การผลิต *N*-acetyl-D-glucosamine โดยใช้เอนไซม์จาก *Aspergillus fumigatus*

นาย ภคภพ เศรษฐเกษตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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PREPARATION OF *N*-ACETYL-D-GLUCOSAMINE USING ENZYME FROM *ASPERGILLUS FUMIGATUS*

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Field of Study	Biotechnology
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ภคภพ เศรษฐเกษตร: การผลิตเอ็น-แอซิทิล-ดี-กลูโคซามีนโดยใช้เอนไซม์จาก *Aspergillus fumigatus* (PRODUCTION OF *N*-ACETYL-D-GLUCOSAMINE USING ENZYME FROM *ASPERGILLUS FUMIGATUS*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. มงคล สุขวัฒนาสินิทธิ์: อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร. รัฐ พิชญางกูร; 57 หน้า.

เอ็น-แอซิทิล-ดี-กลูโคซามีนเป็นองค์ประกอบพื้นฐานของกระดูกอ่อนในสัตว์เลี้ยงลูกด้วยนม ซึ่งเป็นที่รู้ว่าช่วยซ่อมแซมกระดูกอ่อนที่เสื่อม บรรเทาอาการเจ็บและอักเสบในผู้ป่วยที่เป็นโรคออสทีโออาร์ไธรทิส (osteoarthritis) การผลิตเอ็น-แอซิทิล-ดี-กลูโคซามีนด้วยการย่อยไคตินจากแกนหมึก โดยการใช้เอนไซม์ไคติเนสที่เตรียมจากเชื้อรา *Aspergillus fumigatus* การเลี้ยงเชื้อรา *Aspergillus fumigatus* ใน colloidal chitin minimum media ที่พีเอช 3.5 และอุณหภูมิ 40 องศาเซลเซียส เป็นเวลา 3 วัน จะได้เอนไซม์ได้สูงที่สุดโดยให้แอคติวิตีสูงถึง 8.0 $\pm$ 0.4 U/mL ปฏิกิริยาไฮโดรไลซิสของไคตินบดจากแกนหมึก (5 g) โดยเอนไซม์ย่อยไคตินที่ผลิตจาก *Aspergillus fumigatus* ให้ผลิตภัณฑ์เอ็น-แอซิทิล-ดี-กลูโคซามีนมากกว่า 80 % ใน 2 วัน อัตราส่วนที่เหมาะสมของเอนไซม์ต่อไคตินคือ 22 U/g chitin ที่ความเข้มข้นของซับสเตรทที่เหมาะสม 2.5% w/w ไคตินบดที่ได้จากแกนหมึก100 กรัม ใช้ในการไฮโดรไลซิส ที่ อุณหภูมิ 45 องศาเซลเซียส และพีเอช 4 (กรดแอซิติก 2M) โดยใช้เอนไซม์ 2.2 kU หลังจาก 2 วัน ผลิตให้เอ็น-แอซิทิล-ดี-กลูโคซามีนได้ผลผลิต 65% ได้ผลิตภัณฑ์เอ็น-แอซิทิล-ดี-กลูโคซามีนที่มีความบริสุทธิ์สูง (100%) โดยใช้ผงถ่านกัมมันต์ในการดูดสีและตกตะกอนผลิตภัณฑ์ด้วยเอทานอล

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต…….....

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ลายมือชื่อที่ปรึกษาวิทยานิพนธ์ร่วม……...

4872403023 : MAJOR BIOTECHNOLOGY
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PHAKAPOB SETTHAKASET: PRODUCTION OF *N*-ACETYL-D-GLUCOSAMINE USING ENZYME FROM *ASPERGILLUS FUMIGATUS*. THESIS ADVISOR: ASSOC. PROF. MONGKOL SUKWATTASINITT, THESIS CO-ADVISOR: ASSIST. PROF. RATH PICHYANGKURA., 57 pp.

N-acetyl-D-glucosamine (GlcNAc) is one of the basic constituents of mammal cartilage, which is known to help repair deteriorating cartilage and relieve pain and inflammation in osteoarthritis patients. The preparation of GlcNAc in multigram scale was achieved by hydrolyzing squid pen chitin with crude enzyme from *Aspergillus fumigatus.* The enzyme was obtained by cultivation of the *Aspergillus fumigatus* in colloidal chitin minimum media at pH 3.5 and 40 °C. The highest chitinolytic activity of 8.0 ± 0.4 U/mL was obtained after 3 days of cultivation. The hydrolysis of milled squid pen chitin (5 g) with the crude enzymes gave *N*-acetyl-D-glucosamine (GlcNAc) over 80% isolated yield within 2 days. The optimum ratio of enzyme to chitin was found to be 22 U/g chitin and the optimum chitin concentration was 2.5% w/w of the reaction volume. A 100 g scale hydrolysis of squid pen chitin at 45 °C and pH 4, adjusted with 2 M acetic acid, with 2.2 kU of enzyme gave GlcNAc in 65% isolated yield after 2 days of hydrolysis. The high purity of GlcNAc (100%) was obtained by precipitation of the crude GlcNAc in absolute ethanol from its concentrated charcoal decolorized aqueous solution.

Field of Study Biotechnology Student's Signature……..……..….
Academic Year 2008 Advisor's Signature……..…….....
Co-Advisor's Signature…….

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List of Abbreviations

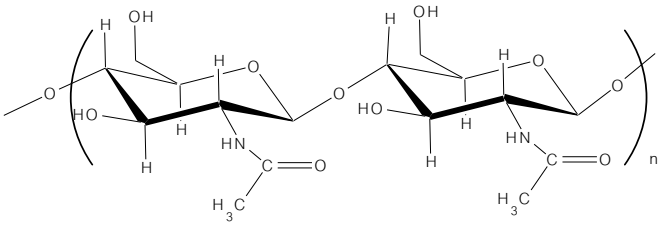
α	alpha
β	beta
ºC	degree celsius
cm	centimeter
DI-water	deionized water
γ	gramma
g	gram (s)
GlcNAc	*N*-acetyl-D-glucosamine
$(GlcNAc)_2$	*N*,*N'*-diacetylchitobiose
$(GlcNAc)_3$	*N*,*N'*,*N''* -triacetyl-chitotriose
$(GlcNAc)_4$	*N*,*N'*,*N''*,*N'''* –tetraacetyl-chitotetrose
GlcN	D-glucosamine
HPLC	High Performance Liquid Chromatography
mg	milligram
M	Molar
min	minute
mL	milliliter
mM	millimolar
mU	milliunit
ppm	part per million
U	unit
μL	microliter
%	percent

CHAPTER I

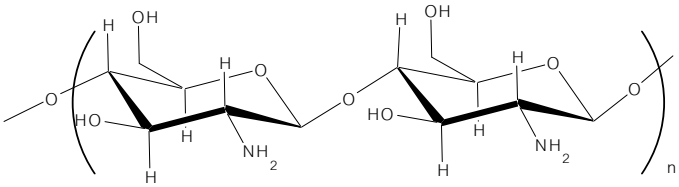
INTRODUCTION

1.1 Chitin

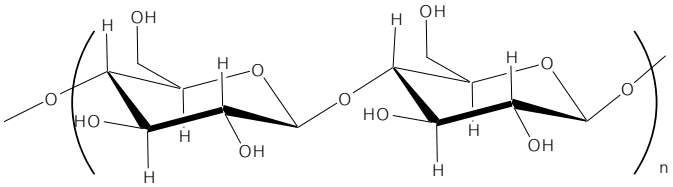
Chitin, a natural homopolymer of β-(1-4) linked *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose), belongs to a large class of amino sugars found in the exoskeletons or cuticles of invertebrates, as well as in the cell wall of many fungi and some algae. The global yield of chitin is amounted to be 1 to 100 billion metric tons, making chitin the second most abundant polysaccharide on the earth. It may be regarded as a derivative of cellulose, the most abundant organic compound, in which the hydroxyl group (-OH) at the second carbon position of the pyranose ring is replaced with an acetamide ($-NHC(=O)CH_3$) group **(Figure 1.1)**.[1]



(a) Chitin



(b) Chitosan



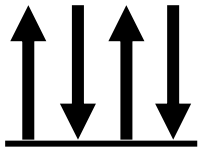
(c) Cellulose

Figure 1.1 Structure of (a) chitin, (b) chitosan and (c) cellulose

The structure refined for α-chitin either by X-ray diffraction[2] or linked atom least-square procedure revealed an antipararell of two adjacent polysaccharide chains (**Figure 1.2**). Half of the hydroxyl groups of sugar ring are bonded to amidic carbonyl groups within the same stack of chains and the other half are bonded to hydroxyl group between the adjacent stacks. The existence of this intersheet bonding is probably responsible for the stability of the α-chitin structure, specifically its inability to swell in water.

The β-chitin is characterized by a pararell arrangement of the polysaccharide chains (**Figure 1.3**). In this arrangement, there is no hydrogen bond between the adjacent chitin stacks. Thus, β-chitin is easily swollen by intercalation of water molecules between the stacks of the chitin chains. In this regard, it is interesting that β-chitin is found exclusively in aquatic organisms.[3] The differences between two forms are slight, however the α-form is more stable. The β-chitin can be converted to the α-chitin by treatment with anhydrous formic acid or strong nitric acid but no known means to date by which this transformation can be reversed.[4,5] The infrared spectra of α-chitin and β-chitin are also essentially similar. It is probable that α-chitin and β-chitin do not differ in any essential chemical manners, since both are readily hydrolyzed by chitinase from a number of sources.[5] The third form, γ-chitin, is a mixture of anitipararell and pararell arrangements of chitin chains.

Although chitin and chitosan are known to have very interesting physiological properties, but there is a doubt concerning their level of absorption in human intestine, their high molecular weights and highly viscous nature may restrict their *in-vivo* uses. Because most animal intestines, especially human gastrointestinal tract, do not possess enzyme such as chitinase and chitosanase which can directly degrade the β-glycosidic linkage in chitin and chitosan. Recently, studies have attracted interest to convert chitin and chitosan to their monomer and oligomers (**Figure 1.4**). The monomers and oligomers of chitin and chitosan have low viscosity due to their small molecular weights and short-chain lengths that allows them to be readily soluble in neutral aqueous solution and absorbed in the *in vivo* system.



α-Chitin

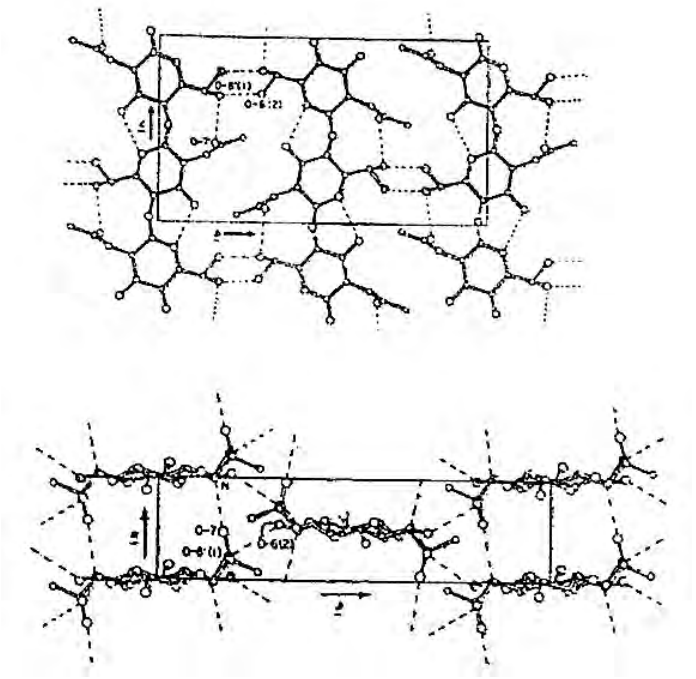
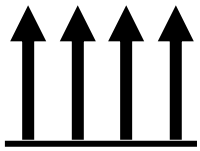


Figure 1.2 Diagrammatic illustration representing the antipararell and the X-ray crystal structure showing the hydrogen bond linkage between to C=OΛNH group of α-chitin.



β-Chitin

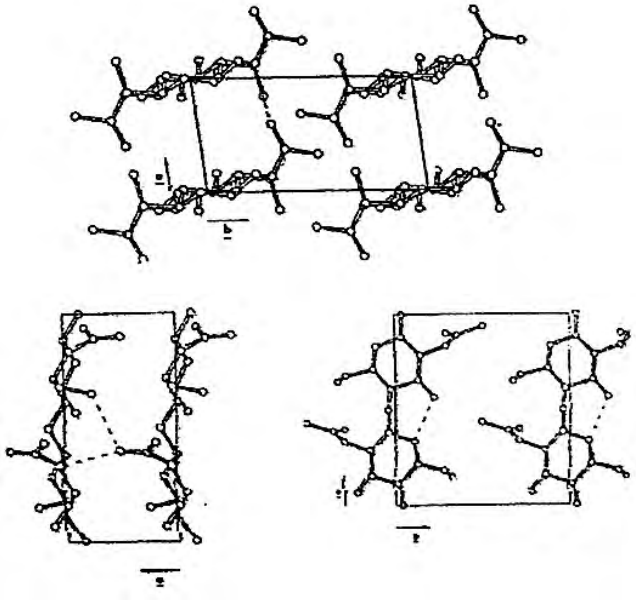


Figure 1.3 Diagrammatic illustration representing the antipararell and the X-ray crystal structure showing the hydrogen bond linkage between to C=OΛNH group of β-chitin.

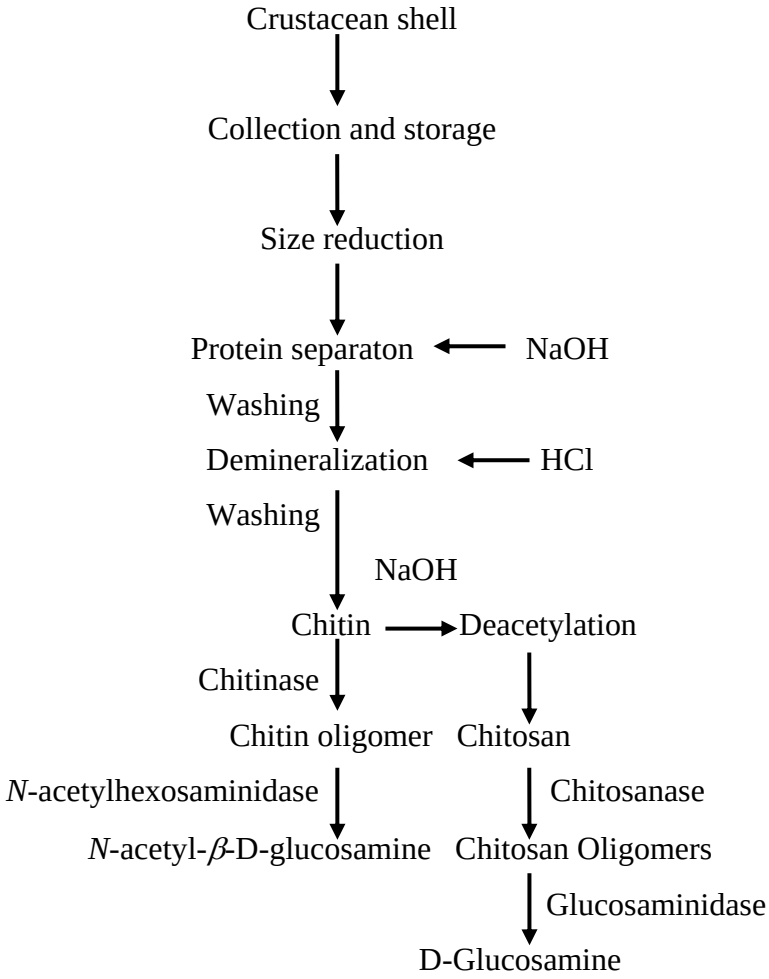


Figure 1.4 Simplified flowsheet for preparation of chitin, chitosan and their oligomers.

1.2 The applications of chitin and their derivatives.

Chitin is a by-product or a waste from crab, shrimp and squid processing industries. However, isolation and preparation of chitin from other marine invertebrate shells have taken place.[6,7] Recent efforts for the use of chitin and chitosan have intensified since efficient utilization of marine biomass resources has become an environmental priority. Applications of chitin and chitosan include a treatment of waste water and heavy metal adsorption in industry, immobilization of enzyme and cells, resin for chromatography, functional membrane in biotechnology, seed coating and animal feed in agriculture, artificial skin, absorbable surgical suture, controlled releasing material for pharmaceutical agents, and wound healing accelerator in the medical field.[6,8,9] Chitin and chitosan have also been developed as new physiological materials lately since possess antitumor activity by immuno-

enhancing, antibacterial activity, hypocholesterolemic activity, and antihypertensive action.[10,11]

Monomers and oligomers of chitin and chitosan have potential applications in the pharmaceutical industry, medical, agriculture, cosmetics and in chemistry as oligo-chitins have been proposed as anti-microbial agents, promoters of plant growth, elicitors of plant resistance, enhancers of the immune response and agents against malignant growth. The monomers, *N*-acetyl-D-glucosamine and D-glucosamine, are candidates as food supplements for the treatment of osteoarthritis[12] and biologically important synthesis building blocks (**Table 1.1**). GlcN and GlcNAc are precursors of the disaccharide units in glycosaminoglycans such as hyaluronic acid, chondroitin sulfate and keratin sulfate, which are necessary for natural repairing and maintaining healthy cartilage and joint function.[13,14] GlcNAc was a part of the makeup of the body tissues and blood vessels.[10,15]

In 1989, S. Hirano and N. Nagao[16] studied relationships between the degree of polymerization (DP) of chitosan and the degree of pathogen inhibition. They showed that chitosan oligomers (DP 2-8) as well as partially hydrolyzed chitosan with a low molecular weight possessed stronger growth inhibition than high molecular weight chitosan against several phytopathogens including *Fusarium oxyporum*, *Phomopsis fukushi*, and *Alternaria alternata*.

On the effects of water-soluble chitin and chitosan oligomers, K. Suzuki *et al*.[17] demonstrated that chitin hexamer, $(GlcNAc)_6$, possessed a strong candiacidal activity. A. Tokoro *et al*.[18] found that $(GlcNAc)_6$ exerted strong growth-inhibitory effect on *Listeria monocytogenes* by elevating the function of cellular immunity.

In 1996, S. Kobayashi and T. Kiyosoda[19] studied chitin, a mucopolysaccharide from invertebrates, which has recently been of great interest in numerous scientific and application fields as a multifunctional substance, activator for immune system, inhibitor of metastases of tumor cells, antibacterial substance, wound-healing materials, additives for cosmetics, drug carrier, health foods, biodegradable polymers, chelating polymer, *etc*.

In 2001, H. Sashiwa *et al*.[20] remarked for D-glucosamine and *N*-acetyl-D-glucosamine (GlcNAc) have attracted much attention owing to their therapeutic activity in osteoarthritis. They have also been evaluated as a food supplement and GlcNAc is more suitable than D-glucosamine for oral administration because of its sweet taste non toxic and water soluble.

In 2007, W. J. Jung *et al*.[21] reported a production of GlcNAc and *N*-acetyl chitooligosaccharides using multi-chitinolytic enzyme complex produced by *Paenibacillus illinoisensis* KJA-424. The effective production of GlcNAc and *N*-acetyl chitooligosaccharides suggested its potential use in industrial applications.

Table 1.1 Application of chitin, chitosan, their monomers and oligomers

Field	**Chitin and chitosan**	**Monomer and oligomers**
Food	Antimicrobial agents	Antimicrobial agents
	Preservative agents	Preservative agents
	Edible film	
Pharmaceutical	Antibacterial infection	Antibacterial infection
	Antitumor agents	Antitumor agents
	Immunopotential agents	Immunopotential agents
	Carrier for drug delivery system	
Medical	Accelerator for wound healing	Osteoarthritis and
	Artificial skin	Inflammatory
	Fiber for absorbable sutures	bowel disease treatment
Nutritional	Dietary fiber	Hypocholesterolemic agents
	Hypocholesterolemic agents	Calcium absorption accelerator
	Antihypertensive agents	*in vitro*
Biotechnological	Carrier for immobilized enzymes and cells	
	Porous beads for bioreactors	
	Resin for chromatography	
	Membrane materials	
Agricultural	Seed coating preparation	Activator of plant cells
	Activator of plant cells	Plant growth
Other	Coagulant for wastewater treatment	Chemistry building blocks
		Cosmetics materials
	Protein recovery preparation in food processing plants	
	Removal of heavy metal from wastewater	
	Cosmetics materials	

1.3 Preparation of *N*-acetyl-D-glucosamine and chitooligosaccharides

N-acetyl-D-glucosamine and D-glucosamine are monomers of chitin and chitosan, respectively. Chitooligosaccharides are the oligomers of either β-(1→4) linked *N*-acetyl-D-glucosamine or D-glucosamine units. Two distinct hydrolytic approaches, chemical hydrolysis and enzymatic hydrolysis, have been applied for preparation of the monomers and chitooligosaccharides from chitin and chitosan.

1.3.1 Chemical Hydrolysis

Chemical method for the preparation of GlcNAc, GlcN, and chitooligosaccharides mostly deals with acid hydrolysis **(Figure 1.5)**.[22-24] Recently, the series of chitooligosaccharides have become commercially available**.** They are usually prepared by hydrolysis of chitin and chitosan with concentrated hydrochloric acid, followed by extensive column chromatographic fractionation.[22] The conventional procedure for their preparation is as follow: 1) acid hydrolysis, 2) neutralization, 3) demineralization, 4) charcoal-celite column fractionation, 5) HPLC fractionation, and 6) lyophilization.[23]

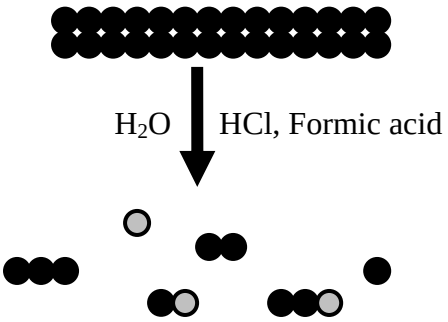


Figure 1.5 Acid hydrolysis of chitin

In 1964, S. Rupley[22] used concentrated hydrochloric acid to digest chitin for preparation a substrate for lysozyme assaying. Moreover, Horowitz *et al.*[24] explained that acid hydrolysis of chitosan with concentrated HCl also led to the production of chitosan oligomers with low degree of polymerization (DP) (monomer to trimer). However, such a simple method, using only concentrated hydrochloric acid associates with some inherent problems such as cost for purification of the products, environmental concerns, and a low yield of the specific product with many by-products.

In 1986, C. Bosso *et al.*[25] noted that fluorohydrolysis of chitin in anhydrous hydrogen fluoride (HF) led to chitin oligomers in almost quantitative yield and conditions can be conveniently monitored in order to optimize the preparation of specific oligomers ranging from 2 to 9 residues. The major products of chitin oligomers obtained are mainly dimer to tetramer and chitinoligomers (β-(1→6)-linked 2-acetamino-2-deoxy-D-glucosyl oligosaccharide) exclusively formed when the solutions of chitin were kept in HF at room temperature for over 10 hrs.

In 1993, K. Kurita *et al.*[26] suggested squid β-chitin as a starting material for simple acetolysis giving rise to the formation of *N*-acetyl chitooligosaccharide peracetates in high yields with good reproducibility.

1.3.2 Enzymatic hydrolysis of chitin and chitosan

Enzyme technology is an interdisciplinary field, and enzymes are routinely used in many environmental-friendly industrial sectors. With the advancement in biotechnology especially in the area of genetics, protein engineering, developments in bioinformatics, and the availability of sequence data have opened a new era of enzyme applications in many industrial processes.

In contrast to chemical hydrolysis, enzymatic hydrolysis of chitin and chitosan has several benefits to produce monomers and oligomers with milder reaction condition. Y. Uchida *et al.*[27] explained that the enzymatic hydrolysis was a useful method for the preparation of oligomers from chitin and chitosan because the yield of the specific products was usually greater in the enzymatic hydrolysis than in the acid hydrolysis.

Chitin is hydrolyzed at the β(1-4) glycosidic bond by both chitinase and lysozyme to *N*-acetyl-D-glucosamine (GlcNAc) **(Figure 1.6)**. There are two types of chitinase : endo-chitinase (E.C.3.2.1.14) which produces the *N*-acetylchitooligosaccharides containing *N*-acetylglucosamine at the reducing end and exo-chitinase (or chitobiase, or β-*N*-acetylhexosaminidase) (E.C.3.2.1.52) which hydrolyzes the chitin oligosaccharide from the nonreducing end to release a monomeric *N*-acetylglucosamine. An alternative pathway involves the deacetylation of chitin to chitosan, which is finally converted to glucosamine residues by the action of chitosanase (EC 3.2.1.132).[28]

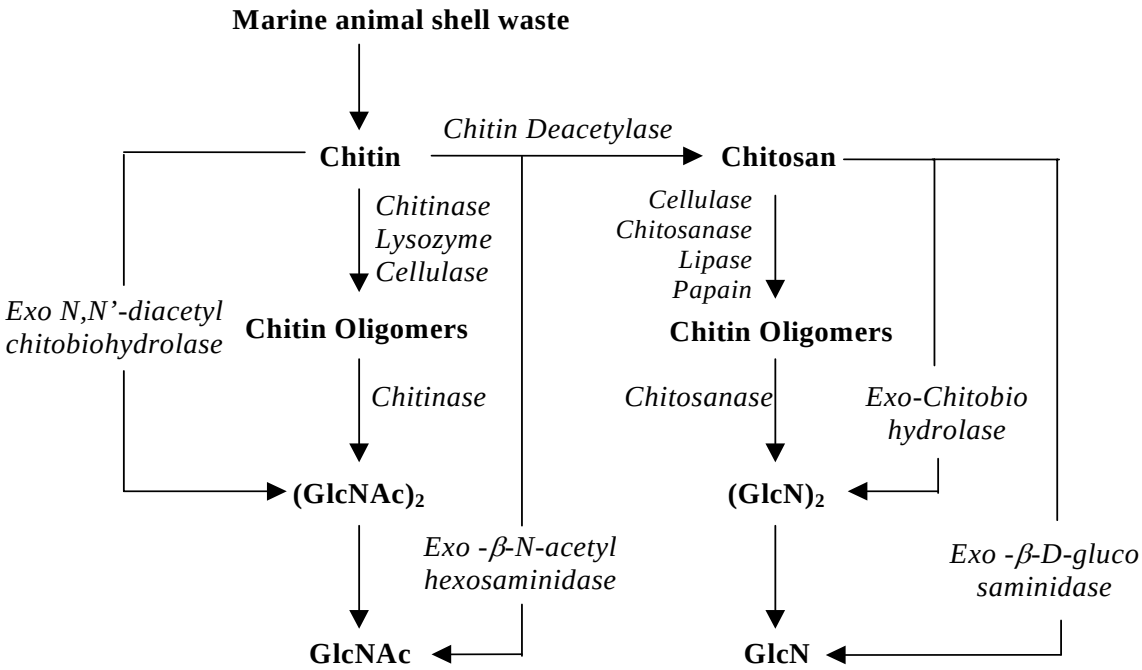


Figure 1.6 Pathway for the conversion of chitin and chitosan into their oligomers by enzymatic means.

Chitin and chitosan degrading enzymes

Based on amino acid sequence similarity, chitinolytic enzymes are grouped into families 18, 19, and 20 of glycosyl hydrolases.[29] Family 18 is diverse in evolutionary terms and contains chitinases from bacteria, fungi, viruses, animals, and some plant chitinases. Family 19 consists of plant chitinases (classes I, II, and IV) and some *Streptomyces* chitinases.[30] The chitinases of the two families, that is, 18 and 19, do not share amino acid sequence similarity. They have completely different 3-D structures and molecular mechanisms and are therefore likely to have evolved from different ancestors.[31] Family 20 includes the β-*N*-acetylhexosaminidases from bacteria, *Streptomycetes*, and humans.

Based on chitinase structure and amino acid sequence, chitinase are separated into different subfamilies, classes or groups. Bacterial chitinases are clearly separated into three major subfamilies, A, B, and C.[32] Subfamily A chitinases have the presence of a third domain corresponding to the insertion of an α+β fold region between the seventh and eighth (α/β)8 barrel. On the other hand, none of the chitinases in subfamilies A and B have this insertion. Several chitinolytic bacteria that possess chitinases belonging to different subfamilies like *Serratia* marcescens[31],

Bacillus circulans WL-12[33] , and *Streptomyces coelicolor* A3(2)[34] are reported.[28] Plant chitinases are divided in five different classes. Class I and Class II chitinases are similar in their catalytic domains. Class I chitinases have a chitin-binding domain. This domain is separated from the catalytic domain by hinge region. Class II chtinases lack both the chitin-binding domain and hinge region. Class III chitinases have higher homology to fungal chitinases than to other plant chitinase classes. Class IV chitinases are similar to class I chitinases but they are smaller in size due to certain deletions. Class V chitinases show some homology with bacterial exochitinases.[35,36]

In general, chitinolytic enzymes can be divided into three categories: exochitinases, demonstrating activity only for the non-reducing end of the chitin chain; endochitinases, which hydrolyze internal β-1,4-glycoside; and β-*N*-acetylglucosaminidase, which cleaves GlcNAc units sequentially from the non-reducing end of the substrate.[37,38] Enzymatic cleavages by chitinase are random in nature, occurring randomly at internal locations over the entire length of the polymer, resulting in the production of soluble, low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose, and chitobiose, with the smallest oligosaccharides being predominant.[39,40]

Different organisms produce chitinase for different purposes based on their own physiology and use. In plants, they appear to play a defensive role against pathogenic or pestiferous organisms.[41] In arthropods, chitinases are involved in cuticle turnover and mobilization and in nutrient digestion.[37,39,40] The role of chitinase in vertebrates is uncertain; however, it may be involved in resistance to fungal infections.[42] For fungi, chitinases apparently help to degrade and mobilize organic matter and probably to antagonize the growth of competitors.[43] In yeasts, chitinases are important for cell separation.[44,45]

Chitinase (EC 3.2.1.14, glycosylhydrolase)

Chitinases hydrolyze β-(1,4)-glycosidic linkage bond randomly within the polymeric chitin chain giving a mixture of *N*-acetylchitooligosaccharide including *N*,*N'*-diacetylchitobiose as a major product and may be with *N*-acetyl-D-glucosamine **(Figure 1.7).** [46]

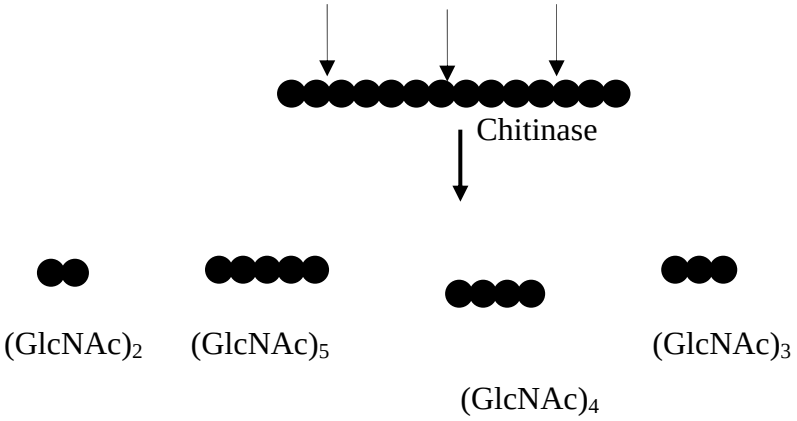


Figure 1.7 The action of chitinase on chitin and its product
●GlcNAc (*N*-acetyl-D-glucosamine) and ○ GlcN (D- Glucosamine)

β-*N*-acetylhexosaminidase (EC 3. 2.1.52, Glycosylhydrolase)

The β-*N*-acetylhexosaminidase (Chitobiase or *N*-acetylglucosaminohydrolase) is the enzyme which hydrolyses terminal, non-reducing GlcNAc residues in chitobiose and higher chitooligosaccharides
(**Figure 1.8)**.[32,47]

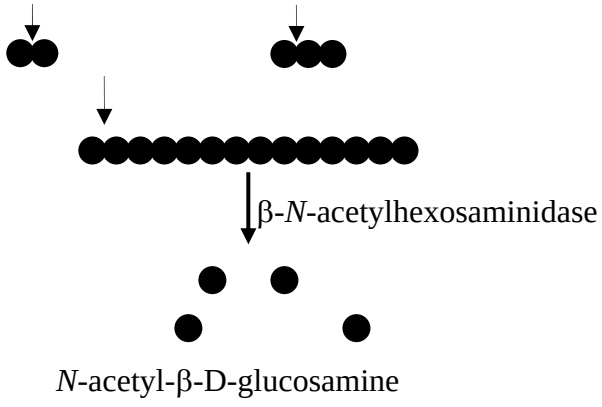


Figure 1.8 The action of chitobiase on chitin and its product

Chitosanase (EC 3.2.1.132, Glycosylhydrolase)

Chitosanases hydrolyzes β-(1,4)-glycosidic linkage bond randomly within the polymeric chitin giving a mixture of chitooligosaccharides as products and may be with D-glucosamine. Chitosanases are found primarily in the families of glycosylhydrolases. Chitosanase is the enzyme which hydrolyses polymer chain of chitin at the GlcNAc-GlcN or GlcN-GlcNAc (**Figure 1.9**)

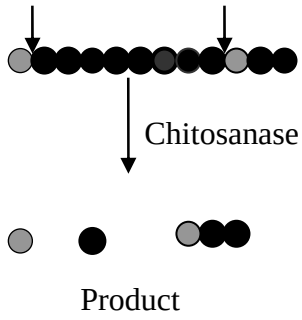


Figure 1.9 The action of chitosanase on chitin and its product
● GlcNAc (*N*-acetyl-D-glucosamine) and ○ GlcN (D-Glucosamine)

Enzymatic preparation of GlcNAc and its oligomers

In 1969, J. Monreal and E. T. Reese[48] reported that *Aspergillus fumigatus* QM 45 could produce chitinase in a medium containing chitin. The maximum chitinase activity was obtained when the fungus was grown in a medium containing 2.0 % chitin at an initial pH 4.5 and ambient temperature of 30 ^{o}C.

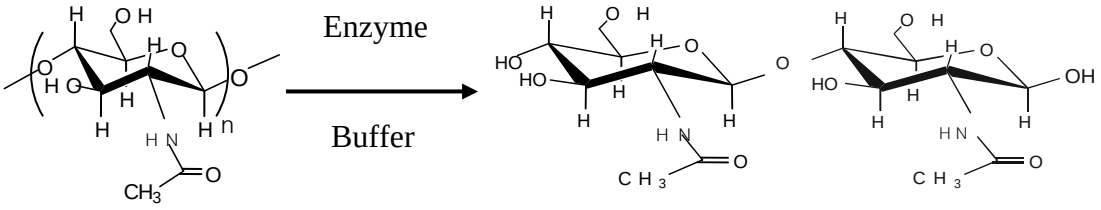
In 1992, S. L. Wang *et al*.[49] reported that *Pseudomonas aeruginosa* was the most potential strain for alkali-tolerant chitinolytic bateria. They showed that maximum chitinase activity could be obtained when the strain was grown aerobically in a medium containing chitinous compound from shrimp and crab shell. The optimum pH and temperature of the enzyme reaction were 7 and 40 ^{o}C and it was stable at pH from 5 to 10 under 60 ^{o}C. *Pseudomonas stutzeri* YPL-1 was reported to produce extracellular chitinase as well as beta-1,3-glucanase which were key enzymes in the decomposition of fungal hyphal cell wall.

In 1993, H. Tsujibo *et al*.[50] found that *Streptomyces thermoviolaceus* OPC-520 produced a thermostable chitinase when it was grown in medium containing colloidal chitin at 50 ^{o}C. The purified chitinase had optimum temperature between 70 ^{o}C and 80 ^{o}C. The optimum pH was 8.0-9.0. Moreover, it was found to be stable in a

wide range of pH and more than 80 % of activity still remained in the pH range between 4.0 to 12.0.

In 1994 S. Aiba[51] suggested that the hydrolyzed sites cannot be regulated by the enzyme in the case of degradation of chitin by chitinase. If chitosan is used as a substrate in a homogeneous state, regulation of the hydrolyzed sites is possible. Chitinase has a specific recognition of GlcNAc residues. When 20% acetylated chitosan was hydrolyzed by *Streptomyces griseus* chitinase for seven days followed by reacetylation with acetic anhydride, the yields of $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$ were 23.5, 25.5, 19.6, and 12.3%, respectively.

In 1997, S. Kobayashi *et al.*[52] reported a study on enzymatic transglycosylation for production of higher oligomers, such as hexamer and heptamer from lower oligomers. *N*, *N'*-diacetylchitobiose was prepared by combining a sugar oxazoline derivative as a glycosyl donor and *N*-acetyl-D-glucosamine as a glycosyl acceptor using chitinase from *Bacillus* sp., a chitin hydrolytic enzyme **(Figure 1.10).**



Chitin *N*, *N'*-diacetylchitobiose

Figure 1.10 Preparation of $(GlcNAc)_2$ by enzymatic transglycosylation.

In 1999, C. A. Haynes *et al.*[53] reported the process for the production of *N*-acetyl-D-glucosamine and chitobiose by hydrolysis of chitin in a two stage reactor. The two–stage reactor comprised two sequential reactors in which the first reactor contained a packed-bed of chitin-containing solid through which an enzyme-containing mobile phase passed and wherein the second reactor was a stirred tank containing a solid-free aqueous solution which was catalyzed by one or more chitobiose degrading enzymes.

In 2001, X. Guoqing *et al.*[54] reported the isolation of extracellular chitinase from *Aspergillus fumigatus* by using ammonium sulfate precipitation followed by DEAE-cellulose chromatography and preparative PAGE. The enzyme was most active at pH 5.0 and 60°C, and was inhibited strongly by Hg^{2+}, Pb^{2+}, Ag^{+}, Fe^{2+}, Mn^{2+}

and Zn^{2+}. The enzyme was stable over a broad pH range 4-8 and below 45 °C. Analysis of the hydrolysis product showed that the enzyme has both endo- and exo-hydrolytic activities.

There are approaches of using the commercially crude enzymes without purification for preparation of the monomer and oligomers of chitin and chitosan. H. Sashiwa *et al*.[55] reported that crude enzyme has some advantage to produce the GlcNAc owing to their low cost and their inclusion of both endo- and exo-type chitinases. These researchers can hydrolyze β-chitin to produce the GlcNAc with high yield (76%) in 8 days when used crude enzyme from Cellulase *Tricoderma viride*. H. Sashiwa *et al.*[56] also reported a digestion of α-chitin with crude enzyme from *Aeromonas hydrophila* H-2330 to give GlcNAc in 77% yield. In addition, R. Pichyangkura *et al*.[57] used crude chitinase form *Burkholderia cepacia* TU09 and *Bacillus lichenniformis* SK-1 to digest the α- and β-chitin powder to give GlcNAc in high yield (>70%). M. Sukwattanasinitt *et al*.[58] studied the utilization of commercial non-chitinase enzyms form fungi to prepare GlcNAc. They found that by using enzyme combination technique, 64% of GlcNAc was obtained within only 4 days with less enzyme used that the results were also inveterated by H. Sashiwa *et al.*[59]

In 2002, N. Rattanakit and T. Tachiki[60] study the optimum condition for chitinase production from *Aspergillus sp*. by isolating basal medium containing ammonium sulphate. The highest yield of enzyme was obtained after 11 days of incubation at 37 °C. The chitinase activity was 110.9 mU/mL and the analysis by ion-exchange column chromatography suggested the presence of at least two chitinolytic enzymes and one *p*-nitrophenyl β-D-*N*-acetylglucosaminide-hydrolyzing enzyme in an extract of the solid-state culture.

In 2005, J. H. Kuk *et al*.[61] reported the production of GlcNAc from chitin by *Aeromonas* sp. GJ-18 crude enzyme. TLC and HPLC analysis revealed that, below 45°C, the product was GlcNAc with a small amount of $(GlcNAc)_2$ and $(GlcNAc)_3$, whereas above 50 °C the major product was $(GlcNAc)_2$. When swollen chitin (100 mg) was incubated with crude enzyme (10 U) at 40 °C, chitin was hydrolyzed to 83.0 and 94.9% yield of GlcNAc within 5 and 9 days, respectively.

In 2006, W. J. Jung *et al*.[62] reported a purification and characterization of an extracellular 104 kDa exo-*β*-D-glucosaminidase from the culture supernatant of *Aspergillus fumigatus* S-26, which showed exceptionally strong chitosanolytic

enzyme activity. The purified enzyme showed optimum pH of 3.0–6.0 and optimum temperature of 50–60 °C. The enzyme was stable between pH 2.0 and 10.0 at 35 °C. The exo-β-D-glucosaminidase was severely inactivated by Cu^{2+} and Hg^{2+} at 10 mM. The enzyme did not degrade chitin, cellulose, and starch. The exo-β-D-glucosaminidase liberated GlcN from chitosan, and GlcN – $(GlcN)_6$ shortened oligomers from $(GlcN)_{2–7}$. This exo-β-D-glucosaminidase exhibited transglycosylation activity.

In 2006, J. H. Kuk *et al.*[63] prepared $(GlcNAc)_2$ from chitin as a major hydrolytic product by controlling the ratio of β-*N*-acetylglucosaminidase to *N,N´*-diacetylchitobiohydrolase activities in the crude enzyme preparation of *Aeromonas* sp. GJ-18. When the enzyme preparation was pre-incubated at 50 °C, β-*N*-acetylglucosa minidase was nearly inactivated, while the *N,N´*-diacetylchitobio hydrolase was still active. Typically, after 7 days of incubation with the substrate chitin, 78.9 and 56.6% of *N,N´*-diacetylchitobiose yields were obtained from swollen α-chitin and powdered β-chitin, respectively, with enzyme preparations that had been pretreated at 50 °C for 60 min.

In the development process for efficient enzymatic hydrolysis of chitin and chitosan, an immobilized enzyme was employed for a continuous production of oligosaccharides. Y. J. Jeon and S. K. Kim[64] also applied an ultra-filtration membrane in enzymatic reactor system for continuous preparation of chitosan oligomers. In addition, K. Matsuoka *et al.*[65] used a dialysis technique in the preparation of *N,N'*-diacetylchitobiose by continuous enzymatic degradation of colliodal chitin with chitinase from *Streptomyces griseus* and the method had potential to be used for large-scale industrial production.

In 2001, N. N. Nawani and B. P. Kapadnis[66] study single step technique of gel filtration was developed for the purification of chitinase from *Serratia marcescens* NK1. chitinase was purified to homogeneity by gel filtration chromatography with 9.2% recovery. The enzyme had a pH optimum of 6.2 and a temperature optimum of 47 °C. It was stable in a wide pH range of 3.0 to 10.0, retaining 60% activity at pH 3.0 and 65% activity at pH 10.5. It retained 70% activity at 28 °C after 72 h and nearly 50% activity at 50 °C up to 24 h.

In 2007, P. M. Kao *et al.*[67] study effects of shear stress and mass transfer on chitinase production by *Paenibacillus* sp. CHE-N1 was carried out in a 5 L stirred-

tank bioreactor. The effects of operation variables including agitation rates, aeration rates and pH values, on the cell growth and chitinase activity yield were evaluated. When the pH was controlled at a constant (6.0-9.0) during the cultivation, lower chitinase production was obtained. The operation conditions at an aeration rate of 3 vvm, an agitation rate of 200 rpm and without pH control could yield an optimal chitinase activity level of 11.8 U/mL.

Chitinases, which hydrolyze chitin, occur in a wide range of organisms including viruses, bacteria, fungi, insects, higher plants, and animals.[68] The roles of chitinases in these organisms are diverse. In vertebrates, chitinases are usually part of the digestive tract. In insects and crustaceans, chitinases are associated with the need for partial degradation of old cuticle. Chitinases have been implicated in plant resistance against fungal pathogens because of their inducible nature and antifungal activities in vitro.[69] Chitinase in fungi is thought to have autolytic, nutritional, and morphogenetic roles. In viruses, chitinases are involved in pathogenesis.[70]

1.4 Microbial

1.4.1 *Aeromonas hydrophila*

Aeromonas hydrophila is a heterotrophic, gram-negative bacterium, mainly found in areas with a warm climate, especially in fresh and marine water. It is the most well known of the six species of *Aeromonas*. It can digest materials such as gelatin, and hemoglobin. It is a very resistant bacterium and very hard to be killed. It can survive either in aerobic or anaerobic environments and resistant to chlorine and refrigeration. Its morphology is straight rod with rounded ends (bacilli to coccibacilli shape) usually from 0.3 to 1 micrometer in width, and 1 to 3 micrometers in length. *Aeromonas hydrophila* does not form endospores, and can grow in temperatures as low as four degrees Celsius. These bacteria are motile by a polar flagella. *Aeromonas hydrophila* is toxic to many organisms. When it enters the body of its victim, it travels through the bloodstream to the first available organ. It produces Aerolysin Cytotoxic Enterotoxin (ACT), a toxin that can cause tissue damage. *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas sobria* are all considered to be “opportunistic pathogens,” meaning they only infect hosts with weakened immune responses. Though *Aeromonas hydrophila* is considered a pathogenic bacterium, scientists have not been able to prove that it actually cause the diseases it is associated with. It is

believed that this bacterium aids in the infection of diseases, but do not cause the diseases themselves.

Taxonomic Classification

Kingdom:	Proteobacteria
Phylum:	Gammaproteobacteria
Class:	Aeromonadales
Genus:	Aeromonas
Species:	A.hydrophila

1.4.2 *Aspergillus fumigatus*

A. fumigatus is a saprotrophic fungus that is widespread in nature, typically found in soil and decaying organic matter such as compost heaps, where it plays an essential role in carbon and nitrogen recycling. Colonies of the fungus produce thousands of minute grey-green conidia (2-3 μm) from conidiophores that readily become airborne. Until recently *Aspergillus fumigatus* was only thought to reproduce asexually, as neither mating nor meiosis had ever been observed in the fungus.

The fungus is capable of growth at 37°C (human body temperature), but can grow at temperatures up to 50°C, with conidia surviving at 70°C - conditions it regularly encounters in self-heating compost heaps. Its spores are ubiquitous in the atmosphere and it is estimated that everybody inhales several hundred spores each day; typically, however, these are quickly eliminated by the immune system in healthy individuals. In immuno-compromised individuals such as transplant patients and people with AIDS or leukaemia the fungus is capable of becoming pathogenic, over-running the hosts weakened defenses and causing a range of diseases generally termed aspergillosis.

Taxonomic Classification

Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Eurotiomycetes
Order:	Eurotiales
Family:	Trichocomaceae
Genus:	Aspergillus
Species:	A.fumigatus

Fungal chitinolytic enzymes could be involved in the growth of the fungus itself. All types of chitinolytic enzyme activities are found in fungi, chitinase, β-*N*-acetyl-D-glucosaminidase and chitosanase. Chitinase can be localized within the cell as soluble cytoplasmic protein, sequestered in microsomes or lyosomal vacuoles or bound to the membrane or cell wall. It can also be found secreted extracellularly.[71]

1.5 Aim of thesis

N-acetyl-D-glucosamine (GlcNAc) has attracted much attention owing to their therapeutic activity in osteoarthritis.[12,53] They have also been evaluated as a food supplement. Comparing to glucosamine salts, GlcNAc is more suitable for oral administration because of its sweet taste. GlcNHCl is mainly produced by acid hydrolysis of chitin in concentrated hydrochloric acid (HCl), which post technical and environmental concerns for acidic waste and low yield (below 65 %). Previous study, squid pen chitin was hydrolyzed by using the crude enzyme from *Aspergillus fumigatus*. After hydrolysis, the solid crude product was analyzed for sugar content by HPLC. The analysis of the crude product showed that the hydrolysis of chitin (6 g) with *Aspergillus fumigatus* enzyme (22 U/g chitin) at pH 3 and 40 °C for 2 days gave 72% yield of GlcNAc with 60% purity. This thesis focused on the suitable condition for effective production of crude chitinolytic enzyme from fungi and its potential use and scale up to 100 g chitin for preparation of *N*-acetyl-D-glucosamine.

CHAPTER II

EXPERIMENTS

Instruments and apparatus

1. High performance liquid chromatograph (HPLC): pump (Waters 600E), autosampler (Waters 917), and diode array detector (Waters 996)
2. Perparative HPLC
3. LC/MS/MS
4. HPLC reverse phase column (Asahipak NH_2P-50, Shodex, Japan)
5. UV-Visible spectrophotometer (Jenway 600)
6. UV-Visible spectrophotometer (Beckman DU 650)
7. pH meter (pHScan3+, Eutech Instruments)
8. Hot-plated magnetic stirrer (Coning)
9. Syringe filter (0.45 μm PTFE, Alltech)
10. Pipette man (P20, P200, and P5000, Gilson)
11. Pipette man (Le100, and Le1000, Nichiryo)
12. Solvent membrane filters (0.45 μm cellulose, Millipore)
13. Freeze-dryer (Freezone 77520, Benchtop, Labconco)
14. Centrifuge (Centuar 2, Sanyo)
15. Ultra-centrifugal mill (Retsch, ZM1000, Germany)
16. Electrical food blender (Cucina HR1791/6, Philips)
17. CHON/S analyser (PE2400 series II, Perkin Elmer)
18. Seemless cellulose tubing (M_w 12000, Wako Chemicals Inc.)
19. Locking dialysis membrane clamps (Membrane Filtration Product Inc.)
20. Vial-capped 1.5mL (MCT-150-C, Axygen)

Chemicals

1. Squid pen β-chitin (Ta-Ming Enterprises, Thailand)
2. *N*-acetyl-D-glucosamine (Fluka Chemicals, Ltd., Switzerland)
3. *N,N'*-diacetylchitobiose (Seikagaku Corporation Co.Ltd., Japan)
4. Glacial acetic acid, analar grade (Merck, Germany)
5. Sodium chloride, analar grade (Merck, Germany)
6. Sodium hydroxide, analar grade (Merck, Germany)
7. Citric acid, analar grade (Merck, Germany)
8. Sodium hydrogen phosphate (Fluka Chemicals, Ltd., Switzerland)
9. Sodium carbonate, analar grade (Carlo Erba, Italy)
10. Hydrochloric acid, Analar grade (Merck, Germany)
11. Potassium hexaferrocyanate, (Merck, Germany)
12. Acetronitrile, chromatography grade (Merck, Germany)
13. Acetyl chloride, analar grade (Aldrich, Germany)
14. Sodium deoxycholate (Merck, Germany)
15. Copper sulphate (Fluka Chemicals, Ltd., Switzerland).
16. Activated charcoal (Fluka Chemicals, Ltd., Switzerland)
17. Absolute ethanol (Merck, Germany)

General procedure

2.1 Preparation of substrates

2.1.1 Squid pen chitin 100 μm

The squid pen chitin (β-chitin) was purchased from Ta-ming enterprise Co., Ltd. The chitin was ground by ultra-centrifugal mill (Retsch, ZM1000, Germany) at the Metallurgy and Materials Science Research Institute, Chulalongkorn University to give the puffy fibrous chitin with diameter about 50 μm and length about 100 μm.

2.1.2 Colloidal chitin

The concentrated hydrochloric acid was gradually added to squid pen chitin (50 g) in a beaker under vigorous stirring. The added amount of hydrochloric acid (~50 mL) was paused when thick viscous slurry was obtained. The viscous slurry was poured as a thin stream into 1 L of a vigorously stirred ice-water mixture forming instantly a fine precipitate. The slurry was kept overnight in a refrigerator at 4 ^{o}C. Afterward the slurry was adjusted to pH 3.5 by 2 M NaOH. Colloidal chitin was separated by centrifugation at 3500 rpm for 15 minutes. The colloidal chitin was washed with DI-water and then it was centrifuged again in order to get the colloidal chitin. The concentration of colloidal chitin was dried by vacuum freeze dry and weighted after it dried.

2.1.3 Swollen chitin

The squid pen chitin (20 g) from Ta-ming enterprise Co., Ltd. was milled using sieve (0.5 mm) to give fibrous chitin and was ground in a food blender with DI-water (10 mL). The addition of DI-water (10 mL) was repeated when the chitin absorbed water and the grinding became inefficient. When the chitin was saturated with the absorbed water, the grinding could be continued efficiently for 20 more minutes.

2.2 Preparation of crude chitinase from microbial

2.2.1 Preparation of Luria-Bertani medium (LB)

The Luria-Bertani broth medium (LB broth) was contained tryptone (1% w/v), yeast extract (0.5% w/v), sodium chloride (1% w/v) to dissolved in water (1 L). Then added agar powder (1.5% w/v) into LB broth and heated it to dissolution to prepare the LB agar. The solution was sterilized under autoclave at 121^{o}C for 15 minutes.

2.2.2 Preparation of potato dextrose agar (PDA)

The potato dextrose broth (PDB) was used for pre-enrichment of freeze dried fungi. The potato dextrose agar (PDA) was used for cultivating of fungi used for enzyme preparation. The PDB was prepared by boiling raw potato cubes ($1\times1\times1$ cm^3, 20 g) in water (1 L) until cooked. After removing of potatoes from the aqueous liquid, the pH of the solution was adjusted with hydrochloric acid and sodium hydroxide to pH 5. Then added agar powder (2% w/v) into PDB and heated it to dissolution to prepare the PDA. The solution was sterilized under autoclave at 121^oC for 15 minutes. Finally, poured PDA into culture plates (20 mL/plate) for the purpose of preparation of cultivation.

2.2.3 Preparation of colloidal chitin minimum medium (CCMM)

Colloidal chitin minimum medium was used for the enzyme production from fungi. The colloidal chitin minimum medium contained KH_2PO_4 (0.1% w/v), $MgSO_4.7H_2O$ (0.05% w/v), $FeSO_4.7H_2O$ (0.005% w/v), urea (0.3% w/v) as a nitrogen source and colloidal chitin (2% dry weight) as a carbon source. These ingredients were mixed in DI-water (1 L). The mixture was adjusted to pH 3.5-5.0 by 1 N NaOH or 1 N CH_3COOH and sterilized under autoclave at 121 ^oC for 15 minutes.

2.2.4 Preparation of crude enzyme from bacteria

The *Aeromonas hydrophila* MOK-1 was received from Assist. Prof. Dr. Rath Pichyangkura, Department of Biochemistry, Faculty of Science, Chulalongkorn University. Bacteria can be picked from LB agar slant culture by scratching the sterile loop across the surface of the culture medium or they can be picked from a liquid culture by immersing the loop in it. In either cases, the loop is used to streak out the cells on the surface of a 1.5% agar/LB plate. Colonies were visible after 12-16 hours of growth at 37°C. A single colony was picked from a streaked plate and inoculated in a 5 or 10 mL LB aliquot. The culture was grown overnight at 37°C with shaking. For chitinolytic production, inoculum culture was added to the CCMM (250ml) at 5% v/v, which contained 1% milled β-chitin or 1% blend α-chitin in 500 mL flask. The culture medium was incubated at 37°C on a rotary shaker at 250 rpm for 24 hours and the sample were taken at 0, 3, 6, 9, 12, 15, 18, 21 and 24 hours for determination of growth curve and enzyme activity. The samples were diluted by 10-10^6 times in water. The diluted samples (0.1 mL) were spread on the LB agar plate and incubated at 37°C for 24 hours, cell growth were counted by plate count technique. Crude

enzyme was separated from fungal cells and solid media by centrifugation at 3,500 rpm for 15 minutes, filtered and analysed for chitinolytic activity by Schale's method**.**

2.2.5 Preparation of crude enzyme from fungi

The fungi *Aspergillus fumigatus*, that was used in this work received from Assoc. Prof. Dr. *Hunsa* Punnapayak, Department of *Botany*, Faculty of Science, *Chulalongkorn* University. Fungal fibrous were cultured on PDA for 2 days or until the growth fungi was observed. The PDA (diameter 0.5 cm × 7 pieces/100 mL) containing fungal filament was inoculated into the CCMM and cultured at 40 ^{o}C on a rotary shaker at 150 rpm for 9 days in flask 250mL and 500mL which contained CCMM 100mL and 200 mL respectively. Colloidal chitin (2% dry weight) was added into the CCMM and the samples were taken at 3, 5, 7, and 9 days. Crude enzyme was separated from fungal cells and solid media by centrifugation at 3500 rpm for 15 minutes, filtrate and analyses of chitinolytic activity by Schale's method**.**

2.3 Protein assaying

2.3.1 Preparation of calibration curve

The stock solution A of standard protein was prepared by dissolving weighed bovine serum albumin (BSA, 5 mg) with DI water (50 mL), and stock solution B was prepared by dissolving weighed bovine serum albumin (BSA, 10 mg) with DI water (50 mL).

Table 2.1 Preparation of BSA standard solutions.

Standard No.	Volumn pipetted μL (stock)	DI-water (μL)	Conc. (μg/mL)
1	200 (A)	800	20
2	400 (A)	600	40
3	600 (A)	400	60
4	800 (A)	200	80
5	1000 (A)	0	100
6	600 (B)	400	120
7	700 (B)	300	140
8	800 (B)	200	160
9	900 (B)	100	180
10	1000 (B)	0	200

The designated amounts of the standard solutions (Table 2.1) were pipetted into clean test tubes. The coomassie bilant blue G 250 reagent (5 mL) was added. The mixture was stirred and waited for 5 min. The absorbance of the solutions was measured by UV-Vis spectrophotometer at 595 nm. The standard curve was obtained by plotting the absorbance in y-axis against the concentration of protein (mg/mL) in x-axis.

2.3.2 Bradford reagent

The coomassie biliant blue dye reagent (100 mg) was dissolved in 95% ethanol (50 mL) and phosphoric acid (85% w/v 100 mL) was added. The reagent was diluted to 1 liter in DI-water when the dye has completely dissolved, and filtered through Whatman #1 paper just before use.

2.3.3 Determination of the protein amount in the enzymes

The enzyme solution (0.1 mL) was pipetted into a clean test tube and Bradford reagent (5 mL) was added. The mixture was stirred and waited for 5 min. before the absorbance was measured at 595 nm by UV-Vis spectrophotometer. The amount of protein was calculated by comparison the absorbance with the standard curve of bovine serum albumin prepared in the previous section.

2.4 Chitinase activity assaying

2.4.1 Preparation of calibration curve

Stock solutions A and B were prepared by dissolving GlcNAc (10.5 mg and 10.7 mg) with DI-water 10 and 20mL, respectively. The stock solution A (4.7466 mM) and stock solution B (2.4185 mM) were diluted to produce standard solutions (1-9) (**Table 2.2**).

Table 2.2 GlcNAc used and concentration of standard solution used for making calibration line in enzyme assaying

Standard No.	Volume (μL) (pipetted stock)	Concentration (μM)	Amount of GlcNAc (μmole)
1	31(B)	49.98	74.97
2	62 (B)	99.96	149.94
3	93 (B)	149.95	224.93
4	124 (B)	199.92	299.88
5	79 (A)	249.98	374.97
6	94 (A)	297.45	446.18
7	110 (A)	348.08	522.12
8	126 (A)	398.71	598.07
9	142 (A)	449.34	674.01

Each standard was added buffer 300 mL pH 4 and the volume was adjusted to 1.5 mL with DI-water. The $K_3Fe(CN)_6$ solution (2 mL of 0.05% w/v in 1 M Na_2CO_3) was pipetted into standard and control tubes, then the mixture was immersed in hot water for 15 min. After cooling to room temperature, The UV-Vis absorbance of standard solutions was measured at 420 nm. The standard curve was obtained by plotting ΔA (Absorbance of the control tube - Absorbance of the standard tube) in Y-axis against the amount of GlcNAc (μmole) in X-axis.

2.4.2 Measurement of chitinolytic activity of the enzyme

The chitinolytic activity of the interested enzyme was assayed by measuring the amount of reducing sugars, equivalent to GlcNAc, produced in a digestion of colloidal chitin with the enzyme according to Schale's Method.

The enzyme (0.025 mL) was pipetted into a clean test tube. Colloidal chitin (0.05 mL, 50mg/mL) was added into the tube. Buffer (1 M, 0.15 mL) which is suitable for each enzyme was added and the reaction volume was adjusted to 1.5 mL by DI-water. The solution was incubated at 45 ^oC for 30 minutes. After the incubation period, the $K_3Fe(CN)_6$ solution (2 mL of 0.1% w/v in 1 M Na_2CO_3) was added into the test tube. The mixture was heated in boiling water for 15 minutes. After cooling at room temperature, small particles were removed from the mixture by centrifugation at 3,500 rpm for 15 minutes.

For the control experiment deactivated enzyme, by heating in boiling water (15 min), was used in place of the active enzyme. The quantity of the reducing sugars was measured by a UV-Vis spectrometer at 420 nm using DI-water as a blank. Both assays and controls were performed in three replicates and the average absorbance (A) was used in the activity determination. The activity unit (U) per volume (mL) of the serum was calculated from the difference of the absorbance (ΔA) between the assay (A_1) and the control (A_0) according to the following equation.

$$\text{Activity (U)} = \mu\text{mole of reducing sugar} / (\text{min} \times \text{mL of serum})$$
$$= (\Delta A/0.207) / (30 \times 0.025)$$

One unit (U) of enzyme activity was defined as the amount of enzyme able to produce reducing sugar equivalent to 1 μmole of GlcNAc per min.

The factor of 0.207 was the slope of the calibration line using GlcNAc as a standard reducing sugar (**section 2.3.1**, **Figure A2**). In this equation, the incubation time was 30 min.

2.5 Product analysis by HPLC

2.5.1 Preparation of calibration curve

Preparation of stock solution I

GlcNAc (2.5 mg) and $(GlcNAc)_2$ (1.9 mg) were dissolved in Milli-Q water (1.0 mL) in each vial**.** 1mL of each solution was mixed together. We got the stock solution which contained 5.65 mM of GlcNAc and 2.24 mM of $(GlcNAc)_2$

Preparation of stock solution II

GlcNAc (3.5 mg) and $(GlcNAc)_2$ (2.5 mg) were dissolved in Milli-Q water (1.0 mL) in each vial**.** 1mL of each solution was mixed together. We got the stock solution which contained 7.91 mM of GlcNAc and 2.95 mM of $(GlcNAc)_2$ Six standard solutions (A, B, C, D, E, and F) were prepared by dilution of I and II.

Table 2.3 Preparation of standard solutions of GlcNAc and $(GlcNAc)_2$

Standard solution	[GlcNAc] (mM)	[$(GlcNAc)_2$] (mM)	Preparation
A	0.095	0.005	Stock solution I (0.03 mL) + H_2O (1.47 mL)
B	0.203	0.094	Stock solution II (0.05 mL) + H_2O (1.45 mL)
C	0.475	0.247	Stock solution I (0.10 mL) + H_2O (0.90 mL)
D	1.020	0.472	Stock solution II (0.20 mL) + H_2O (1.00 mL)
E	1.900	0.988	Stock solution I (0.40 mL) + H_2O (0.60 mL)
F	3.050	1.420	Stock solution II (0.50 mL) + H_2O (0.50 mL)

Each standard (0.300 mL) was mixed with acetonitrile (0.700 mL) and filtered through a 45 μm PTFE membrane filter. Standard solutions (20 μL) were injected into HPLC (pump Waters 600E, Autosampler Waters 917 and diode array detector waters 996) and detected at 210 nm. The mobile phase was acetonitrile:water (70:30) at a flow rate of 1.0mL/min. The Asahipak NH_2P-50, Shodex (Japan) column was used as the stationary phase. The calibration curve was obtained by plotting the peak area (mV*sec) as the Y-axis against the concentration of GlcNAc (mM) and $(GlcNAc)_2$ (mM) as the X-axis.

2.5.2 Analysis of products in hydrolysates

After the designated time, each hydrolysate (1 mL) was pipetted into a 2 mL plastic capped vial. The mixture was boiled for 15 min and centrifuged at 2,000 rpm for 20 minutes. The supernatant (100 μL) was diluted by Milli-Q water (900 μL). The solution (0.300 mL) was pipetted out and mixed with acetronitrile (0.700 mL). The solution was filtered though a 0.45 μm PTFE filter before injecting into the HPLC. The same instrument system and condition as previously described in the preparation of calibration line (**section 2.4.1**) was used in the analysis of the products. The GlcNAc and $(GlcNAc)_2$ were detected at a retention time of 5.6 and 6.5 min respectively. The peak areas were used to calculate the amount of the GlcNAc and $(GlcNAc)_2$ according to following equation:

$$[\text{GlcNAc}]\ (\text{mM}) = \frac{PeakArea}{336.87} \times \text{dilution factor , and}$$

$$[(GlcNAc)_2]\ (mM) = \frac{PeakArea}{611.27} \times \text{dilution factor}$$

The factors of 336.87 and 611.27 were obtained from the slope of the calibration lines of GlcNAc and $(GlcNAc)_2$ respectively (**Figure A3**).

2.5.3 Determination purity of *N*-acetyl-D-glucosamine (GlcNAc)

The GlcNAc sample (5 mg) was dissolved in Milli-Q water (1.0 mL) in each vial**.** The solution (0.300 mL) was pipetted out and mixed with acetronitrile (0.700 mL). The solution was filtered though a 0.45 μm PTFE filter before injecting into the HPLC. The same instrument system and condition as previously described in the preparation of calibration line (**section 2.4.1**) was used in the analysis of the products. The amount of GlcNAc was determined against the calibration line of the corresponding standard and the %purity was calculated according to the following equation.

$$\%\text{purity} = \frac{\text{HPLC weight} \times 100}{\text{Sample weight}}$$

The proton NMR was used to measured purity . The GlcNAc was dissolved in dimethyl sulfoxide (DMSO) and ^{1}H NMR spectra was taken on Varian Mercury+ 400 NMR spectrometer.

2.6 Hydrolysis of squid pen chitin condition

2.6.1 Optimum pH at various temperatures for production GlcNAc

The study of the optimum pH for chitinolytic enzyme hydrolysis chitin at different temperature was observed. Crude enzyme (0.025 mL) was pipetted into test tube that contained colloidal chitin (0.1 mL) and Phosphate buffer pH 3, 3.5, 4, 4.5, 5, 5.5 (0.275 mL) and adjusted volume to 1.5mL by DI-water. The reaction mixtures was incubated at each temperature (30, 35, 40, 45, 50, 55 ^{o}C) in water bath for 30 minutes. The reaction of mixtures was separated by centrifugation at 3000 rpm for 15 minutes and analyzed chitinolytic activity by Schale's method**.**

2.6.2 Chitinolytic susceptibility of different substrates

The enzyme was incubated with various type of substrates (10 mg) which were colloidal chitin, mill squid pen chitin, swollen squid pen chitin, ground squid pen chitin and ground shrimp chitin. Each reaction consisted of chitinolytic enzyme (0.25mL) and Phosphate buffer pH 4 (0.5 M, 0.275 mL) which was adjusted volume of the reaction to 1.5mL by DI-water in test tube. The reaction was incubated at 45 ^{o}C for 30 minutes. The reaction of mixtures was centrifugated at 3000 rpm for 15 minutes and analyzed (chitinolytic activity) by Schale's method**.**

2.6.3 Stability enzyme at various temperatures

Crude enzyme was pre-incubated at various temperatures 40, 45, 50 ^{o}C in water bath for 63 hours. Samples were taken at 0, 1, 3, 7, 15, 31, 63 hours. Crude enzyme was measured enzyme activity in test tube which contained colloidal chitin. Afterward crude enzyme was adjusted pH 4 and incubated at 45 ^{o}C in water bath for 30 minutes. The reaction of mixtures was separated solid media by centrifugation at 3000 rpm for 15 minutes and analyzed chitinolytic activity by Schale's method**.**

2.6.4 Purification of *N*-acetyl-D-glucosamine (GlcNAc)

After hydrolysis, the reaction of mixture was heated in boiling water for 15 minutes to deactivate the enzyme. The remaining chitin was removed by filtration and then decolorized with activated charcoal (20 g/100 g initial chitin). The decolorizing solution stirred at room temperature for 30 minutes. The solution was filtered to remove insoluble residue and activated charcoal, clear filtrate was evaporated to recover GlcNAc solids. The solids was obtained and washed with absolute ethanol (100 mL/100 g initial chitin), sonicated for 30 minutes at room temperature and filtered. The white solids was obtained and dried under vacuum to produce the final product, GlcNAc.

2.7 Hydrolysis of squid pen chitin in flask scale

2.7.1 Hydrolysis of squid pen chitin in various volume

Squid pen chitin (5 g) was incubated with crude enzyme of *Aspergillus fumigatus* (22 U/g chitin). Then pH was adjusted by 1 M acetic acid to pH 4. After that the volume was adjusted the various volume 50 to 250 mL by DI-water. The mixture was incubated at 45 ^{o}C for 2 days. After incubation, the reaction of mixture was heated in the boiling water for 15 minutes to deactivate the enzyme. The

remaining chitin was removed by filtration and Crude product was kept in solid form after evaporation.

2.7.2 Hydrolysis of squid pen chitin using different amount of enzyme

Squid pen chitin (5 g) was incubated with the crude enzyme from *Aspergillus fumigatus* at various enzyme/chitin ratios (22, 28, 33, 39, 44, 50 U/g chitin). The reaction mixture was adjusted to the volume of 200 mL by DI-water. Then pH was adjusted to 4 by 1 M acetic acid. The mixture was incubated at 45 °C for 2 days. After the incubation period, the reaction mixture was heated in the boiling water bath for 15 minutes to deactivate the enzyme. The remaining chitin was removed by filtration and the crude product was kept in the solid form after evaporation. The purification of GlcNAc was conducted according to the procedure described in section 2.5.4 and the purity of GlcNAc was determined by the method described in section 2.4.3.

2.8 Hydrolysis of chitin in large scale

2.8.1 Bacth fermentation

Squid pen chitin (100 g) was incubated with the enzyme (22 U/g chitin) in 10 L fermentor and the volume was adjusted by DI-water to 4,000 mL. Then pH was adjusted by 1 M acetic acid to pH 4. The mixture was incubated at 45 °C for 2 days and the reaction mixture was then heated in a boiling water bath for 15 minutes to deactivate the enzyme. The remaining chitin was removed by centrifugation of reaction mixture at 3000 rpm for 15 minutes and crude product was kept in solid form after evaporation. The purification of GlcNAc was conducted according to the procedure described in section 2.5.4 and the purity of GlcNAc was determined by the method described in section 2.4.3

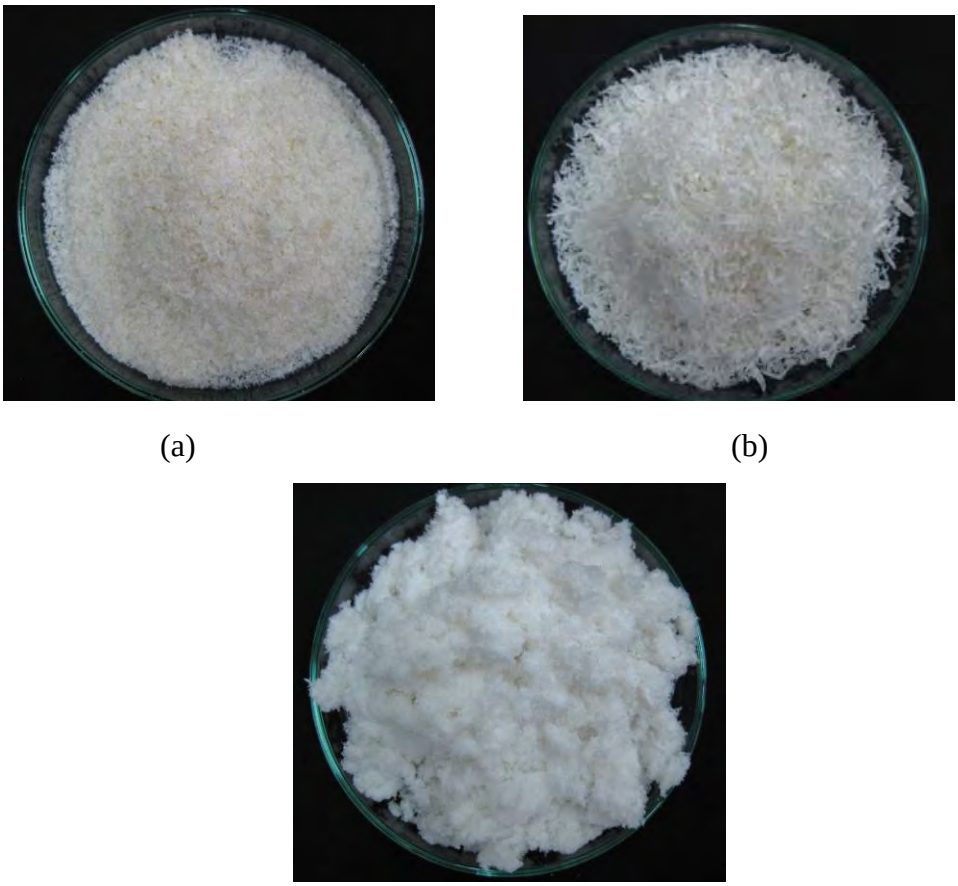
CHAPTER III

RESULTS AND DISCUSSION

3.1 Substrates and enzymes

3.1.1 Substrate preparation

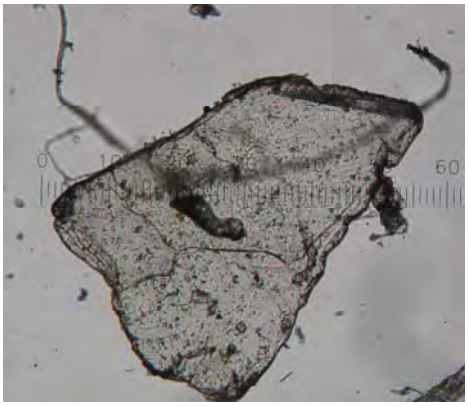
The shrimp chitin and squid pen chitin substrates were prepared by grinding strip chitin with food blender **(Figure 3.1a and 3.1b)** and an ultra-centrifugal mill (Retsch, ZM1000, Germany) to give fibrous chitin with an average size of 100 μm width and 1 mm length **(Figure 3.1c and 3.2f)**.



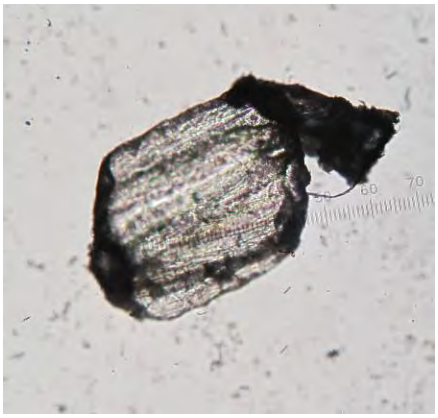
(a) (b)

(c)

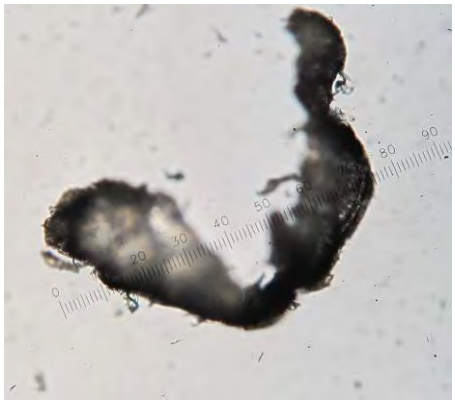
Figure 3.1 Photographs of (a) shrimp chitin (α-chitin), (b) squid pen chitin (β-chitin) and (c) milled squid pen chitin



(d)



(e)



(f)

Figure 3.2 Photographs of (d) ground shrimp chitin (α-chitin), (e) ground squid pen chitin (β-chitin) and (f) milled squid pen chitin under optical microscope (one scale unit equals to 100 μm).

The colloidal chitin was prepared under acidic condition according to the literature procedure.[72] In the final step, white slurry chitin was obtained. The concentration of colloidal chitin determined by drying in vacuum was ~100 mg/g. This colloidal chitin was stored at 4 °C for further use in all experiments throughout this thesis.

3.1.2 Enzyme prepared from microbial

***Aeromonas hydrophila* MOK-1**

Aeromonas hydrophila was cultivated in chitin minimum medium (CMM) using milled squid pen chitin and ground shrimp chitin as a carbon source. The growth of *Aeromonas hydrophila* cultured in milled squid pen chitin was comparable to the one cultured in the ground shrimp chitin. Growth curve was highest about 2,000 CFU/mL at 12 h.

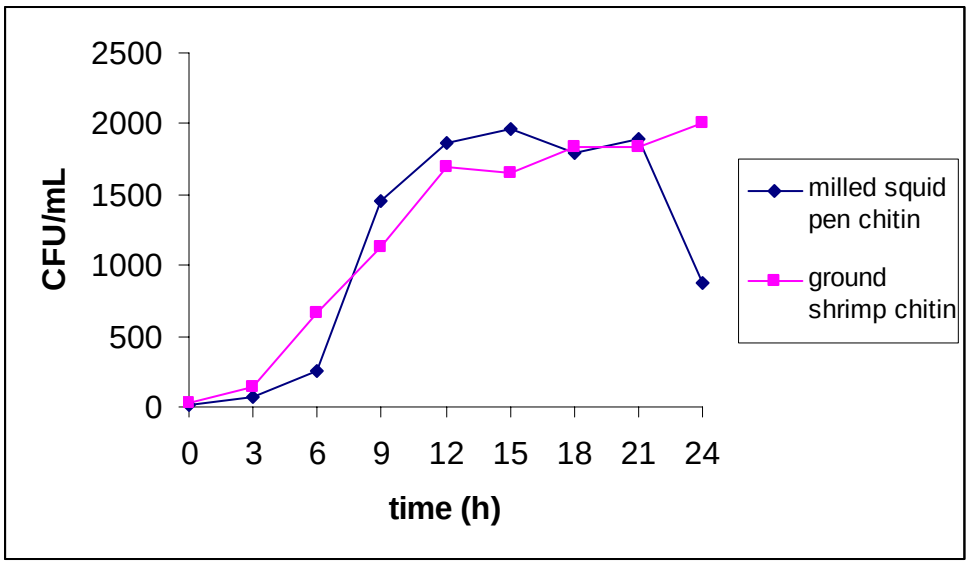


Figure 3.3 Growth curve of *Aeromonas hydrophila* was cultured in CMM (milled squid pen chitin and ground shrimp chitin) at 37 °C for various times.

Crude enzyme obtained from *Aeromonas hydrophila* cultivated in CMM, using milled squid pen chitin and ground shrimp chitin substrate, gave rather low chitinolytic activity. The highest activity determined by Schale's method was obtained after 15 h. *Aeromonas hydrophila* cultivated with milled squid pen chitin substrate produced crude enzyme containing chitinolytic activity lower than 250 mU_{cc}/mL (1 U_{cc} of enzyme was defined as the amount of enzyme which catalyses the release of 1 μmol of reducing sugar per minute under assay conditions using colloidal chitin as the substrate).

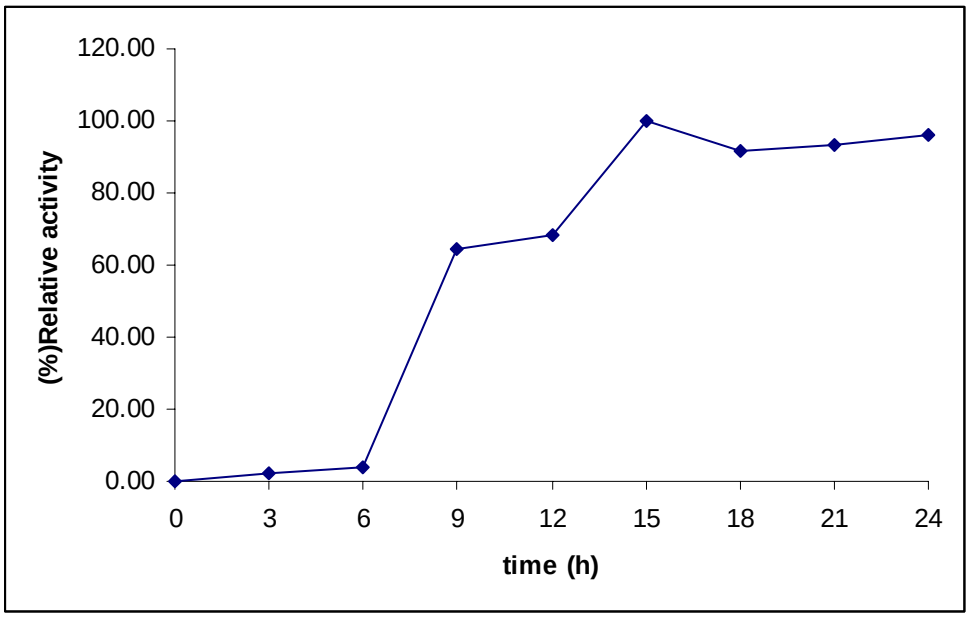


Figure 3.4 Chitinolytic activity of crude enzyme obtained from cultivation of *Aeromonas hydrophila* at 37°C for various times.

Aspergillus fumigatus

The chitinolytic activities of crude enzyme from *Aspergillus fumigatus* (*A. fumigatus*) determined by Schale's method was typically more than 2.2 U_{cc}/mL (1 U_{cc} of enzyme was defined as the amount of enzyme which catalyses the release of 1 μmol of reducing sugar per minute under assay conditions using colloidal chitin as the substrate). The protein quantity in the crude enzyme determined by Bradford's method was about 160 – 200 μg/mL.

- Effect of harvesting time on enzyme activity

The fungus was cultivated on CCMM 100 and 200 mL containing 20 and 40 g colloidal chitin in a 250 and 500 mL flask, respectively. After three days, the chitinolytic activities obtained from both cultivations were relatively constant and not much difference. The chitinolytic activities of the enzyme produced after three days were 8.0 ± 0.4 U_{cc}/mL and 5.6 ± 0.4 U_{cc}/mL for the incubation in the 250 and 500 mL flasks, respectively.

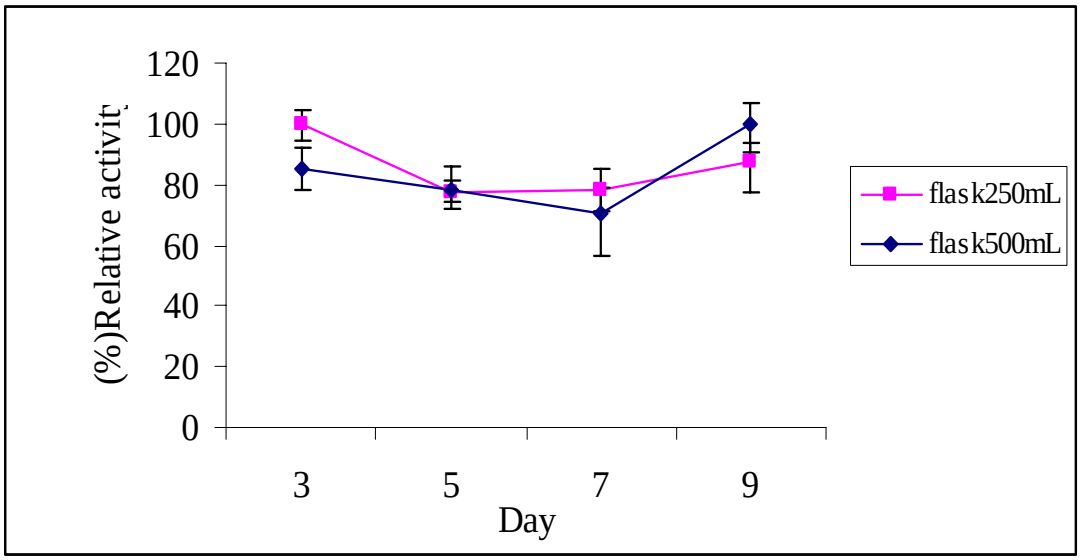


Figure 3.5 Chitinolytic activity of crude enzyme obtained from cultivation of *Aspergillus fumigatus* in 250 and 500 mL flasks at pH 3.5 and 40 °C for various times. Data are shown as the average of three assays from one cultivation..The error bar represents the maximum and minimum values obtained from the assays.

- Effect of culturing pH on the chitinolytic activity obtained

The chitinolytic enzyme was prepared at various pH in the range of 3 to 5.5 by cultivation of *Aspergillus fumigatus* on CCMM 100 mL in a 250 mL flask. Within the cultivation pH range of 3 to 5, the chitinolytic activity of the crude enzyme obtained rarely varied **(Figure 3.6)**. Figure 3.4 also shows that pH 3.5 is the optimum pH for cultivation of the fungus to produce the highest chitinolytic activity of 3.1± 0.15 U_{cc}/mL. The low pH response in a window of 3 to 5 indicated that some pH fluctuation during cultivation would not affect the production of chitinolytic enzyme from *Aspergillus fumigatus*.

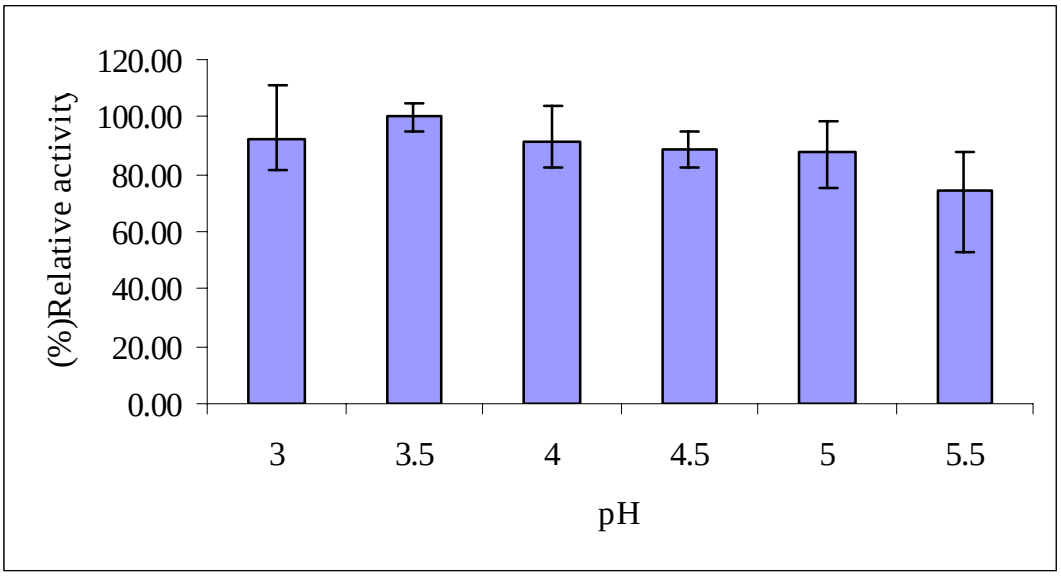


Figure 3.6 Chitinolytic activity of crude enzyme obtained from *Aspergillus fumigatus* cultivated in colloidal chitin minimum media at various pH and 40 °C after 5 days of cultivation. Data are shown as the average of three assays for one cultivation. The error bars represent the maximum and minimum values obtained from the analyses.

3.2 Condition optimization for the hydrolysis of chitin with enzyme from *Aspergillus fumigatus*

3.2.1 Chitinolytic susceptibility of various chitin substrates

Five different substrates, four from squid pen chitin (β-chitin) and one from shrimp (α–chitin) were studied for chitinolytic susceptibility. For shrimp chitin, it was ground by food blender. Four substrates from squid pen chitin were prepared by grinding it with food blender (ground squid pen chitin), milling it with an ultra-centrifugal mill (milled squid pen chitin), saturation of milled squid pen chitin in food blender with water (swollen squid pen chitin, see section **2.1.3** for detailed procedure) and acid dissolution and neutralization to form colloidal chitin (colloidal squid pen chitin, see section **2.1.2** for detailed procedure). The substrates were hydrolyzed with the crude enzymes from *Aspergillus fumigatus.* The results revealed that squid pen chitin possessed much higher chitinolytic susceptibility toward the ensemble of chitinolytic enzymes from *Aspergillus fumigatus* than that of shrimp chitin. The colloidal chitin was the best substrate. The swollen squid pen chitin and milled squid pen chitin were hydrolysed at 40% and 30% of the colloidal squid pen chitin, respectively. While Ground squid pen chitin and ground shrimp chitin showed less

than 10% chitinolytic susceptibility of the colloidal squid pen chitin. It is common for β-chitin to have greater chitinolytic susceptibility than shrimp chitin as the squid pen chitin has looser packing of chitin chains in the crystalline domains comparing to the shrimp chitin. Although, the colloidal chitin has the highest chitinolytic susceptibility, the preparation of this substrate is time consuming and produces a lot of waste from concentrated hydrochloric used and its neutralization. Using ultra-centrifugal mill is the much simpler substrate pretreatment of squid pen chitin that the milled squid pen chitin is selected to be used in the subsequent preparation of GlcNAc,

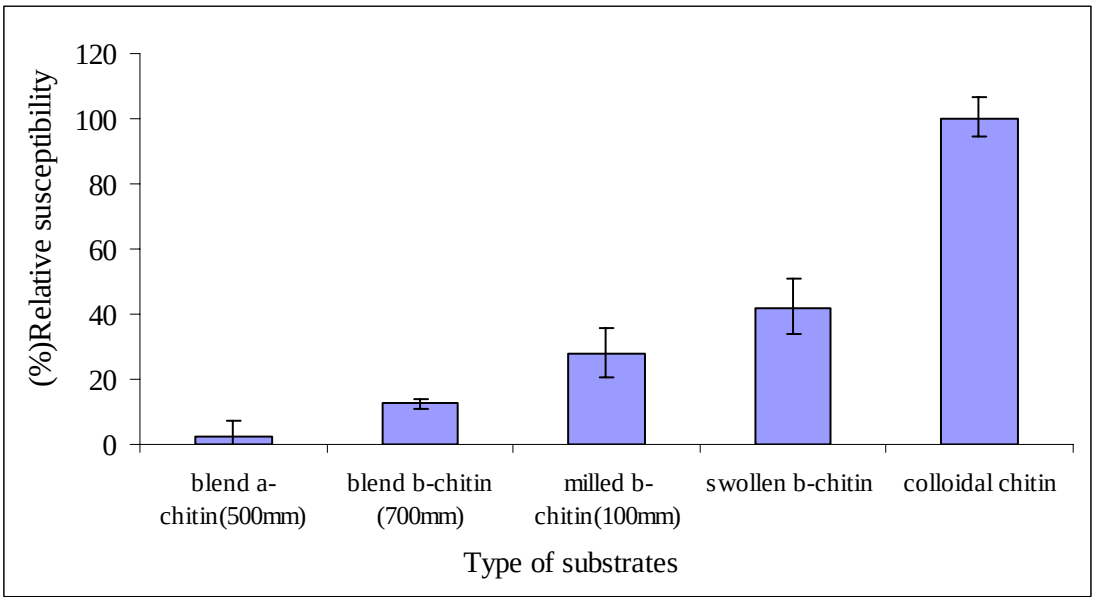


Figure 3.7 Chitinolytic susceptibility of various chitin substrates toward the crude enzyme from *Aspergillus fumigatus* incubated at pH 4 and 45 °C assayed after 30 minutes of incubation. Data are shown as the average of three assays for one cultivation. The error bars represent the maximum and minimum values obtained from the analyses.

3.2.2 Optimum pH for the GlcNAc production from the colloidal chitin substrate by the crude enzyme

The optimum pH for the hydrolysis of colloidal chitin by the crude enzyme from *Aspergillus fumigatus* showed interesting temperature dependence. The optimum pH was 3.5 at the hydrolysis temperature of 35 and 40°C and it became higher at higher temperatures (Figure 3.8). At the hydrolysis temperature of 45 and 50°C, the optimum pH was 4.0 that increased to 4.5 at 55°C. The chitinolytic activity at the optimum pH also increased with the hydrolysis temperature upto 50°C where

the highest chitinolytic activity of 4.6 ± 0.1 U_{cc}/mL was observed. At 55°C, the activity at the optimum pH started to drop slightly which may due to the lost of the enzyme stability at higher temperature

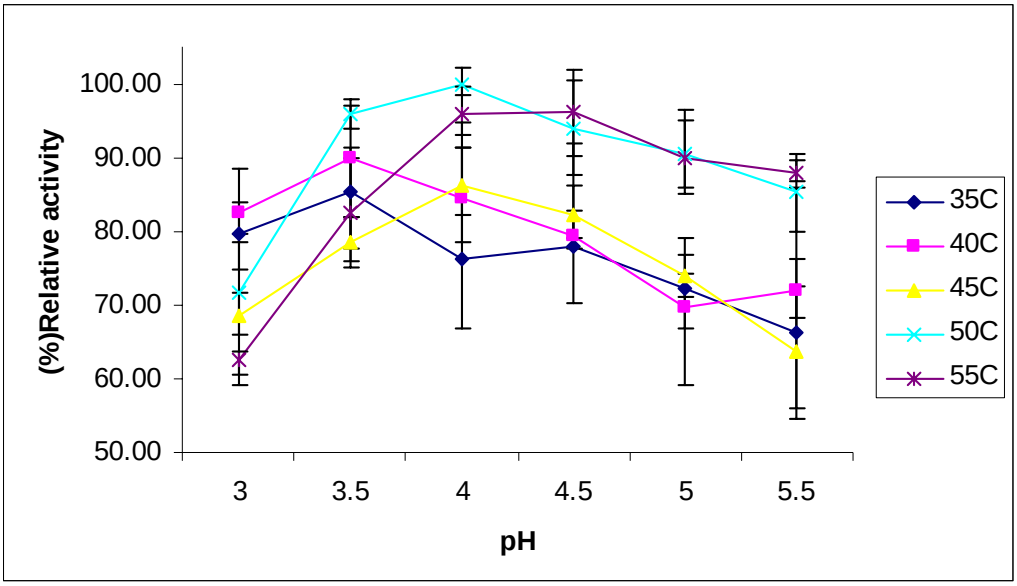


Figure 3.8 Relative chitinolytic activity of the crude enzyme from *Aspergillus fumigatus* on colloidal chitin tested at various pH and temperatures with hydrolysis time of 30 minutes. Data are shown as the average of thee assays for one cultivation. The error bars represent the maximum and minimum values obtained from the analyses.

3.2.3 The temperature dependence of the enzyme stability

The temperature dependence of the enzyme stability was studied by incubating the enzyme at various temperatures and pH 4.0 for 63 hours. The chitinolytic activity of the enzyme was assayed at each time interval. The enzyme was relatively stable at 40 and 45 °C retaining at least 90% of its activity up to 63 hours **(Figure 3.9)**. The enzyme however lost its activity considerably at 50 °C with only 50% of its activity remained after 63 hours of incubation. Hence, the optimal temperature the hydrolysis of colloidal chitin by the enzyme should be 45 °C when both activity and stability are taken into account. At the optimum pH and temperature the hydrolysis of colloidal chitin, the disappearance of milled squid pen chitin substrate, indicating the completion of hydrolysis, was generally observed after 2 days.

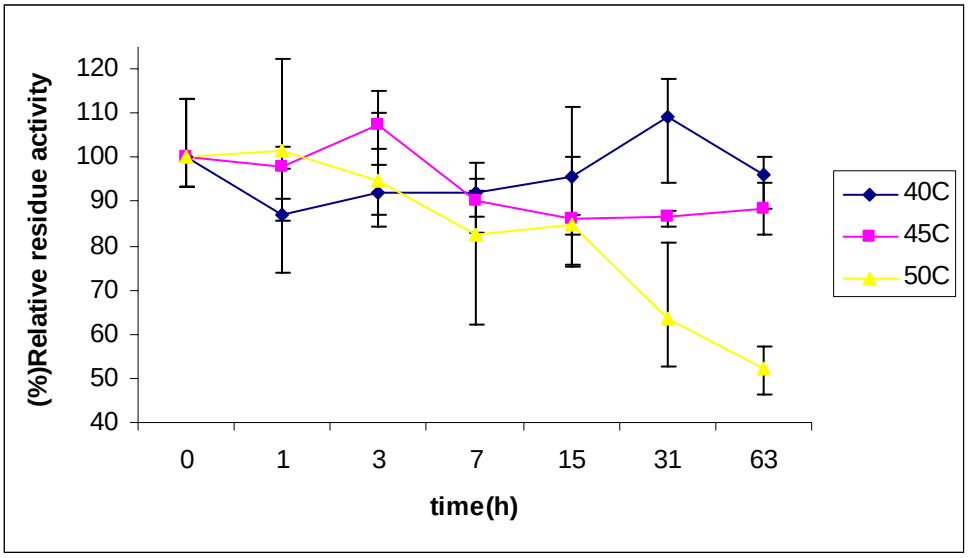


Figure 3.9 The time dependence of the chitinolytic activity of the crude enzyme from *Aspergillus fumigatus* incubated at pH 4 and various temperatures showing the stability of the enzyme. Data are shown as the average of thee assays for one cultivation. The error bars represent the maximum and minimum values obtained from the analyses.

3.3 Preparation of GlcNAc from enzymatic hydrolysis of squid pen chitin

3.3.1 Preparation of GlcNAc in 500 mL flask

- **Effects of chitin concentrations**

The milled squid pen chitin (5 g) was hydrolyzed by crude enzyme from *Aspergillus fumigatus* (22U_{mc}/g) in a 500 mL flask. The pH of the reaction medium was adjusted to 4 by 1 M acetic acid. The volume of the reaction medium was adjusted by DI-water to 50, 100, 150, 200 and 250 mL. The reaction mixture was incubated at 45 °C for 2 days and the reaction mixture was then heated in boiling water to deactivate the enzyme. The solid residue was removed by centrifugation at 3000 rpm for 15 minutes and the centrifugate was evaporated to afford the crude product**.** The crude yield of GlcNAc increased as the concentration of chitin decreased from 10% to 3.3% (w/v) and became relatively constant from 3.3% to 2.0% (w/v) that provided 73-75% yield of crude GlcNAc **(Table 3.1)**. The chitin concentration of 2.5% (w/v) was thus used for further study.

Table 3.1 GlcNAc production hydrolyse squid pen chitin in various volumes

flask	Chitin concentration (% w/v)	Crude product (g)	Crude yield (%)
1	10.0	2.9	54
2	5.0	3.6	66
3	3.3	4.0	73
4	2.5	4.1	75
5	2.0	4.1	75

The reaction was carried out with milled squid pen chitin 5 g and crude enzyme 22 U/g at 45°C, pH 4 for 2 days.

- **Effects of the amount of crude enzyme used**

The milled squid pen chitin (5 g) was incubated with 110, 140, 165, 195, 220, 250 U_{mc} (1 U_{mc} of enzyme activity was defined as the amount of enzyme which catalyses the release of 1 μmol of reducing sugar per minute under assay conditions using milled squid pen chitin as the substrate) of crude enzyme from *Aspergillus fumigatus*. The reaction volume was adjusted to 200 mL using DI-water. Then pH of the reaction mixture was adjusted to 4 by 1 M acetic acid. The mixture was incubated at 45°C for 2 days and the crude product was obtained by using the same procedure described above. The crude yield increased with the amount of enzyme used and the highest yield of 87% was obtained with 250 U_{mc} of enzyme or 50 U_{mc} of enzyme per 1 g of chitin (Table 3.2). It is also important to note that the increase of the enzyme from 22 U_{mc}/g to 50U_{mc}/g increased the yield of GlcNAc significantly lower than 2 folds suggesting that enzyme was almost saturated with the substrate.

Table 3.2 GlcNAc production hydrolyse squid pen chitin in different amount of enzyme

flask	enzyme(U_{mc}/g)	Crude product (g)	Crude yield (%)
1	22	3.9	72
2	28	4.2	77
3	33	4.3	78
4	39	4.4	80
5	44	4.5	83
6	50	4.7	87

The reaction was carried out with milled squid pen chitin 5 g in 200 mL of the reaction mixture at 45°C, pH 4 for 2 days .

3.3.2 Preparation of GlcNAc in 5 L fermenter

Milled squid pen chitin (100 g) was incubated using crude enzyme from *Aspergillus fumigatus* at 22 U_{mc}/g chitin. The volume was adjusted to 4 L using DI-water. The pH was adjust to 4 (acetic acid 2 M) and incubated at 45 °C for 2 days. The reaction mixture was evaporated and reprecipitated by absolute ethanol from the concentrated aqueous solution. The GlcNAc was isolated in 68 %yield with 100% purity.

3.4 Purity analysis of GlcNAc

The purities of isolated GlcNAc was determined by using two different techniques; high performance liquid chromatography (HPLC) and nuclear magnetic resonance (^{1}H NMR). In the HPLC technique, the analysis showed that GlcNAc isolated by precipitation with absolute ethanol had 89% purity. The purity colud be improved to 100% by decolorization with activated charcoal.

The determination of purity by using ^{1}H NMR technique was performed by comparing the ^{1}H NMR spectrum of the isolated product to the spectrum of the corresponding standard GlcNAc. The spectrum of GlcNAc obtained from the precipitation follow with decolorization was identical to that of the standard GlcNAc confirming high purity of the isolated product **(Figure 3.10)**

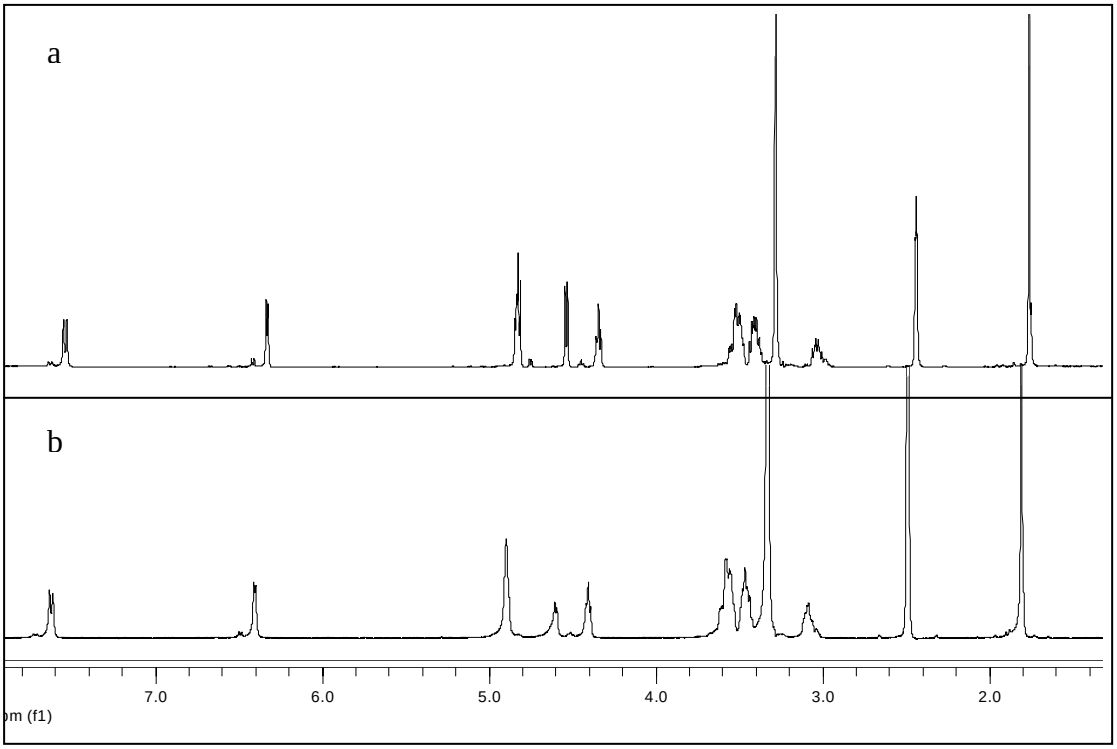


Figure 3.10 NMR spectrum of standard GlcNAc (a) and precipitated GlcNAc (b)

Suggestion for future work

Monitoring the reaction pH, it was found that the reaction pH had changed from 4 to 4.6 toward the end of the reaction. The periodic addition of acetic acid may help controlling the pH closer to 4 and keeping the enzyme activity. The higher yield of GlcNAc can be produced with higher amount of the crude enzyme used. Big fermentor is required to produce large amount of enzyme and a larger reactor equipped with good agitation system is required for kilogram scale production of GlcNAc. A continous flow reactor may also be used to recycling the eynzyme. However, appropriate design is necessary to prevent clogging of the flow system due to fine particles of solid chitin produced in the reaction.

CHAPTER IV

CONCLUSIONS

As one of the most ubiquitous of the airborne saprophytic fungi, *Aspergillus fumigatus* has been shown to be a pathogen causing pneumonia and other fatal invasive infections. *Aspergillus fumigatus* is known to produce of extracellular chitinolytic enzymes, especially when being cultivated by using colloidal chitin minimum medium (CCMM) as a carbon source and urea as a nitrogen source. The optimum cultivating temperature was 40 °C and optimum pH was 3.5 that gives the crude enzyme with chitinolytic activity reaching the maximum of 8.0 ± 0.4 U/mL after 3 days of cultivation. The chitinolytic enzymes produced from *Aspergillus fumigatus* was active in wide pH range of 3-5.5. At 35-40 °C, the optimum pH was 3.5 and moved slightly higher to pH 4 as the temperature increased to 45-50. The enzyme was relatively stable at 45 °C and pH 4, retaining at least 90% of its activity up to 63 hours. The squid pen chitin chitin showed higher enzymatic susceptibility than the shrimp chitin. The chitinolytic susceptibility of squid pen chitin could also been increased by 5 times when the size of the substrate was reduced from chitin flake to colloidal chitin. The hydrolysis of squid pen chitin was used optimum substrate concentration at 5 g chitin per volume 200 mL and optimum ratio of enzyme to chitin more than 44 U/g chitin. This condition used to hydrolyzed squid pen chitin to *N*-acetyl-D-glucosamine with over 80% isolated yield.

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APPENDIX

APPENDIX A

1. Prepartion calibration curve of protein assay by Bradford's method.

Table A1 The absorbance of standard bovine serum albumin (BSA) used for plotting the calibration line in Figure A1

standard No.	amount of protein (μg/mL)	ΔAbsorbance
1	0	0.000
2	20	0.017
3	40	0.033
4	60	0.048
5	80	0.063
6	100	0.079
7	120	0.120
8	140	0.144
9	160	0.166
10	180	0.182
11	200	0.201

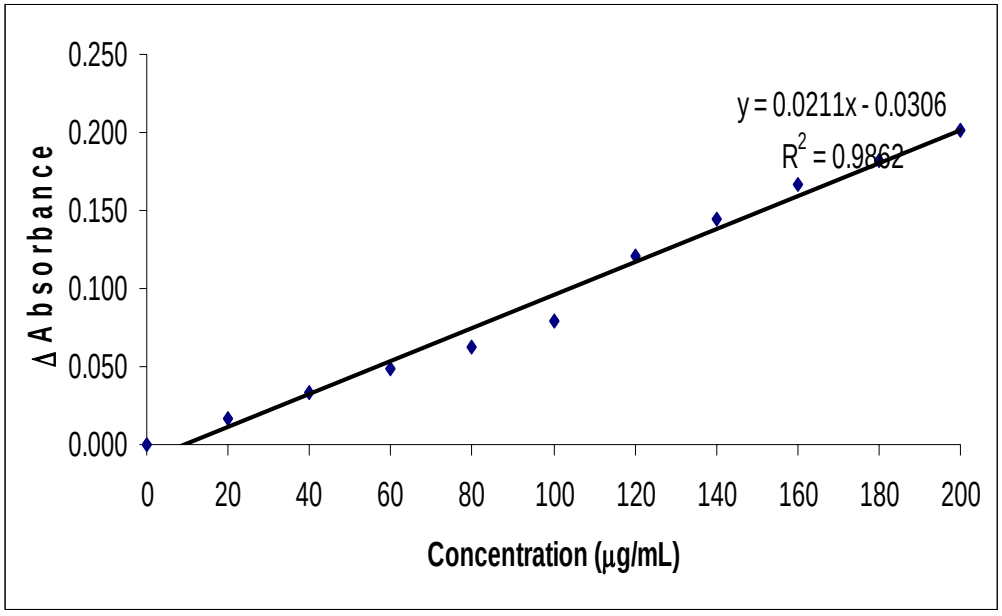

Figure A1 The calibration line for BSA assaying by Bradford's colorimetric method at optical density (absorbance) at 595 nm..

2. Prepartion calibration curve of N-acetyl-D-glucosamine for chitinolytic enzyme assay by colorimetric method.

Calibration curve for GlcNAc was made by determining the absorbance value at 420 nm of standard GlcNAc according to the method of Schales. Using potassium ferrocyanate ($KFe_2(CN)_6$) 0.5 g in 0.5 M sodium carbonate (Na_2CO_3) 1 L.

Table A2 The amount of standard solution of GlcNAc and ΔAbsorbance

standard No.	amount of GlcNAc (μmole)	ΔAbsorbance
1	0.0000	0.000
2	0.0499	0.104
3	0.0999	0.208
4	0.1499	0.310
5	0.1999	0.405
6	0.2499	0.499
7	0.2974	0.599
8	0.3480	0.682
9	0.3987	0.780

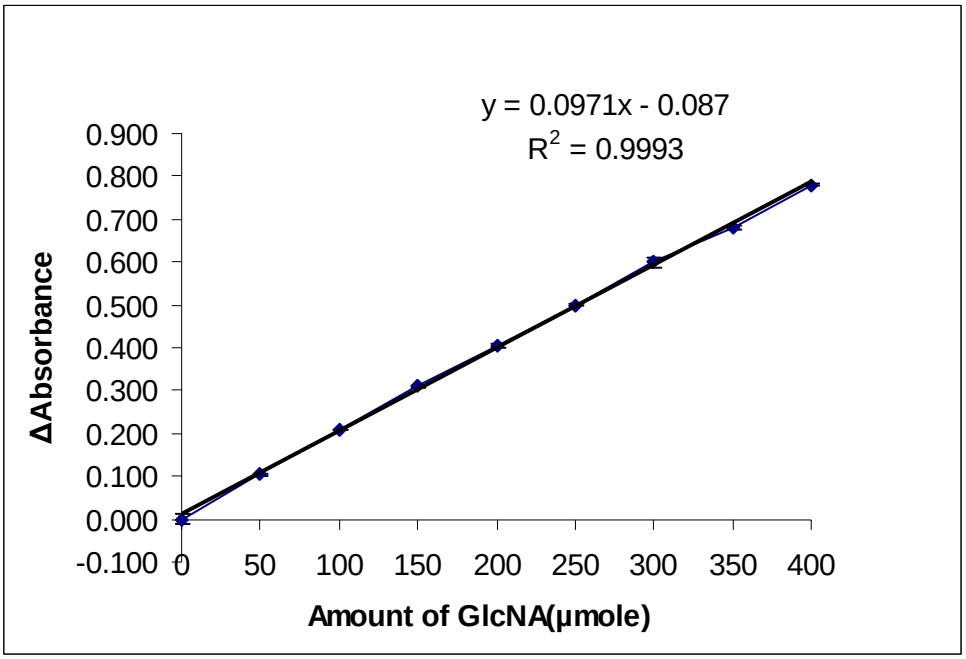

Figure A2 Correlation between amount of standard of *N*-acetyl-D-glucosamine and optical density (absorbance) at 420 nm.

Calibration curve for GlcNAc was made by determining the absorbance value at 420 nm of standard GlcNAc according to the method of Schales. Using potassium ferrocyanate ($KFe_2(CN)_6$) 1 g in 0.5 M sodium carbonate (Na_2CO_3) 1 L.

Table A3 The amount of standard solution of GlcNAc and ΔAbsorbance

standard No.	amount of GlcNAc (μmole)	ΔAbsorbance
1	0.0000	0.000
2	0.0999	0.233
3	0.1999	0.442
4	0.2998	0.658
5	0.3998	0.872
6	0.4998	1.083
7	0.5980	1.270
8	0.6993	1.465
9	0.7974	1.663

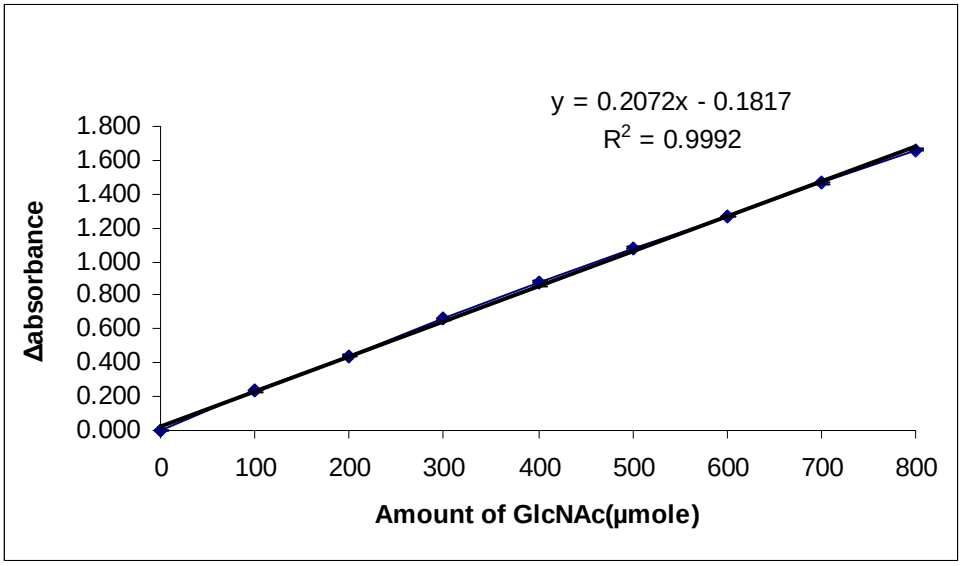

Figure A3 Correlation between amount of standard of *N*-acetyl-D-glucosamine and optical density (absorbance) at 420 nm.

3. Preparation the calibration curve of *N*-acetyl-D-glucosamine for HPLC analysis

Calibration curve of GlcNAc was made by varying the concentration and measuring the peak area by HPLC.

Table A4 The concentration of standard solution of GlcNAc and peak area.

standard No.	Conc. GlcNAc (mM)	Peak Area (mV*Sec)
1	0.10	9.504
2	0.20	33.962
3	0.50	136.64
4	1.00	309.344
5	2.00	653.099
6	3.00	977.349

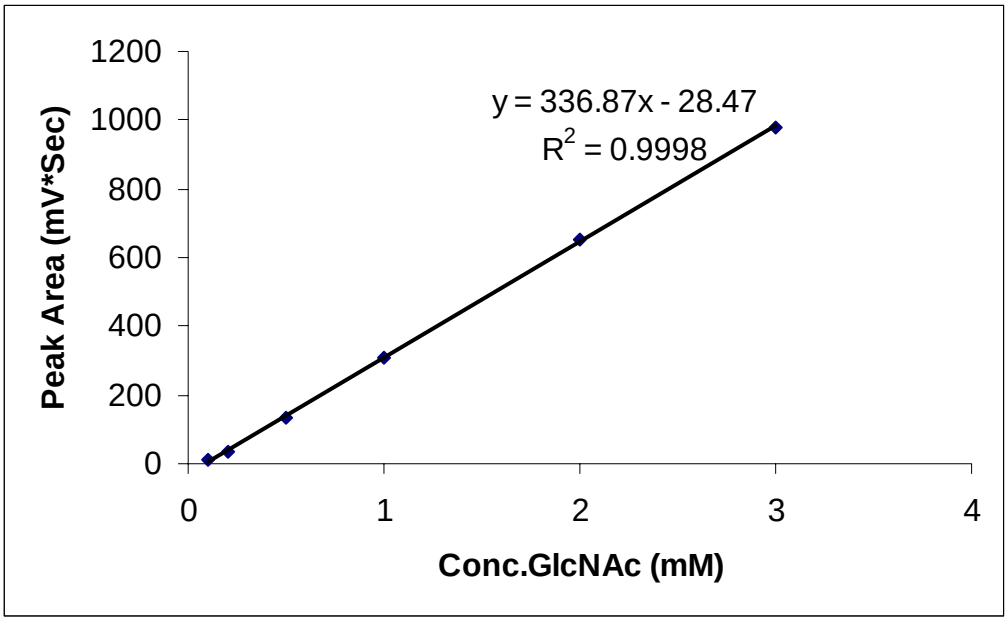

Figure A4 Correlation between concentration of standard *N*-acetyl-D-glucosamine and peak area by HPLC.

VITAE

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