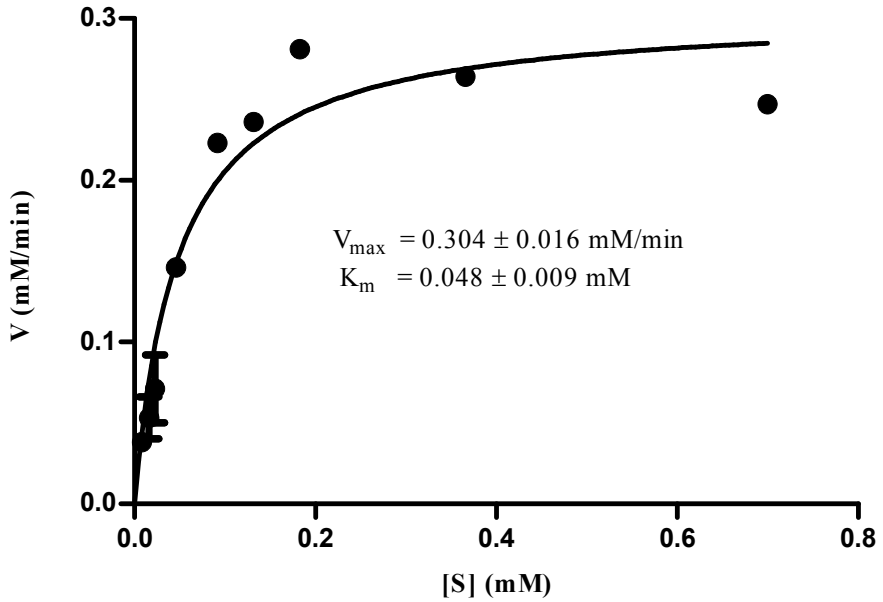

Figure 5A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi13 in sodium phosphate buffer pH 6.0 (duplicate).

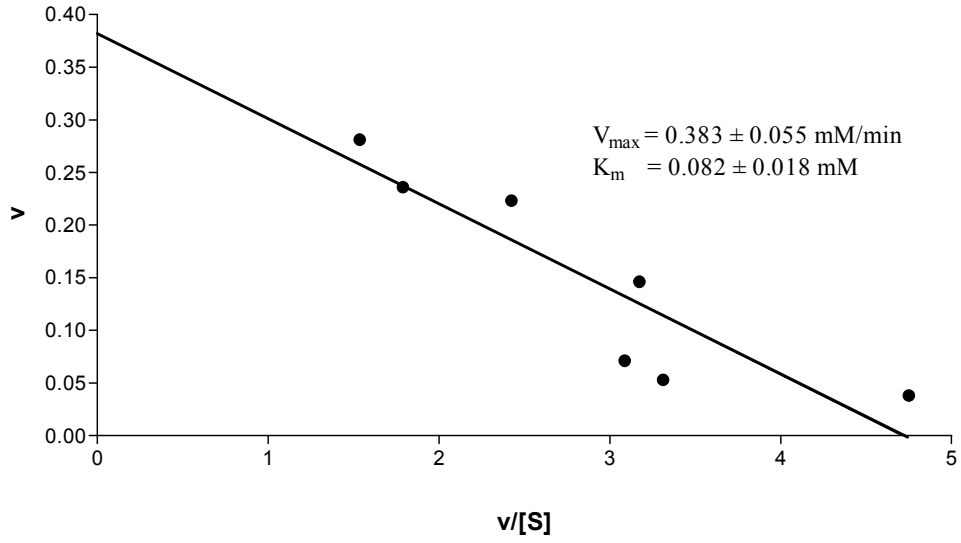

Figure 6A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi13 in sodium phosphate buffer pH 6.0 (duplicate).

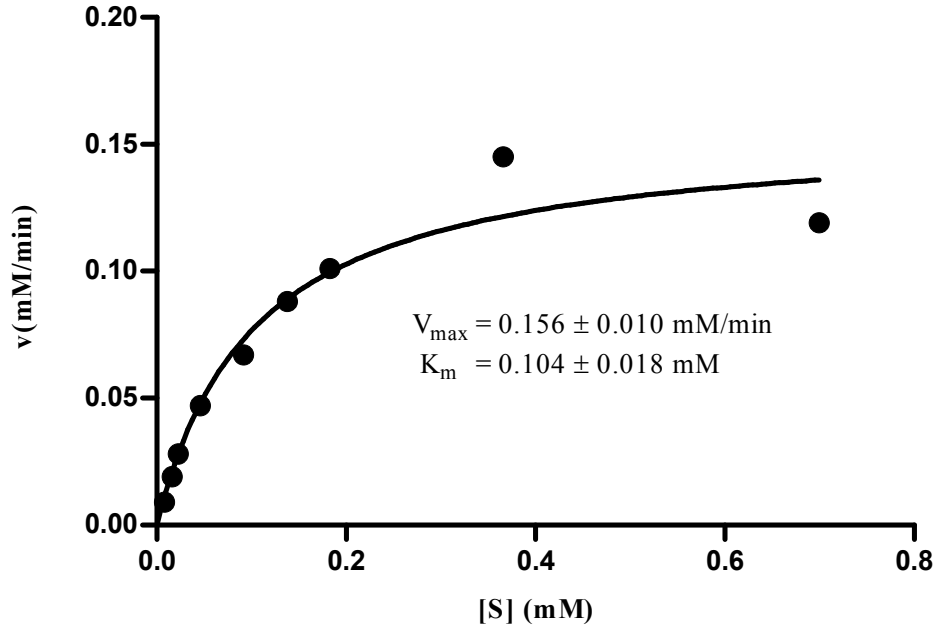

Figure 7A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi13 in glycine-HCl buffer pH 3.0.

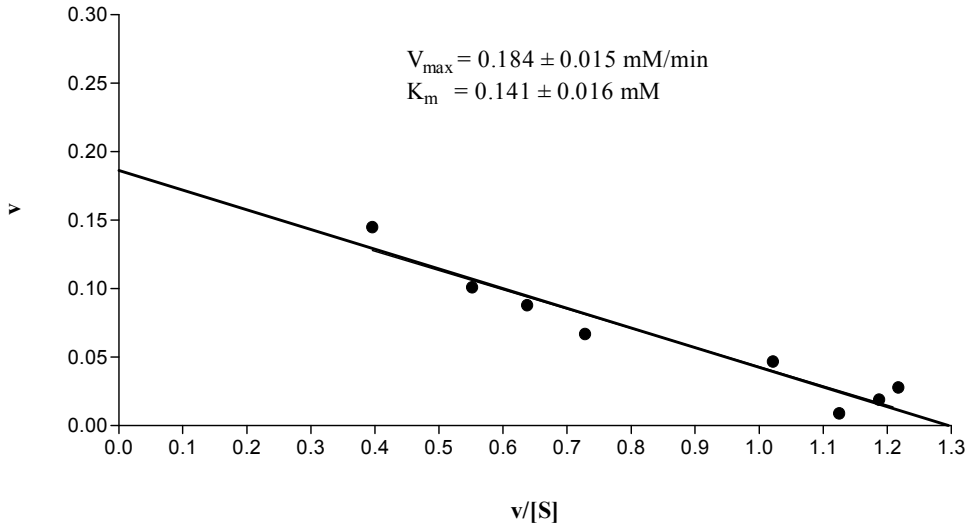

Figure 8A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi13 in glycine-HCl buffer pH 3.0.

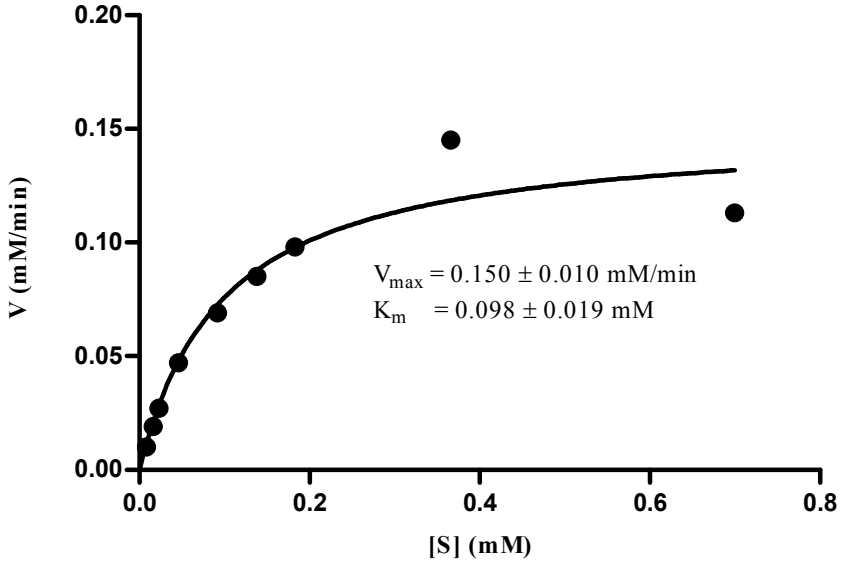

Figure 9A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi13 in glycine-HCl buffer pH 3.0 (duplicate).

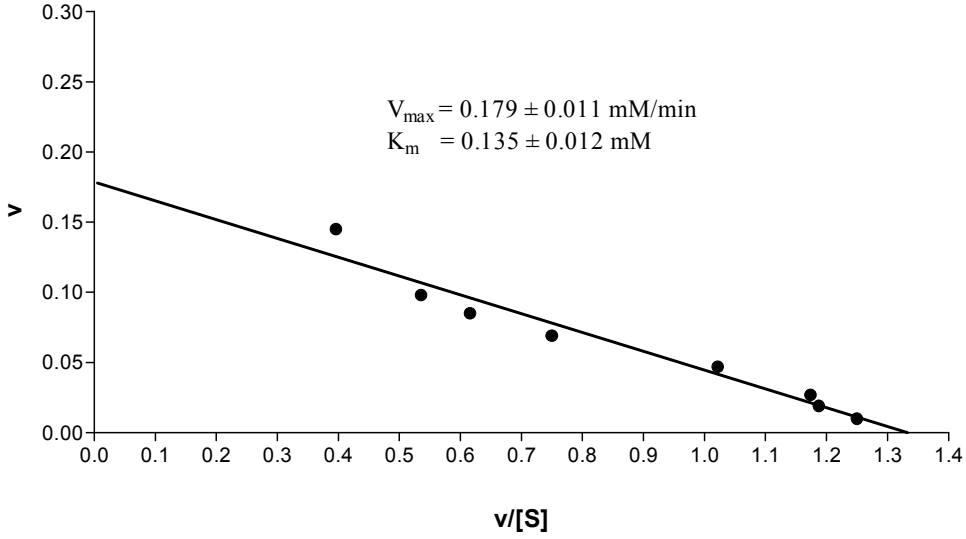

Figure 10A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi13 in glycine-HCl buffer pH 3.0 (duplicate).

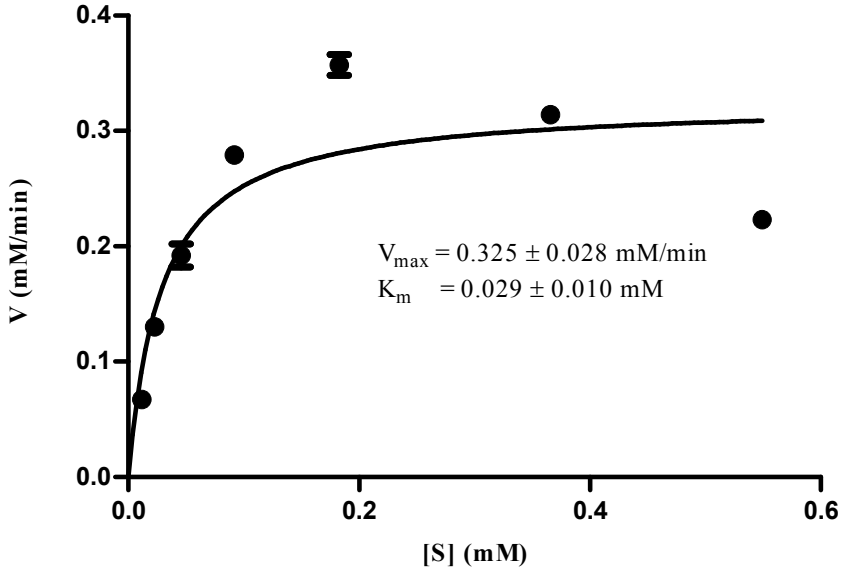

Figure 11A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi8785 in sodium phosphate buffer pH 6.0.

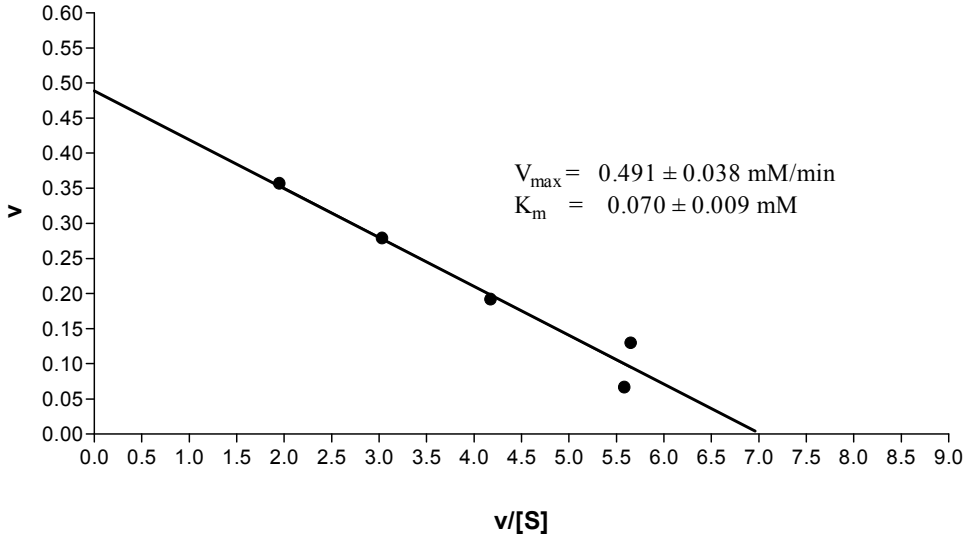

Figure 12A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi8785 in sodium phosphate buffer pH 6.0.

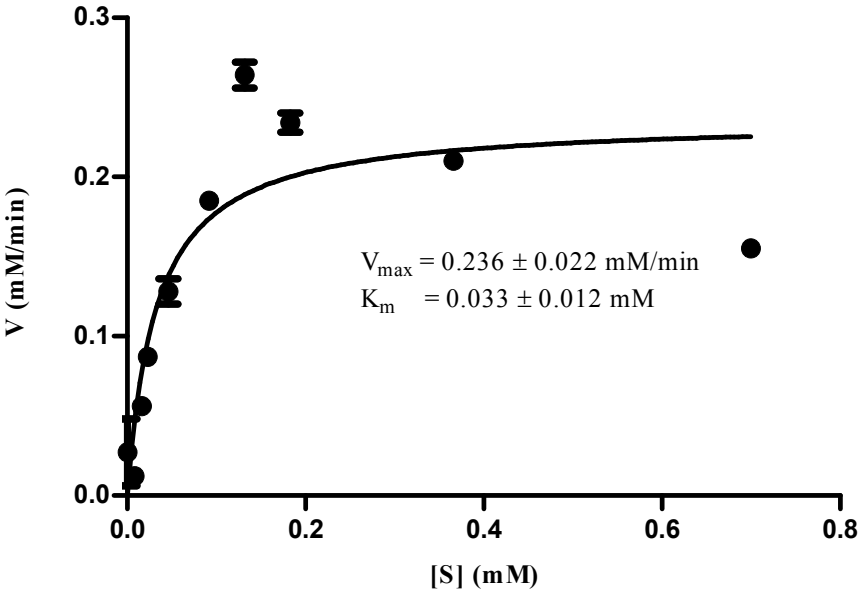

Figure 13A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi8785 in sodium phosphate buffer pH 6.0 (duplicate).

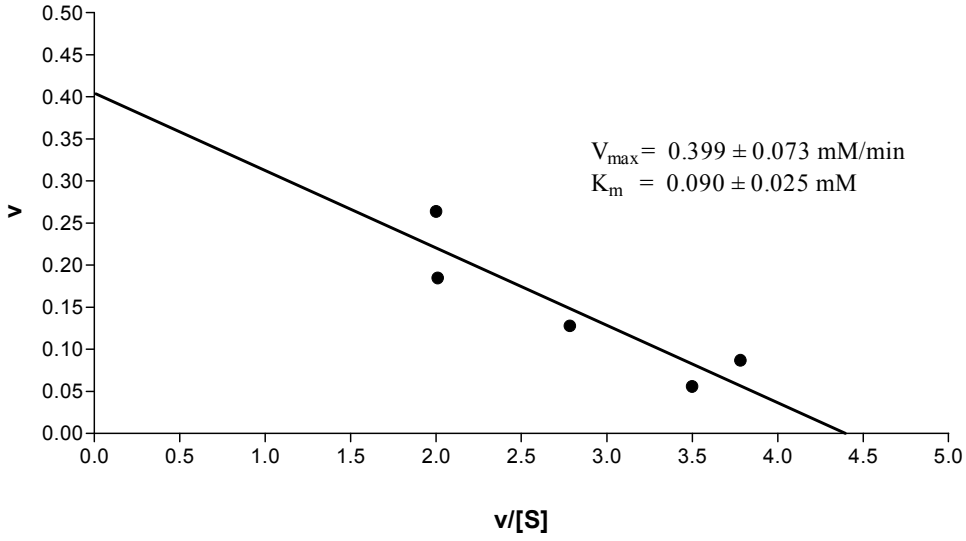

Figure 14A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi8785 in sodium phosphate buffer pH 6.0 (duplicate).

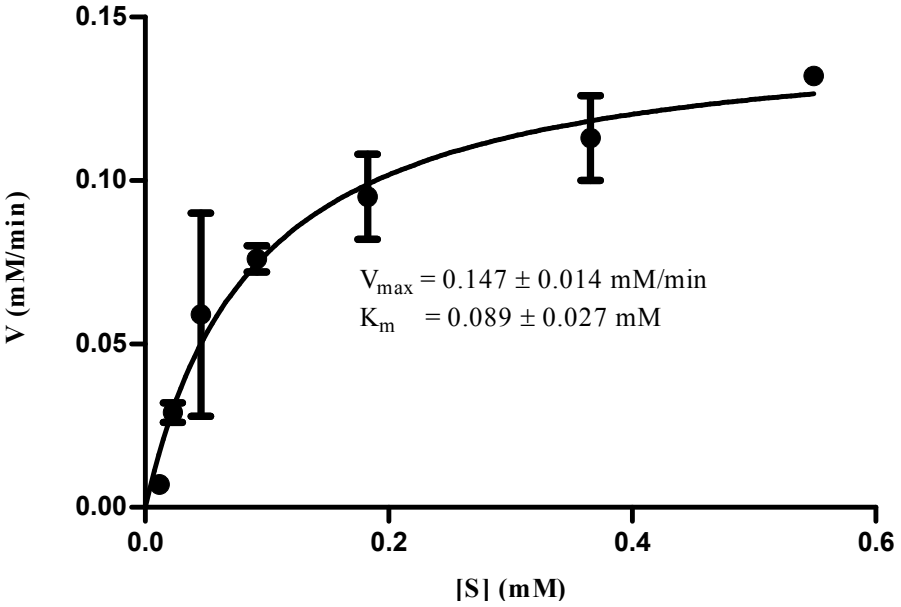

Figure 15A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi8785 in glycine-HCl buffer pH 3.0.

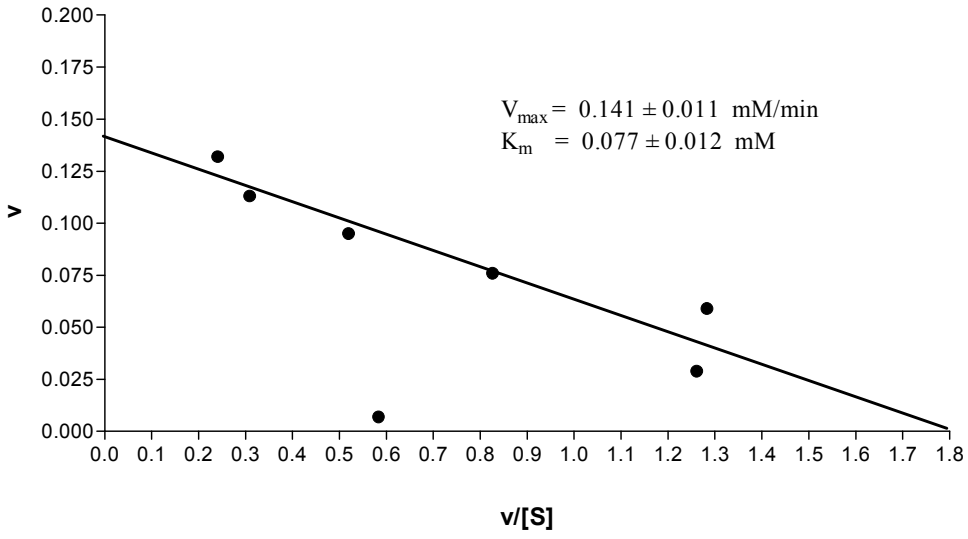

Figure 16A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi8785 in glycine-HCl buffer pH 3.0.

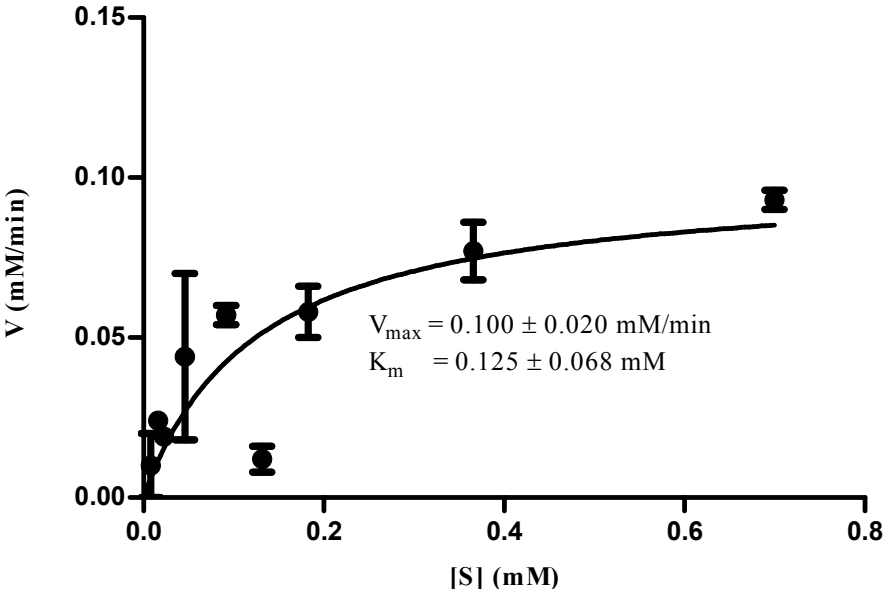

Figure 17A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi8785 in glycine-HCl buffer pH 3.0 (duplicate).

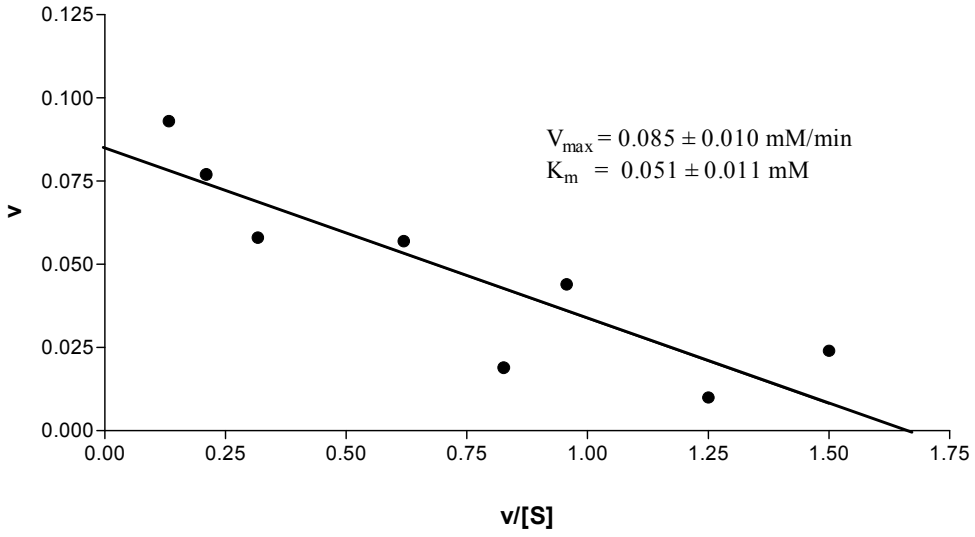

Figure 18A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by pFChi8785 in glycine-HCl buffer pH 3.0 (duplicate).

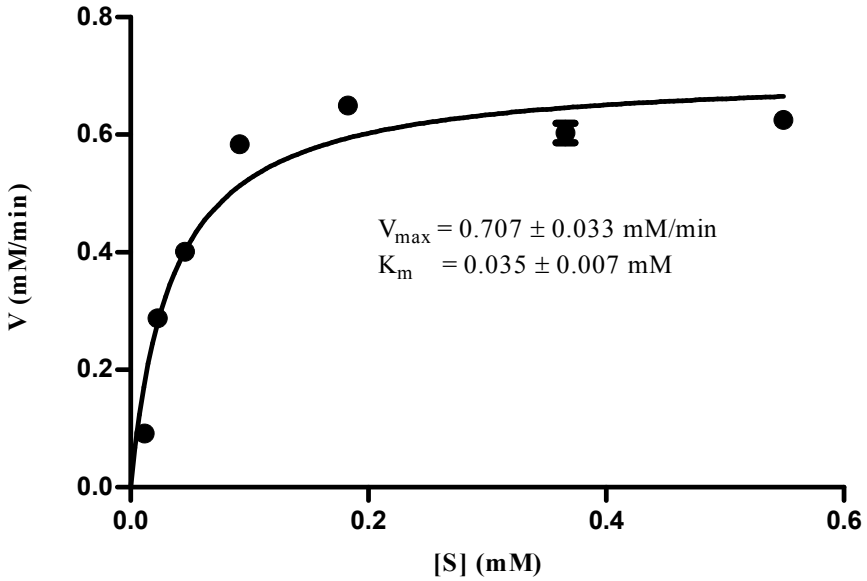

Figure 19A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by 128mt in sodium phosphate buffer pH 6.0.

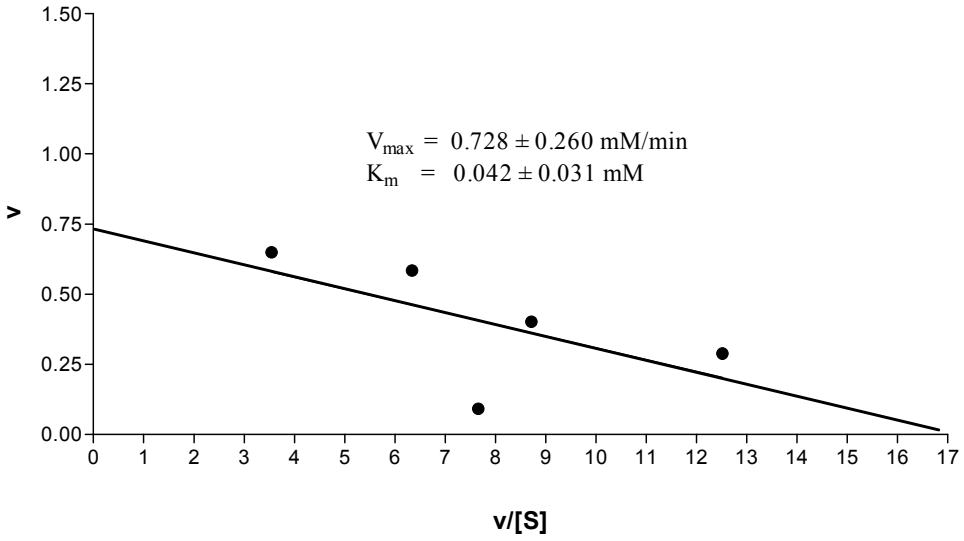

Figure 20A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by 128mt in sodium phosphate buffer pH 6.0.

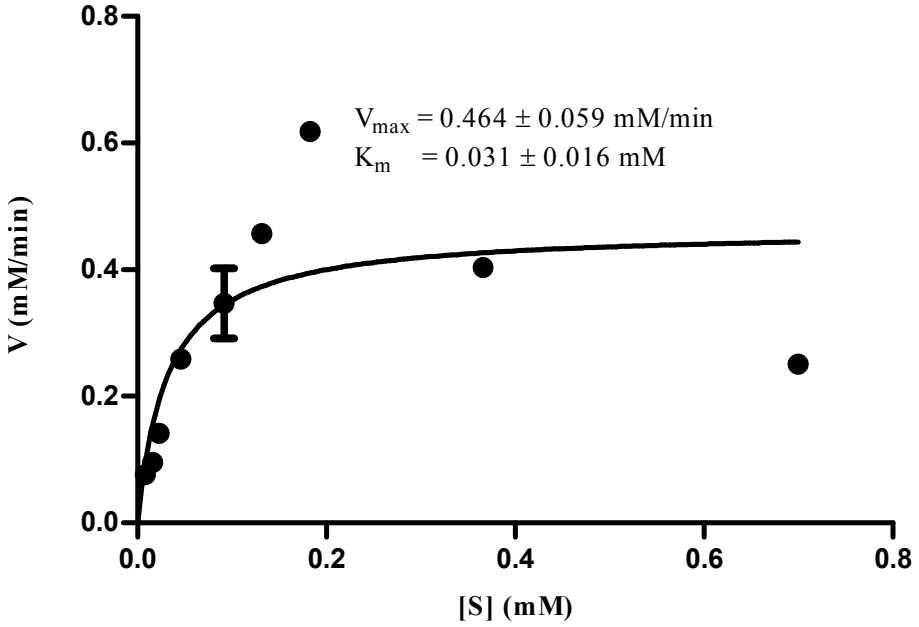

Figure 21A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by 128mt in sodium phosphate buffer pH 6.0 (duplicate).

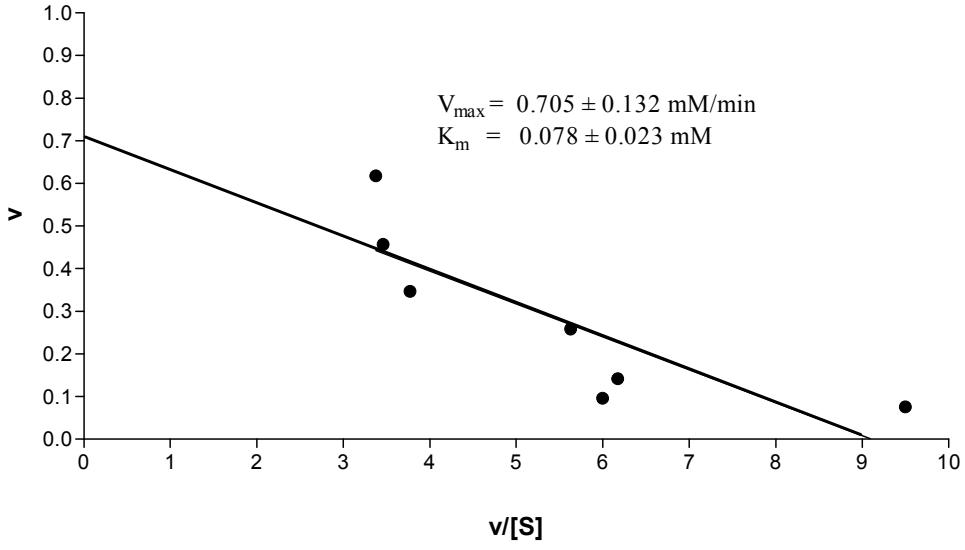

Figure 22A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by 128mt in sodium phosphate buffer pH 6.0 (duplicate).

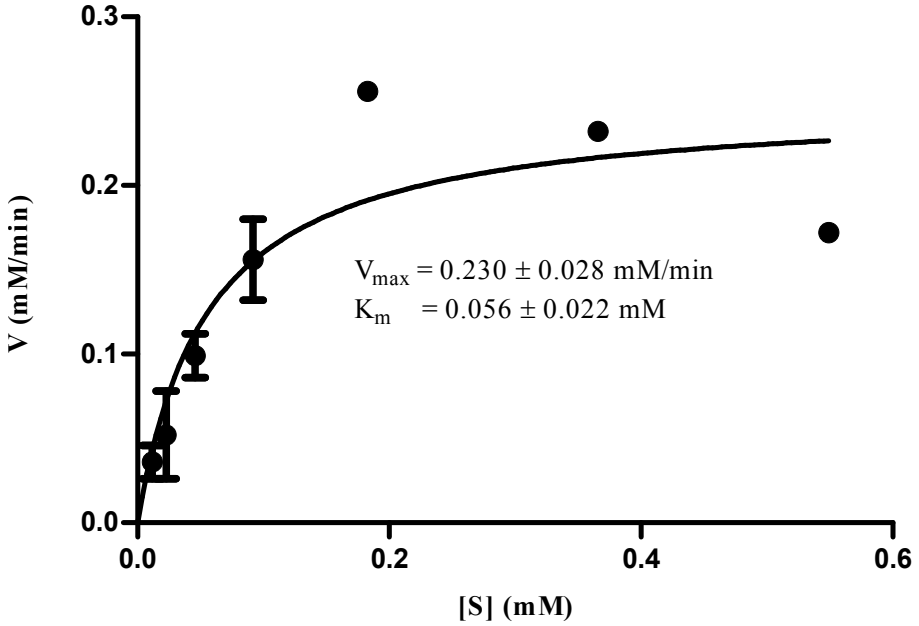

Figure 23A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by 128mt in glycine-HCl buffer pH 3.0.

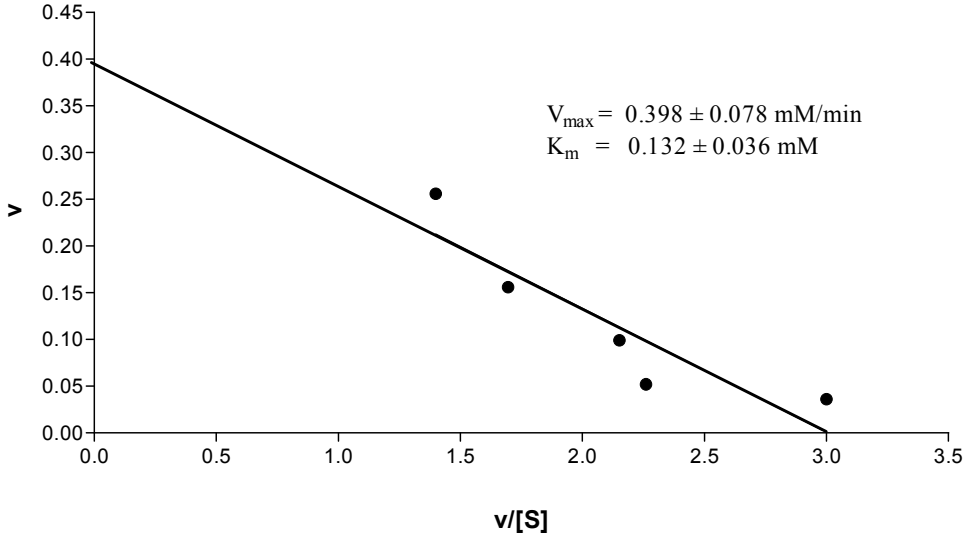

Figure 24A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by 128mt in glycine-HCl buffer pH 3.0.

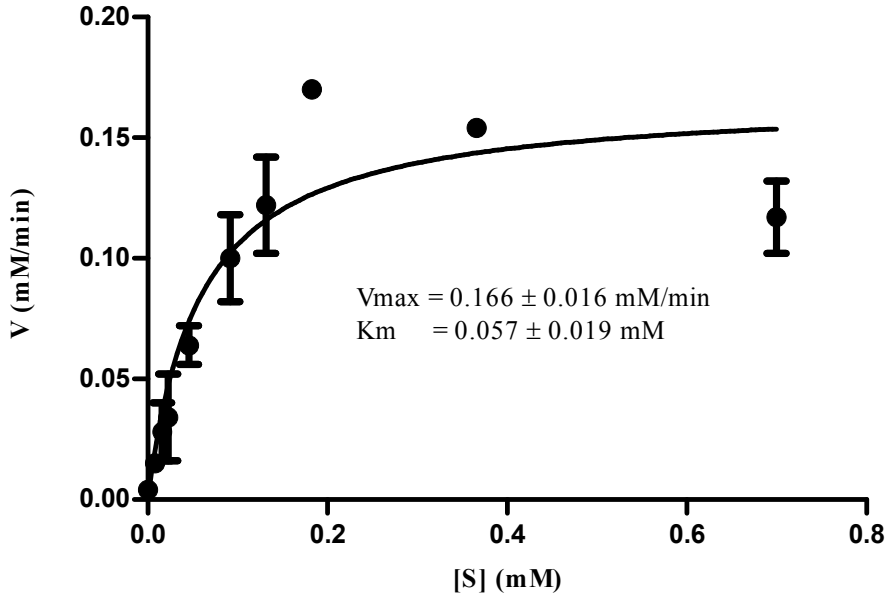

Figure 25A. Michaelis-Menten plot for hydrolysis of *p*-nitrophenyl chitobiose by 128mt in glycine-HCl buffer pH 3.0 (duplicate).

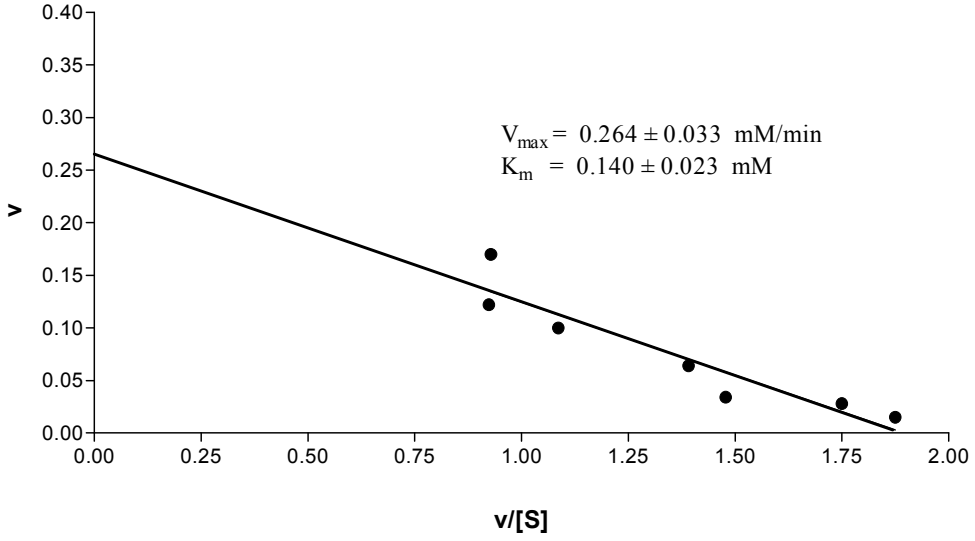

Figure 26A. Edie-Hofstee plot for hydrolysis of *p*-nitrophenyl chitobiose by 128mt in glycine-HCl buffer pH 3.0 (duplicate).

BIOGRAPHY

Puntarika Pesatcha was born on June 12, 1980 in Burirum, Thailand. She graduated with a Bachelor degree from Department of Biotechnology, Faculty of Technology, Khonkaen University of Technology, Khonkaen University, in 2003. After graduation, she has opportunity to study master degree in school of Biotechnology, Institute of Agricultural at Suranaree University of Technology. She conducted to research in the topic of directed evolution of chitinase for industrial application as her thesis work.