CHITINOLYTIC ENZYMES OF *Bacillus licheniformis* ; PARTIAL PURIFICATION , CHARACTERIZATION AND MOSQUITO LARVICIDAL PROPERTY

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ชื่อวิทยานิพนธ์ การแยกและศึกษาเอนไซม์ที่สามารถย่อยสลายไคติน จาก *Bacillus licheniformis* และความสามารถในการฆ่าลูกน้ำยุงของเอนไซม์

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บทคัดย่อ

แบคทีเรีย *Bacillus licheniformis* สายพันธุ์ที่แยกได้จากห้องปฏิบัติการสามารถผลิตเอนไซม์ไคตินเนสได้เป็นจำนวนมากเมื่อแบคทีเรียชนิดนี้ถูกเพาะเลี้ยงในอาหารเลี้ยงเชื้อที่มีไคติน เป็นส่วนผสมเมื่อเซลล์เข้าสู่ช่วง stationary phase โดยที่เอนไซม์ไคตินเนสที่ผลิตได้มีอยู่อย่างน้อย 5 ชนิดและมีขนาด 70.8, 68.1, 63.7, 53.0 และ 49.5 kDa เอนไซม์ขนาด 70.8 kDa ถูกแยกให้บริสุทธิ์โดยใช้วิธี fractionation ของส่วนน้ำเพาะเชื้อของแบคทีเรียชนิดนี้ด้วยแอมโมเนียม ซัลเฟต ช่วง 35-65 เปอร์เซ็นต์ และ column chromatography ด้วย CM- และ DEAE-sephadex matrix ตามลำดับ จากนั้นจึงมีการศึกษาคุณสมบัติ physicochemical ในด้านต่างๆ พบว่า เอนไซม์มี optimal temperature ที่ 60°C และ activity ค่อนข้างคงทน เมื่อเก็บที่ 40-50°C และพบว่า activity ของเอนไซม์จะลดลงอย่างมากเมื่อเอนไซม์อยู่ในสารละลายที่มีภาวะเป็นกรด,ด่าง หรือ อุณหภูมิสูง ($\geq 70^{\circ}C$), activity จะคงทนมากเมื่อเอนไซม์ถูกเก็บอยู่ในน้ำกลั่น จากการศึกษา enzyme kinetics ได้ค่า Michaelis's constant และ maximum velocity (V_m) ของเอนไซม์ชนิดนี้มีค่า

3.33 เปอร์เซ็นต์ และ 781.90 mU/ml/mg ตามลำดับ จากการทดสอบความสามารถในการฆ่าลูกน้ำยุง *Aedes aegypti* ของ crude fraction ของเอนไซม์ไคตินเนสซึ่งเตรียมโดยวิธี fractionation ด้วย แอมโมเนียม ซัลเฟต ช่วง 35-65 เปอร์เซ็นต์ และเอนไซม์ไคตินเนส ขนาด 70.8 kDa ที่บริสุทธิ์ ได้ค่า LC_{50} ของ crude fraction มีค่าเท่ากับ 60.67 mU/ml และพบการเสริมประสิทธิภาพในการฆ่าลูกน้ำยุง *Aedes aegypti* เมื่อมีการทดสอบ crude fraction นี้ร่วมกับ *Bacillus thuringiensis* subsp. *israelensis* สายพันธุ์ 4Q2-72 และ crude fraction จะสูญเสียคุณสมบัติในการฆ่าลูกน้ำยุงถ้าถูกต้มที่ 100°C เป็นเวลา 10 นาที และจากการศึกษาผลของเอนไซม์ไคตินเนสขนาด 70.8 kDa ที่บริสุทธิ์ในการฆ่าลูกน้ำยุง *Aedes aegypti* พบว่า ค่าLC_{50} ของเอนไซม์ชนิดนี้มีค่าเท่ากับ 66.67 mU/ml

Thesis Title Chitinolytic Enzymes of *Bacillus licheniformis* ; Partial Purification, Characterization and Mosquito Larvicidal Property

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ABSTRACT

The *Bacillus licheniformis*, isolated by our laboratory, produced chitinolytic enzymes when cells grown in medium supplemented with crude chitin entered the stationary phase. This *Bacillus licheniformis* strain produced at least 5 types of chitinolytic enzymes and their molecular weights were 70.8, 68.1, 63.7, 53.0, and 49.5 kDa. The chitinolytic enzyme from culture supernatant of cells grown in medium supplemented with chitin was purified by fractionation with ammonium sulfate ranges from 35-65 percent and passed through column chromatography with CM- and 2 columns of DEAE-sephadex matrix respectively. The pure fraction of chitinase with molecular weight of 70.8 kDa was obtained. Results of physicochemical properties of purified enzyme showed optimal temperature of 60°C, relatively stable when kept at 40 and 50°C with

optimal pH of 5.0. Its activity was rapidly lost when kept in strong acid, strong alkali or high temperature (≥ 70°C), but very stable in deionized distilled water. Enzyme kinetics study showed that Michaelis's constant (K_m) and maximum velocity (V_m) value were 3.33 % (w/v) and 781.90 mU/ml/mg of enzyme, respectively. Crude fraction and purified 70.8 kDa chitinolytic enzyme were assayed for toxicity against mosquito larvae. The LC_{50} of crude chitinolytic enzymes against 2nd instar of *Aedes aegypti* larvae was 60.67 mU/ml. These enzymes enhanced toxicity effect when assayed against mosquito larvae in combination with *Bacillus thuringiensis* subsp. *israelensis* strain 4Q2-72. The crude enzyme fraction alone was completely lost its larvicidal property upon heating at 100°C for 10 min. The LC_{50} of purified 70.8 kDa chitinolytic enzyme against 2nd instar of *Aedes aegypti* larvae was equal to 66.67 mU/ml.

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LIST OF ABBREVIATIONS

^{o}C	degree Celsius
DNA	deoxyribonucleic acid
g	gram
µg	microgram
µl	microlitre
l	litre
ml	millilitre
mg	milligram
rpm	revolution per minute
mM	millimolar
M	Molar
nm	nanometre
cm	centimetre
min	minute
hr	hour
O.D.	optical density
B.	*Bacillus*
E.coli	*Escherichia coli*
kDa	kilodalton
et al.	Et. alii (Latin), and others.
Fig.	figure
etc.	Et cetera (Latin), other things.
i.e.	*id est*
subsp.	subspecies

LIST OF ABBREVIATIONS

(continued)

U	unit
w/v	weight by volume
v/v	volume by volume
g	gravity force
M.W.	molecular weight
std.	standard
spp.	species
RNA	ribonucleic acid
UV	ultraviolet

CHAPTER I
INTRODUCTION

Research and development of microbial agents for controlling insect vectors is rapidly progressing by using knowledge in genetic engineering and biotechnology. The rationale behind this is to find alternative methods to replace chemical insecticides. Since chemical insecticides are also toxic to non-target organisms and in addition have long and persistent residual activities in nature agents (Payne, 1988; Moffat *et al.*, 1991).

Bacillus thuringiensis subsp. *israelensis* (*B.t.i.*) has been widely used for mosquito larvae and black flies control (Maramorosch, 1985). *Bacillus thuringiensis* subsp. *israelensis* is a gram-positive bacterium which produces spherical shape parasporal crystalline inclusions during the sporulation stage (Aronson *et al.*, 1986). This spherical inclusion is called δ-endotoxin. It is believed that the δ-endotoxin is solubilized in the alkaline pH of the mosquito larva midgut and release the toxic proteins after proteolytic activation. The activated polypeptides then bind to epithelial cell and cause cell lysis. The larva stops feeding, paralyses and dies (Porter *et al.*, 1993). The δ-endotoxin is composed of at least five polypeptides with molecular weight of approximately 134, 128, 58, 70 and 27 kDa (Whiteley and Schnepf, 1986; Porter *et al.*, 1993). It has been shown that each of these proteins exhibits mosquito larvicidal property except the 27 kDa protein (Porter *et al.*, 1993). The δ-endotoxin is most active against *Aedes* mosquitoes, with less active against *Culex*

species whereas it has least toxicity or inactive against *Anopheles* species (Aronson *et al.*, 1986).

The improvement to increase the toxicity and efficiency of *B.t.i.* has been attempted by many methods such as the modification of the field formulations (Ali *et al.*, 1994), expression of *B.t.i.* toxin genes in the novel hosts (de Marsac *et al.*, 1987; Angsuthanasombat and Panyim, 1989; Chungjatupornchai, 1990; Murphy and Stephens Jr., 1992; Thanabalu *et al.*, 1992; Rak *et al.*, 1993) or applying in combination with the other agents (Manasherob *et al.*, 1994; Chui *et al.*, 1995). The modification of toxin genes has been done using various approaches, *i.e.*, using a stronger promoters either alone or in tandem, optimizing the strength of ribosome binding sites and downstream placement of bacterial enhancer and terminators sequences (Gentz *et al.*, 1981; Wong and Chang, 1986).

Chitin, a linear polymer of β-1,4-N-acetyl-D-glucosamine, is an abundant polysaccharide in nature. It is present in diatoms, fungi, protozoans, arachnids, insects (including mosquito), crustaceans, nematodes and other invertebrates (Muzzarelli, 1977). The chitin is found in the peritrophic membrane of both larval stage and adult of mosquitoes (Wigglesworth, 1972; Richards and Davies, 1977; Richards and Richards, 1977). The peritrophic membrane is formed as a membranous tubular sheath enclosed the epithelium cells and surrounding the ingested food in the mid gut (Wigglesworth, 1964; Eiseman and Binnington, 1994). The peritrophic membrane is a barrier to prevent pathogens and food particles in the gut of mosquito (Wigglesworth, 1964; Eiseman and Binnington, 1994).

Chitinolytic enzymes, *i.e.*, endochitinases, exochitinases (EC 3.2.1.14), β-N-acetylglucosaminidases (EC 3.2.130), chitobiases and chitosanases (EC 3.2 1.99) are the enzyme system that use chitin and its derivative such as chitosan and N-acetyl-D-glucosamine oligomers as the substrate. These enzymes are produced in chitin-containing organisms such as insect, crustaceans, marine invertebrates, nematodes, algae and fungi for using in their morphogenesis and exoskeleton development. In the insectivorous animals, *i.e.*, fishes, amphibians, reptiles, birds and mammals produce chitinolytic enzymes to digest chitin as the nutrition source (Flach *et al.*, 1992). The higher plants produce chitinolytic enzymes for defense mechanisms. A wide variety of bacteria produce chitinolytic enzymes for using enzymatic product as the carbon source (Flach *et al.*, 1992). Malarial parasites also produces the enzyme to digest the peritrophic membrane in the mosquito midgut during penetration (Huber *et al.*, 1991; Shahabuddin, 1993). Chitinase enzymes were also used as the adjuvants for insecticidal activity. The biological activity of *Bacillus thuringiensis* and baculovirus against the spruce budworm and gypsy moth respectively was enhanced with the addition of a chitinase preparation and the host exposed to the microbial agent and chitinase combination died more rapidly than exposed to the microbial agent alone (Smirnoff, 1971; Smirnoff, 1973; Morris, 1976; Shapiro, 1987). Since, chitinase disrupted the chitinous material of the insect gut (peritrophic membrane) resulting in greater penetration of agent into the host's hemolymph and subsequently caused septicemia (Smirnoff, 1974; Brandt *et al.*, 1978 and Gunner *et al.*, 1985).

The objective of this study was aimed at studying the chitinase enzymes produced by *Bacillus licheniformis*. The bacteria was isolated by screening on the chitin containing medium. The correlation between growth and chitinolytic enzymes production of *Bacillus licheniformis* will be studied. The molecular weight of all chitinase isozymes will be determined by SDS-PAGE and activity detection methods on polyacrylamide gel. The *Bacillus licheniformis* chitinolytic enzymes will be purified by fractionation and ion exchange chromatography methods. This purified chitinolytic enzyme will be used to characterize the physicochemical properties of the enzyme such as optimal temperature, optimal pH, temperature stability, pH stability and enzyme kinetics. Both crude and pure fractions of *Bacillus licheniformis* chitinolytic enzymes will be tested alone or in combination with *Bacillus thuringiensis* subsp. *israelensis* for the killing activity against *Aedes aegypti* larvae.

CHAPTER II
BACKGROUND

1 The mosquito larvicidal agents

Mosquitoes transmit some of the world's most serious diseases. The most important disease vectors are members of the subfamilies Culicidae and Anopheliae. There are 30 genera in the Culicidae subfamily but the medically important mosquitoes are *Culex*, *Aedes*, *Mansonia* and *Armigeres* (Porter *et al.*, 1993). Members of the genus *Aedes* are vicious biters and transmit yellow fever and dengue haemorrhagic fever as well as filariasis in several parts of the world (Porter *et al.*, 1993). *Culex quinquefasciatus* is an important vector of filariasis whereas *Culex tritaeniorhynchus* transmits Japanese encephalitis. *Anopheles* mosquitoes transmit malarial and filarial parasites (*Wucheria bancrofti* and *Brugia malayi*), and a few arboviruses (Service, 1986). It has been estimated that there are 90 million cases of human lymphatic filariasis worldwide (Porter *et al.*, 1993). The most common and debilitating disease transmitted by mosquitoes is malaria which is caused by 4 *Plasmodium* species (Porter *et al.*, 1993). Approximately 350 to 450 million people live in highly malarious areas, and malaria has an annual incidence of about 270 million cases and kill over 1 million children in Africa alone (Cowley *et al.*, 1993).

The future of mosquito borne diseases in the world will depend on the success of three different approaches; treatment of the diseases

by drugs, prevention of the diseases by vaccination and drugs and interference with mosquito vectors by control measures, repellents, etc. The treatment by drugs and prevention by vaccines may solve many problems in the industrialized world but not in less developed or developing countries. There only efficient mosquito control can hold the promise for the future.

These mosquito control methods can be done by two programs; mosquito adults control program and mosquito larvae control program. Chemical agents, *i.e.*, dichlorodiphenyltrichloroethane (DDT), gammaxane, fenthion, malathion, naled, permethrin, resmethrin and chlordane have been used as the method of choice for mosquito control over the last 45 years (Miller, 1992; Tietze *et al.*, 1993; Tidwell *et al.*, 1994). However, the mosquito borne diseases are on the rise again in many tropical areas (Miller, 1992). In addition the emergence of chemical pesticides coupled with a clearer appreciation of the long term detrimental effects of powerful chemicals to nonpest insects and concern about accumulation of pesticides in the food chain and environment has highlighted the need to quickly develop an alternative measures (Payne, 1988; Moffat *et al.*, 1991). The other method that are developing such as mosquito adult sterilization for reducing the larval population. The mosquito larvae control program has been classified into three main groups.

1.1 Environmental development methods

1.1.1 Filling and draining

The filling of mosquito breeding places with soil, rock or rubbish is the most permanent operations. It is of particular value in the elimination of small depressions that do not require a great deal of material, whereas, the draining of mosquito breeding places may be accomplished by open ditching, subsoil drainage, pumping and diking with use of tide gates. The choice of these methods depends upon many factors such as relative cost, terrain, soil type and extent of mosquito breeding area (Pratt and Littig, 1971).

Monomolecular films covering larger bodies of water can be used to interfere with mosquito breeding (Maramorosch, 1985).

1.1.2 Management of water

Management of water is of tremendous important in controlling mosquito production on man-made impoundments, farm ponds, sewage stabilization ponds, borrow pits, salt marshes and irrigated land (Pratt and Littig, 1971).

1.2 Chemical agents methods

A mosquito larvicidal chemical agent is a chemical used as an agent to destroy the larvae of mosquitoes. They are classified into three groups depending on their chemical compounds, inorganic, natural organic and synthetic organic (Pratt and Littig, 1971). They are divided into three groups base on the way in which the chemical enters the body of the insects and cause either stomach poisons

(ingested and absorbed through the alimentary system), contact poisons (penetrate the body wall) and respiratory poisons (fumigants and volatile chemicals enter the insect through the spiracles or breathing pores) (Pratt and Littig, 1971).

The important chemical materials such as Paris green is used as stomach poisons. It must be ingested by the larvae as they feed in treated waters. Methoxychlor, BHC and some of the organic phosphorus insecticides are also act as stomach poisons, but their primary action is as contact poisons and respiratory poisons which penetrate the body wall or the respiratory tract (Pratt and Littig, 1971). Larviciding oil, diflubenzuron, methopene, temephos and kerosene can be used as mosquito larvicidal agent (Tietze *et al.*, 1993).

If areas cannot be drained or filled at reasonable cost, chemical agents is often selected as the method of choice. Chemical agents is of primary importance in areas where immediate control of pest or disease carrying mosquitoes is necessary, particularly in cases of extensive flooding following natural disasters such as hurricanes or prolonged rainy seasons (Pratt and Littig, 1971).

As the case of mosquito adults control with chemical agents, the using of chemical pesticides leads to many problems such as detrimental effects to nontarget living things, accumulation of pesticides in the food chain, environment and the water pollution. These problems are leading to the need of developing an alternative measures. It has been recommended that a promising alternative measure is the application of biological control agents (Payne, 1988; Moffat *et al.*, 1991).

1.3 Biological agents methods

Biological insecticides including fungi, nematodes, bacteria, viruses can be used alone or in combination with inexpensive natural compounds (Schmutterer *et al.*, 1980) and will pose but little environmental hazards which will most likely be the future approach (Bulla, 1973; Burges, 1981; Kurstak, 1982; Rice, 1983).

There are many naturally occurring predators, plants, parasites and pathogens of vector insects including viruses, rickettsiae, fungi and bacteria which vary greatly in their mode of infection, site of replication and mechanism of pathogenicity (Maramorosch, 1985).

The World Health Organization of the United Nations has listed the biological control agents against mosquito larvae in order of priority since 1984 (Maramorosch, 1985). The priority is based on the results obtained in the field as well as in the laboratory. The most promising of all agents is *Bacillus thuringiensis israelensis* (H-14). There is no doubt that it has the greatest potential in controlling mosquitoes, not only when used alone, but, perhaps even more so, in integrated control. *Bacillus sphaericus* is considered to be the next one in order of preference (Yousten, 1984). The fungus, ***Lagenidium giganteum*** is also included in the first group (Maramorosch, 1985). The second group comprises certain larvivorous fish as well as nematodes (Maramorosch, 1985). The third group contains *Bacillus thuringiensis* strains other than H-14, as well as other larvivorous fish, *Toxorhynchites* mosquitoes (Focks *et al.*, 1982; Bailey *et al.*, 1983; Smittle and Focks, 1986), *Coelomomyces* fungi and snails. The fourth group is concerned with the control of tsetse flies, the predacious cyclops and *Turbellaria*

flatworms (Maramorosch, 1985). Some of certain snail predators are also included in this group. The committee lists iridescent, nuclear polyhedrosis viruses and *Metarrhizium anisopliae* under no priority or little potential (Maramorosch, 1985).

1.3.1 Bacterial agents

Certain entomopathogenic bacteria, particularly members of the genus *Bacillus* produce protoxin crystals during sporulation. These proteins are deposited as inclusions alongside the spore and are highly toxic to susceptible insects which ingest them. The protoxins dissolve in the alkaline pH of the mosquito larvae midgut, where they are proteolytically activated and bind to epithelial cell membranes in the brush border. The cells are destroyed, the larva stops feeding, paralyzed and dies (Porter *et al.*, 1993). Among the new generation of biological control agents which are currently being deployed for control of mosquitoes are strains of *Bacillus sphaericus* and *Bacillus thuringiensis*.

1.3.1.1 *Bacillus thuringiensis* subspecies *israelensis*

Depending on the particular strain of *Bacillus thuringiensis*, the protein toxins of *Bacillus thuringiensis* are classified into five classes base on characteristic of nucleotide sequences and insecticidal spectra (Porter *et al.*, 1993). Class I or Cry I is consisting of toxin specific to Lepidoptera including butterflies and caterpillars. Class II or Cry II is consisting of toxin specific to Lepidoptera and Diptera . However the dipteran activity of this class

is low compared to class IV and easily overlooked. Class III or Cry III is consisting of toxin specific to Coleoptera, *i.e.*, beetles. Class IV or Cry IV is consisting of toxin specific to Diptera species especially mosquitoes and blackflies. Class V or Cry V is consisting of toxin specific to Lepidoptera and Coleoptera species. Each class of protein consists of several subclasses (Hofte and Whiteley, 1989; Tailor *et al.*, 1992). Each class of toxin effectively kills a narrow range of target insects, and in recent years new *Bacillus thuringiensis* strains have been discovered with a target range including organisms other than insects (Aronson *et al.*, 1986; Feitelson *et al.*, 1992). For nearly 30 years, various *Bacillus thuringiensis* strains have been successfully used as biopesticides, mostly against agriculturally important caterpillar pests (Moffat, 1991). The molecular potency is found to be higher when compared with that of chemical pesticides, *i.e.*, about 300 times higher than that of synthetic pyrethroids or 80,000 times higher than that of organophosphates (Porter *et al.*, 1993). The toxin is degraded in a few days after their action is complete and the bacteria appears to be safe for other insects, animals and the environment (Davidson, 1982; Laird *et al.*, 1990).

A strain of *Bacillus thuringiensis* with a high larvicidal activity and was found to be specific for mosquitoes has been isolated from the soil of a mosquito breeding site in Israel by Goldberg and Margalit (Goldberg and Margalit, 1977). On the basis of flagella antigen, this strain is classified as *Bacillus thuringiensis* subspecies *israelensis* or *B.t.i.* (de Barjac, 1978). Since then, many workers have reported the occurrence of this strain in various regions of the world (Zhang *et al.*, 1984; Shim *et al.*, 1990).

It has been shown that the toxins of *Bacillus thuringiensis* subsp. *israelensis* are produced during sporulation. It is deposited as an endosporal crystal and is most active against *Aedes* mosquitoes. with less toxicity to *Culex* species and least to *Anopheles* species (Aronson *et al.*, 1986).

B.t.i. toxic proteins and genes

Bacillus thuringiensis subsp. *israelensis* produces three major inclusion types during sporulation which are toxic for mosquito larvae (Whiteley and Schnepf, 1986). The crystals within the inclusions are composed of at least five polypeptides of approximately 134 kDa, 128 kDa, 58 kDa, 70 kDa and 27 kDa. The new terminologies for 134, 128, 58, 70 and 27 kDa are CryIVA, CryIVB, CryIVC, CryIVD and CytA respectively. The sizes of these proteins lie in the range 98 to 145, 93 to 135, ≈58, 65 to 72 and 25 to 28 kDa respectively (Porter *et al.*, 1993). The CytA protein has hemolytic and no or marginal mosquitocidal activity. In contrast, the CryIVA, CryIVB, CryIVC, CryIVD proteins are all toxic to mosquito larvae. None of the individual crystal proteins is as toxic as the intact crystal complex containing all the proteins (Hofte and Whiteley, 1989). One explanation is that synergism may be important for toxicity. *Bacillus thuringiensis* subsp. *israelensis* toxins are glycoproteins containing approximately 1.0 % neutral sugars and 1.7 % amino sugars. It has been suspected that N-acetylglucosamine containing oligosaccharides may be important for full activity of the δ-endotoxins (Pfannenstiel *et al.*, 1990). The toxic domain of the CryIVA, CryIVB, CryIVC and CryIVD protoxins resides in the N-

terminal half of each protein and is equivalent to the gut-activated core toxin whereas the more highly conserved C-terminal half of each protoxin is not required for toxicity or host range. It is probable that the high conservation of C-terminal amino acid sequences is required for some other function such as the formation of crystalline inclusions (Porter *et al.*, 1993). The sequences of CryIVA, CryIVB and CryIVC toxin in N-terminal half share significant homology which is restricted largely to five distinct regions named blocks 1 to 5 (Hofte and Whiteley, 1989). The CryIVD toxin has extensive homology with the other classes only within block 1. The *cryIVA*, *cryIVB*, *cryIVC* and *cryIVD* toxin gene of *Bacillus thuringiensis* subsp. *israelensis* together with the gene coding for the CytA cytolytic protein are all located on the same 72 MDa plasmid (Porter *et al.*, 1993).

Toxicity assessment

Field trials with various formulations of *Bacillus thuringiensis* subsp. *israelensis* against *Aedes*, *Anopheles* and *Culex* mosquitoes have demonstrated their safety and potential for controlling mosquito larvae (Lacey and Undeen, 1986). However operation success against the three major mosquito genera, *Aedes*, *Anopheles* and *Culex*, has been limited and confined mainly to temperate regions of the world where these insects are merely a nuisance. Mosquitocidal bacteria currently represent a tiny fraction of the biopesticide market, which in turn is still only a small fraction of the annual worldwide pesticide market (Porter *et al.*, 1993).

Bacillus thuringiensis subsp. *israelensis* does not survive long in highly polluted water and is particularly prone to UV inactivation in strong sunlight (Mulligan *et al.*, 1980; Mulla, 1990). Nevertheless only in the case of *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus* still have the most field trials against mosquitoes.

Formulations of *Bacillus thuringiensis* subsp. *israelensis* which have been successfully used in the laboratory trials and in the field are liquid concentrates, powders (dry , primary, technical or wettable) granules; corncob formulations (Ali *et al.*, 1994), briquettes and encapsulated forms. These formulations generally cause high rate of mortality in susceptible mosquito species but the rapid settling of the bacterial spores leads to a relatively short duration of control under normal field conditions ranging from 1 to 4 weeks (Porter *et al.*, 1993). There are several possible ways to overcome the settling problem and enhance larval mortality. The floating formulations by microlipid droplet encapsulation method make equally effective against *Anopheles* and *Culex* larvae, unlike the nonencapsulated method which give much lower activity against *Anopheles* larvae (Cheung and Hammock, 1985). Mosquitocidal toxin genes have been expressed in bacteria which normally exist at or near the water surface such as cyanobacteria and *Caulobacter* species (de Marsac *et al.*, 1987; Angsuthanasombat and Panyim, 1989; Chungjatupornchai, 1990; Murphy and Stephens Jr., 1992; Thanabalu *et al.*, 1992; Rak *et al.*, 1993). The ubiquitous photosynthetic cyanobacteria exist at or near the water surface. These bacteria are adaptable to both freshwater and saltwater environments, they have a

wide temperature tolerance and being protoautotrophs that have limited nutritional requirements (Rippka *et al.*. 1979). *Caulobacter* species are found in almost every aquatic habitat and within these habitats they are found predominantly in the regions at or close to the water surface (Poindexter, 1981). In the flagellate swarmer stage, *Caulobacter* species are motile, thus allowing distribution through the habitat. In both the swarmer and the stalked stages, they are capable of attachment to solid particles at or near the water surface. *Caulobacter* species are able to persist and grow in environments with low nutrient concentrations (Poindexter, 1981). Slow release formulations of *Bacillus thuringiensis* subsp. *israelensis* have been tried with limited effectiveness (Novak *et al.*, 1985).

The other main limitations of natural strains of *Bacillus thuringiensis* subsp. *israelensis* which have discouraged their development are the high costs of fermentation, the insufficient toxicity and narrow host range compared with that of the chemical mosquitocidal agents (Porter *et al.*, 1993). The second problem may be solved by improvement the expression levels of the toxins in *Bacillus thuringiensis* subsp. *israelensis* by the use of stronger promoters either alone or in tandem, by optimizing the strength of ribosome binding sites (Vellanoweth and Rabinowitz, 1992) and by downstream placement of bacterial enhancer and terminators sequences (Gentz *et al.*, 1981; Wong and Chang, 1986) or using the *Bacillus thuringiensis* subsp. *israelensis* spores combine with the other agents such as protozoan (Manasherob *et al.*, 1994) for enhance the toxicity. It has been shown that the toxicity of *Bacillus thuringiensis* subsp. *israelensis* in mosquito larvae is enhanced by

encapsulation in the protozoan *Tetrahymena pyriformis*. The *Aedes aegypti* larvae which fed on *Tetrahymena pyriformis* loaded with *Bacillus thuringiensis* subsp. *israelensis* died about three times faster than when fed on the same concentrations of *B.t.i.* alone due to ingestion of higher toxin concentrations, reflected by shorter death times of exposed populations. The combination between *B.t.i.* and teflubenzuron is also allowed the higher toxicity than *B.t.i.* alone (Chui *et al.*, 1995). The last problem of narrow host range of *B.t.i.* is probably addressed by the genetic engineering of strains expressing combinations of new and existing mosquitocidal toxins and the engineering of increased potency, host range and persistence. This approach will also have an impact on the cost of application (Porter *et al.*, 1993).

1.3.1.2 The other *B.t.* strains

Many workers have reported the occurrence of other highly mosquitocidal *Bacillus thuringiensis* strains. They are *Bacillus thuringiensis* subsp. *morrisoni* PG-14 (Padua *et al*, 1984) and subsp. *medellin* (Orduz *et al.*, 1992; Orduz *et al.*, 1994) which occur in various regions of the world. However, it is generally accepted that the mosquito specific *Bacillus thuringiensis* with moderate to low toxicities is also disseminated in natural environments. The latter strains are *Bacillus thuringiensis* subsp. *darmstadiensis* 73-E10-2 (Padua *et al.*, 1980), *kyushuensis* (Ohba and Aizawa, 1979; Knowles *et al.*, 1992), *fukuokaensis* (Yu *et al.*, 1991), *aizawai* (Haider *et al.*, 1987), *galleria* (Ahmad *et al.*, 1989), *amagiensis* (Ishii and Ohba, 1993b), *canadensis*, *darmstadiensis*,

shandongiensis (Ishii and Ohba, 1993a) and subsp. *kurstaki* HD-1 that can kill mosquitoes as well as certain lepidopteran larvae. The mosquitocidal toxin of *B. thuringiensis* subsp. *kurstaki* HD-1 is classified into class II of toxicity of *Bacillus thuringiensis* (Yamamoto and McLaughlin, 1981).

1.3.1.3 *Bacillus sphaericus* and other bacteria

The highly toxic strains of *Bacillus sphaericus* used in the field produce two proteins with masses of 51.4 and 41.9 kDa. Both proteins are jointly responsible for toxicity (Broadwell *et al.*, 1990; Davidson *et al.*, 1990). These toxins are produced at the onset of sporulation and accumulate in parasporal crystalline inclusions (Baumann *et al.*, 1985). The lower toxic strains of *Bacillus sphaericus* do not produce the 51.4 and 41.9 kDa (binary) toxins. Their toxicities have only about 1,000 fold lower than that of the high toxicity strains. The toxin appears during vegetative growth and is cell associated. The molecular weight of toxin is approximately 100.6 kDa and composed with 870 amino acids (Porter *et al.*, 1993). These toxic system is also contained in the highly toxic strains of *Bacillus sphaericus*. It has been shown that *Bacillus sphaericus* is generally most toxic for mosquitoes of the genus *Culex*, with less activity against *Anopheles* species and least activity against *Aedes* species (Thiery and de Barjac, 1989). The most toxic strains as recommended by WHO are *B. sphaericus* strains 1953 and 2362. Another genus that contain mosquitocidal toxins are *Pseudomonas fluorescens* (Murty *et al.*, 1994) and *Clostridium bifermentans* subsp. *malaysia* (Charles *et al.*, 1990).

1.3.2 Fungal agents

The fungi species of the genus *Coelomomyces*, *Culicinomyces clavasporus* and *Lagenidium giganteum* (Lacey and Orr, 1994; Federici, 1995) occurs in many species of mosquitoes and may ultimately have a potential role in mosquito control programs. However, the field trials are needed to be done.

1.3.3 Botanical agents

Botanical mosquitocidal agents may provide a safe and effective short term control technology for larvae and adult mosquitoes (Sukumar *et al.*, 1991). Among the more promising candidates are phytochemicals extracted from *Tagetes* spp. in the family *Asteraceae*. Extracts of *Tagetes* spp. are effective against larvae and adults of *Aedes aegypti* (Maradufu *et al.*, 1978; Aronson *et al.*, 1981; Green *et al.*, 1991; Perich *et al.*, 1994) and *Culex tritaeniorhynchus* Giles larvae (Singh *et al.*, 1987). For centuries, crude extracts from the neem tree *Azadirachta indica* have been used in Asia and parts of Africa to control insect pests (Schmutterer *et al.*, 1980).

1.3.4 Protozoan agents

The protozoan parasites, *i.e.*, *Nosema algerae* (Federici, 1995), *Thelohania* and *Lankesteria*, have been studied as an alternative method for controlling mosquitoes. Some of information on pest control by protozoan agents have been documented by Pratt and Littig (Pratt and Littig, 1971).

1.3.5 Predator agents

Several kinds of predator agents have been studied and reported. Some of them demonstrate a good potential for mosquito control. The most promising candidates are larvivorous fish, *i.e.*, *Gambusia affinis*, *Tilapia zilli* and *Oreochromis niloticus*. It has been shown that *Toxorhynchites* mosquitoes especially *Toxorhynchites splendens* and *Toxorhynchites rutilus rutilus* can control mosquito larvae under certain conditions (Panicker and Bai, 1983, Focks *et al.*, 1982; Bailey *et al.*, 1983; Smittle and Focks, 1986; Miyagi *et al.*, 1992; Asimeng and Mutinga, 1993; Amalraj and Das 1994; Lacey and Orr, 1994; Federici, 1995). Other promising predator agents that demonstrate a good potential for mosquito control in the laboratory are mermithid nematodes species *Romanomermis culicivorax* (Lacey and Orr, 1994; Federici, 1995) and *Notonecta*, predatory cyclopoid copepods; *Mesocyclops longisetus* (Lacey and Orr, 1994; Tietze *et al.*, 1994), snails and *Turbellaria* flatworms.

2 The gut barrier, peritrophic membrane

The lining of the animals gut are delicate and readily injured by hard particles of food that they digested. In most other animals, the gut cells protect themselves by secreting a covering of slime or mucous. But the most arthropods including mosquito do not secrete mucous in the intestine and the chief component of the protective cuticle of the insect is chitin which can be regarded as a tough solid sort of mucous. The fore gut is formed in development by an ingrowth from the surface and lined by a chitinous cuticle like that

which covers the surface of the body. The hind gut also is similarity lined with cuticle. The intervening section where the greater part of digestion and absorption takes places has no cuticle, but it is generally protected by a thin delicate detached membrane of chitin that is called the peritrophic membrane (Wigglesworth, 1964; Eiseman and Binnington, 1994). This peritrophic membrane term was called over 100 years ago by Balbiani in 1890 (Ramos *et al.*, 1994). The concept of membrane at present has since changed to that of a lipid bilayer, which the peritrophic membrane is clearly not. Thus, the term peritrophic matrix better describes this type of structure (Ramos *et al.*, 1994).

There are two types of peritrophic membrane and they are formed in two different ways (Waterhouse, 1953; Wigglesworth, 1972). The first type is found in Acridiids, Odonata, Phasmids Ephemeroptera, caterpillars, *Apis*, *Vespa* and their larvae, *Tenebrio*, *Hydrophilus* and other beetles. The membrane is made up of concentric lamellae which is independent or loosely attached to one another. It is produced by the separation of thin sheets from part or all of the general surface of the cells throughout the length of the mid gut (Wigglesworth, 1972). The second type is found in the larvae and adults of some Diptera, in the earwig and perhaps in termites, and consists of a single uniforms layer. It is secreted in viscous form by a group of cells near the junction of fore and mid gut (anterior limit of the mid gut) and extruded by a muscular press in this region through an annular cleft between the esophageal invagination and the mid gut, and in so doing solidifies to form a homologous tube. Finally, the

peritrophic membrane of this type is always of uniform circumference throughout its length (Wigglesworth, 1972).

The peritrophic membrane has the same components as the inner layers of the cuticle. It is consisting of chitin with protein incorporated in it. The chitin content of the membrane in different insects ranges from 3.7-12.9 percent and the protein content varies from 21-47 percent. The remainder of the dry weight is probably made up of other mucopolysaccharides (Wigglesworth, 1972). The glycogen in the mid gut cells of many insects may contribute to the formation of the membrane. So that, the permeability of the peritrophic membrane varies somewhat from one insect to another (Wigglesworth, 1972).

The peritrophic membrane in mosquito

The peritrophic membrane or matrix is formed as a membranous tubular sheath enclosed the epithelium cells and surrounding the ingested food in the mid gut of mosquito in larvae and adult stage (Wigglesworth, 1972; Richards and Davies, 1977; Richards and Richards, 1977). In mosquito larvae the membrane is secreted by a narrow band of cells at the anterior end of the mid gut and is formed as a continuous tube. Whereas, in mosquito adults all mid gut columnar cells secrete the substance of the membrane which is deposited around the ingested blood mass (Clements, 1992; Ramos *et al.*, 1994). A ultrastructural study produced direct evidence that the peritrophic membrane is a barrier to infection in adult stage of *Aedes aegypti* mosquito. The formations of peritrophic membrane in adult stage of *Aedes aegypti* mosquito starts immediately after the

blood meal, but about 12 hours elapse before it attains a mature structure (Freyvogel and Staubli, 1965; Perrone and Spielamn, 1988)

3 Chitin and chitinolytic enzymes system

3.1 Chitin and its derivatives

3.1.1 Definition

Chitin is a polymer of unbranched chains of β-1, 4 linked 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine, GlcNAc) and is related to cellulose with the C-2 hydroxyl groups replaced by acetamido residues (Figure 1). The chitin substance is the most abundant renewable natural resource after cellulose (Deshpande, 1986; Gooday, 1991; Nicol, 1991). It is estimated that the worldwide annual recovery of chitin from the processing of marine invertebrates is 37,300 tonnes (Nicol 1991). Chitin is widely distributed in nature such as in insect exoskeletons, shells of crustaceans, marine invertebrates, algae and fungal cell walls (Muzzarelli, 1977).

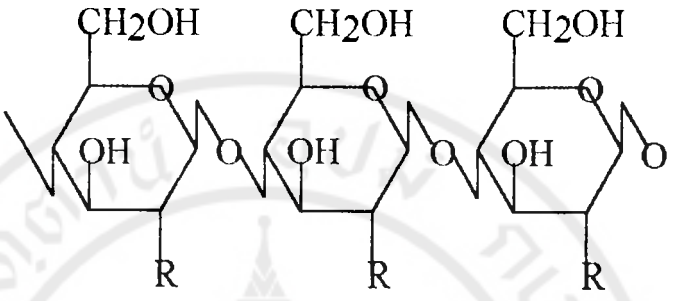
The partially deacetylated derivative of chitin is chitosan and corresponding to the D-glucosamine polymer (Figure 1A).

The glycol chitin is a soluble form of chitin (Figure 1B) and can be prepared by acetylation of glycol chitosan. The colloidal chitin is a small particle size of chitin that can be prepared by solubilization with strong acid and resolubilization with alcohol.

3.1.2 The applications of chitin and its derivatives

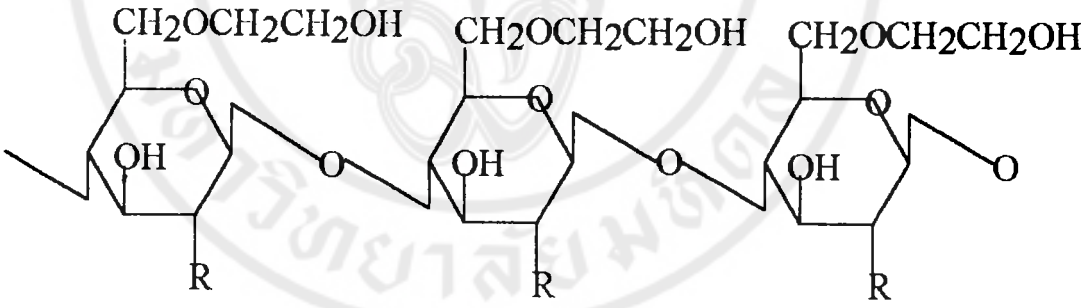
Chitin and chitosan and their respective monomers have significant potential applications in the pharmaceutical industry and in

A



Chitin (R = $NHCOCH_3$)
Chitosan (R = NH_2)
Cellulose (R = OH)

B



Glycol chitin (R = $NHCOCH_3$)
Glycol chitosan (R = NH_2)

Figure 1. The chemical structure of chitin, chitosan and cellulose (A), glycol chitin and glycol chitosan (B).

medicine. They can be used in making of contact lenses, artificial kidney membranes, antifungal preparations, biodegradable pharmaceutical carriers, blood anticoagglulants, microbiological media, wound healing accelerators and dressings for burns (Muzzarelli, 1977) In the agricultural industry, seeds can be protected from fungi by using a capsule containing chitin and chitosan derivatives. The cosmetic industry makes shampoos, gels, creams and even sponges with chitin and chitosan. In the food industry, they are used in the preparation of fruit juices or soluble coffee. Moreover, in the other industrial production, chitin and chitosan are used as chelating polymers for harmful metals (i.e. Hg, Pb and Cu), paper and textile additives, textile finishes, photographic products and processes, coagglulants for suspensions, water purifying systems and protein recovery processes (Muzzarelli, 1977). In addition, some shorter chitin oligosaccharides could have an inhibitory effect on the development of cancerous tumors (Flach *et al.*, 1992). Finally, a glycol chitin is used in the assays which need a soluble substrate (Trudel and Asselin, 1989) whereas colloidal chitin is used in the sensitivity assays of enzymatic activity (Reissig *et al.*, 1955; Boller *et al.*, 1983).

3.2 Chitinolytic enzymes system

A chitinases enzyme has been described since 1911 by Bernard (Flach *et al.*, 1992) who have found a thermosensitive and diffusable antifungal factor in orchid bulbs. Chitinases or chitinolytic enzymes are defined as enzymes cleaving a bond between the C1 and C4 of two consecutive N-acetyl-D-glucosamines of chitin. The

nomenclature system of chitinolytic enzymes is currently reclassified. Flach *et al.* (1992) have characterized the chitinolytic enzymes as endochitinases (EC 3.2.1.14), exochitinases (EC 3.2.1.14), β-N-acetylglucosaminidases (EC 3.2.1.30) and chitobiases (3.2.1.52) (Flach *et al.*, 1992). β-N-acetylglucosaminidases is usually defined as an enzyme releasing N-acetyl-D-glucosamines monomers from terminal non reducing ends of chitin and exochitinases are classified as enzyme releasing chitobiose (N-acetyl-D-glucosamines dimer) whereas endochitinases are classified as an enzyme splitting within the chitin polymer (Flach *et al.*, 1992). Chitobiase is defined as an enzyme releasing N-acetyl-D-glucosamines monomers from chitobiose. It has been demonstrated that some chitinases also display a more or less pronounced lysozyme activity (EC 3.2.1.17) corresponding to the cleavage of a glycosidic bond between the C1 of N-acetylmuramic acid (MurNAc) and the C4 of N-acetyl-D-glucosamine in the bacterial peptidoglycan (Jolles and Jolles, 1984). It has been shown that transglycosidase activities are associated with exochitinase activities (Flach *et al.*, 1992). The chitinolytic enzymes system is divided into two main classes; endochitinases (EC 3.2.1.14) and β-N-acetylglucosaminidases (EC 3.2.1.30) by Shaikh and Deshpande (1993). β-N-acetylglucosaminidases is also termed as chitobiases or N-acetylhexosaminidases (EC 3.2.1.52) (Shaikh and Deshpande, 1993). The existence of a third class of enzyme, exochitinase, has also been suggested. Endochitinases randomly hydrolyse GlcNAc polymers and eventually giving diacetylchitobiose as the major product together with some triacetylchitotriose. Though the β-N-actylglucosaminidases act preferentially on a dimer,

diacetylchitobiose, some enzymes in this group cleave GlcNAc units from the non reducing ends of chitin chains. The exochitinases also catalyse processive release of diacetylchitobiose (or GlcNAc) units from the non reducing ends of chitin chains. However, the classification of exochitinase and endochitinase enzymes depends mainly on the nature of the substrate. For example, the *Streptomyces* chitinase complex degrades pure crystalline β-chitin of diatom spines only from the non reducing ends to yield diacetylchitobiose, whereas colloidal chitin is degraded by this chitinase to a mixture of oligomers and diacetylchitobiose (Shaikh and Deshpande, 1993).

Chitosanases (EC 3.2.1.99) are a class of endo-acting enzymes which hydrolyse chitosan to a mixture of glucosamine oligomers in which the degree of polymerization (DP) is between two and eight (Ohtakara, 1988; Uchida and Ohtakara, 1988). Chitosanases are distinguished from chitinases by their substrate specificity.

3.2.1 Activity determination of chitinolytic enzymes

Several techniques are available for detecting of chitinolytic enzymes activity. Chitinase activities can be assayed by monitoring changes in the molecular size of a substrate by viscosity measurement or reduction of turbidity measurement, determination of chitooligosaccharides for endochitinases or of N-acetyl-D-glucosamine for β-N-acetylglucosaminidases (Flach *et al.*, 1992). These determinations can be performed by measurements of reducing power, by using ρ-dimethylaminobenzaldehyde after enzymatic conversion of the oligosaccharides into monosaccharides (Reissig *et al.*, 1955;

Boller *et al.*, 1983), or using [(^{3}H)acetyl]-chitin as substrate (Wood and Kellogg, 1988). Chitinases which have chitobiase or β-N-acetylglucosaminidase activity can hydrolyse PNP-N,N',N'',N''',N''''-pentaacetyl-β-chitopentaoside (Usui *et al.*, 1988; Vasseur *et al.*, 1990) or equivalent di-, tri-, or tetraosides (Roberts and Selitrennikoff, 1988; Benhamou and Asselin, 1989). Some endochitinases have lysozyme activity, which can easily be measured by lysis of a *Micrococcus luteus* suspension. The activity can be detected on polyacrylamide gels (Trudel and Asselin, 1989) or isoelectric focusing gels (Hendy *et al.*, 1990) using glycol chitin or a fluorescent derivative as substrate. The chitinolytic enzymes are able to detect by covering electrophoresis gels with filter paper impregnated with 4-methylumbelliferyl-β-D-N,N'-diacetylchitobioside or 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotriose followed by photography of gels under ultraviolet illumination after removal of the paper (Trudel *et al.*, 1989).

3.2.2 Chitinolytic enzymes source in nature

Chitinolytic enzymes are commonly produced in chitin-containing organisms, *i.e.*, insect, crustaceans, marine invertebrates, nematodes, algae and fungi for using in their morphogenesis and exoskeleton development (Flach *et al.*, 1992; Shaikh and Deshpande, 1993). However, in some other organisms which do not contain chitin also produce chitinolytic enzymes; for example, a wide variety of bacteria, insectivorous animals and higher plants (Flach *et al.*, 1992). The higher plants developing these enzymatic systems for defense mechanisms in response to pathogens and biotic stresses (Flach *et al.*,

1992). Chitinases are secreted by the pancreas and the gastric mucosa of insectivorous animals, *i.e.*, fishes, amphibians and reptiles, as well as by the gastric mucosa of some insectivorous birds and mammals for using chitin as the nutrition source. Bacteria develop these enzymatic systems for using enzymatic product (N-acetyl-D-glucosamine) that was degraded from chitin as the carbon source (Flach *et al.*, 1992; Shaikh and Deshpande, 1993).

3.2.2.1 Plant chitinases

Plant chitinases are widely studied by several investigators. They are of interest partly due to the probable absence of chitin natural substrates in the plant itself. Chitinases are therefore considered as a plant defense against pathogens (Flach *et al.*, 1992). Chitinases are present either constitutively, specific steps of plant development or after induction. It has been shown that the latex contains large amounts of chitinase constitutively in *Hevea* (Martin, 1991). The induction factors of plant chitinase enzymes are numerous abiotic agents, *i.e.*, ethylene treatment, salicylic acid, salt solutions, ozone, UV light, chemical induction or wounding and by biotic factors such as fungi, bacteria, viruses, viroids, fungal cell wall components and oligosaccharides (Punja and Zhang, 1993). The mechanisms of the inductions of these factors are not completely elucidated (Flach *et al.*, 1992).

These chitinase enzymes can be found in any organs of plant ; *i.e.*, leaf, root, seed and close contact site with fungal cells. In these organs, plant chitinases can be found in any organelles or fluid ; *i.e.*, vacuole, golgi cisternae, extracellular fluid, apoplastic

compartments. Different classes of plant chitinases are distinguishable by molecular, biochemical and physicochemical criteria. Thus, plant chitinases may differ in substrate binding characteristics, localization within the cell and specific activities (Punja and Zhang, 1993). The plant chitinases are classified into five classes (class I-V) based on amino acid sequence features (Shinshi *et al.*, 1990; Collinge *et at.*, 1993; Melchers *et al.*, 1994). Class I chitinases are enzymes with an N-terminal cysteine-rich domain of about 40 amino acids and a highly conserved main structure. This class possess a leucine-rich or valine-rich signal peptide and can be acidic or basic proteins. Class II chitinases lack the N-terminal cysteine-rich domain but have high amino acid sequence identity to the main structure of class I chitinases. This class seem to be acidic proteins. Class III chitinases show no sequence similarity to enzymes in class I or II and can be acidic or basic proteins. Class IV chitinases contain a cysteine-rich domain and a conserved main structure which resemble those of class I chitinases but are significantly smaller due to the deletion. The amino acid sequence identity of class IV chitinases with class I chitinases is only 41-47 % compared with 59-63 % identity between the individual chitinases of class IV. The class I and II enzymes are serologically related, while class I and IV enzymes are serologically distinguishable. Class V chitinases show sequence homologous to exochitinases of *Bacillus circulans*, *Serratia marcescens* and *Streptomyces plicatus* bacteria with no sequence similarity to the previous classes.

3 2.2.2 Fungal chitinases

Fungal chitinolytic enzymes could be involved in the growth of the fungus itself. All types of chitinolytic enzymes activities are found in fungi ; *i.e.*, endochitinase, β-N-acetylglucosaminidase and exochitinase. Chitinases can be soluble, probably sequestered in microsomal, lysosomal vacuoles, membrane-bound or wall-bound and in culture supernatant (Flach *et al.*, 1992).

3.2.2.3 Bacterial chitinases

Bacteria play a large role in chitin mineralisation, for example in marine waters and sediments, but not all species are able to hydrolyse chitin. Chitinase enzymes are found in some strains of *Aeromonas* (Ueda *et al.*, 1994), *Serratia marcescens* (Monreal and Reese, 1969), *Vibrio alginolyticus* (Aribisala and Gooday, 1978), *Vibrio vulnicius, Vibrio harveyi, Chromobacterium, Enterobacter, Klebsiella, Pseudomonas, Serratia liquefaciens* and *Clostridium.* (Clarke and Tracey, 1956; Shaikh and Deshpande, 1993) and many species of *Bacillus* such as *licheniformis* (Takayanagi *et al.*, 1991), *amyloliquefaciens, subtilis* and *circulans* (Cody, 1989; Sabry, 1992) also produce these enzymes.

Chitinase produced in *Bacillus circulans* and *Serratia marcescens* are the most of interested and studied by many groups of scientist. In *Bacillus circulans,* at least six major chitinases (A1, A2, B1, B2, C and D) have so far been found in the culture supernatant when induced with chitin (Watanabe *et al.*, 1990a). Among these enzymes, the chitinase A1 has the most important role in degradation of chitin to chitobiose $(GlcNAc)_2$. The *chiA* and *chiD*

genes that encode the precursor of chitinase A1 and D respectively have been cloned and sequenced (Watanabe *et al.*, 1990b; Watanabe *et al.*, 1992; Watanabe *et al.*, 1994b). The action on partially N-acetylated chitosan and site directed mutagenesis of *Bacillus circulans* chitinases enzyme were also studied (Watanabe *et al.*, 1994 a; Mitsutomi *et al.*, 1995). The *Serratia marcescens* chitinase enzymes system is extracellular and is composed of five chitinolytic proteins with subunit molecular weights of 21,000, 36,000, 48,000, 52,000 and 57,000 respectively (Flach *et al.*, 1992). The activity type of the enzymes are endochitinase and chitobiase (Monreal and Reese, 1969). The chitinase gene has been successfully transformed to some organisms such as *Lactococcus lactis* and *Lactobacillus plantarum* (Brurberg *et al.*, 1994).

3.2.2.4 Marine invertebrate chitinases

The chitinase enzymes activities of marine invertebrates such as *Euphasia superba* and *Meganyctiphanes norvegica* in the lower temperature range are still high, suggesting a functional adaptation to low temperature in the seawater (Spindler and Buchholz, 1988).

3.2.2.5 Insect chitinases

Several insect chitinases are glycoproteins (Funke *et al.*, 1989; Kramerov *et al.*, 1985). The molecular weights of insect chitinolytic enzymes usually range between 40,000 and 150,000 respectively (Flach *et al.*, 1992). The chitinolytic enzymes are found

principally in the integument, molting fluid, hemolymph and alimentary canal.

3.2.2.6 Protozoan chitinases

Malaria parasites (ookinetes) appear to digest the peritrophic membrane in the mosquito midgut by using chitinase enzymes during penetration and transmission (Huber *et al.*, 1991; Shahabuddin, 1993; Shahabuddin and Kaslow, 1994).

3.2.2.7 Fish chitinases

Chitinases are also found in the digestive tract of some fishes feeding on invertebrates such as in stomach of Japanese eel (Kono *et al.*, 1990).

3.2.3 Applications of chitinolytic enzymes

Chitinolytic enzymes are used in the fungal protoplast preparation, chito-oligosaccharides preparation, detection the chitin or chitosan location of interested cells. Chitinolytic enzymes are used as the adjuvants for fungicidal and insecticidal agents (Smirnoff, 1971; Smirnoff, 1973; Morris, 1976; Shapiro, 1987; Flach *et al.*, 1992; Shaikh and Deshpande, 1993).

CHAPTER III
MATERIALS AND METHODS

1 Chemicals and reagents

All chemicals used throughout this study were analytical grade or the purest grade available.

Glacial acetic acid, ammonium sulfate, boric acid, calcium chloride, disodium hydrogen phosphate (Na_2HPO_4), ethanol, glycerol ($C_3H_8O_3$), hydrochloric acid, magnesium chloride, manganese chloride, methanol, phosphoric acid, potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen phthalate, potassium hydroxide, sodium acetate (CH_3COONa), sodium azide, sodium chloride, sodium dihydrogen phosphate (NaH_2PO_4), sodium hydroxide and Triton X-100 were obtained from Merck (Germany).

Acetic anhydride, N-acetyl-D-glucosamine, ammonium persulfate, bromphenol blue, bovine serum albumin, calcofluor white M2R, chitin, Coomassie brilliant blue G-250, ρ-dimethylamino benzaldehyde (ρDAB), disodium ethylene diamine tetraacetic acid (EDTA), glycol chitosan, β-mercaptoethanol, N,N'-methylene bisacrylamide, potassium chloride, potassium tetraborate ($K_2B_4O_7$), sodium dodecyl sulfate (SDS), and Tris [(hydroxymethyl) aminomethane] were purchased from Sigma (USA).

Acrylamide and N,N,N',N'-tetramethylethylenediamine (TEMED), were obtained from GIBCO BRL (USA).

All bacteriological media were obtained from Difco Laboratory (USA), agar from OXOID (England) and sucrose from Amresco (USA).

2 Preparation of colloidal and glycol chitin

2.1 Colloidal chitin preparation

The colloidal chitin was prepared from commercial chitin (Sigma). It was prepared by dissolving 20 g of crude chitin with 300 ml of 37 % hydrochloric acid (12 N) and stirred at 4°C for overnight. The suspension was added with 2 litters of 95 % (v/v) ethanol, mixed vigorously and left standing at -20°C for overnight. The acid hydrolyzed chitin was collected by centrifugation at 6,000 rpm for 10 min. The precipitate was washed many times with distilled water until the pH value reach 7 and finally the solution was lyophilized for 24 hrs at -150°F.

2.2 Glycol chitin preparation

The glycol chitin was obtained by acetylation of glycol chitosan. Five grams of glycol chitosan was dissolved in 100 ml of 10 % (v/v) glacial acetic acid by grinding in a mortar. The viscous solution was allowed to stand overnight at 22°C, 450 ml of absolute methanol was slowly added and the solution was vacuum filtered through a Whatman No. 4 filter paper. The filtrate was transferred into a beaker and 7.5 ml of acetic anhydride was added with magnetic bar and stirring for a few minutes. The resulting slurry gel was allowed to stand for 30 min at room temperature and then cut into

small pieces. The liquids eluting from the gel pieces were discarded. Gel pieces were transferred to a blender, covered with the excess volume of absolute methanol and homogenized for 4 min at the highest speed. This suspension was centrifuged at 16,000 g for 5 min at 4°C. The gelatinous pellet was resuspended in about 1 volume of absolute methanol, homogenized and centrifuged as in the preceding step. The pellet was resuspended in distilled water (500 ml) containing 0.02 % (w/v) sodium azide and homogenized for 4 min. The final 1 % (w/v) stock solution of glycol chitin was obtained and it was kept at 4°C.

3 Microorganisms and culturing procedures

All pure cultures of *E.coli*, *Bacillus thuringiensis* subsp. *israelensis* (*B.t.i.*) and *Bacillus licheniformis* were kept as stock cultures at 4°C after streaking and growing at 37°C on LB (Luria-Bertani) agar (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar). The preparation of frozen stock cultures was done by growing in LB broth consisting of 1.0% tryptone, 0.5% yeast extract and 0.5% NaCl in rotary shaker until the growth of bacteria reached the exponential phase. Subsequently, a 1.5 ml aliquot of a mixture consisting of two volumes of cell cultures and one volume of 45% (v/v) glycerol was slowly freezed and kept at -70°C.

3.1 Bacterial culturing for chitinolytic enzymes production or mosquitocidal assay.

3.1.1 *E.coli* and *E.coli* carrying chitinase gene

The pure culture of the original strain *E.coli* JM109 and *E.coli* strain that carrying a plasmid with chitinase gene from *Aeromonas hydrophila* were maintained by growing in LB broth at 37°C in rotary shaker for overnight. An overnight grown *E.coli* preculture was transferred to nutrient broth in the amount to make 1% of the final volume of the medium. The nutrient broth consisting of 0.3% Bacto beef extract and 0.5% Bacto peptone was used. The growing condition of this bacteria in NB without chitin was used as negative control for enzyme production. The bacteria were grown in a 50% concentration of NB broth containing 0.1% (w/v) colloidal chitin. This medium was called 1/2 NBC broth. The 50% concentration of NB broth is consisting of 0.15% Bacto beef extract and 0.25% Bacto peptone. The cultures were incubated at 37°C for 48 hrs and centrifuged at 8,000 rpm for 10 min. The enzyme activity was determined directly from the cell pellet.

3.1.2 Growth curve and enzymatic production of *Bacillus licheniformis*

The correlation between growth curve and enzymatic production of the *Bacillus licheniformis* was studied. A pure culture of this bacteria was grown by shaking overnight in LB broth at 37°C. A active preculture was transferred to 1/2 NB broth containing 0.1% chitin (1/2 NBC) in the amount to make 1% in a total volume and

incubated at 37°C in a shaker. A 3 ml sample of the broth culture was taken at various intervals: i.e., 0, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 48 and 72 hrs of culturing time. The colony forming unit and chitinolytic enzymes production from each sample were determined. The enzymatic activity assay was determined from specimens derived from centrifugation of the 2 ml cell culture at 10,000 rpm for 10 min. A 1.5 ml of supernatant was then concentrated by evaporation in a UNIVAPO 100 H (UNIEQUIP) at 4°C for 1 hr until the volume was reduced to 200 µl. The chitinolytic enzyme activity from each concentrated supernatant was determined by using colorimetric method enzymatic assay.

In the case of large scale enzymes production, *Bacillus licheniformis* was grown in LB broth for overnight at 37°C in rotary shaker. The bacteria was subcultured into 1/2 NB broth containing 0.1 % chitin (1/2 NBC) in the amount to make 1% final concentration. The culture was grown for 48 hrs at 37°C in rotary shaker. As a negative control, the bacteria was grown in NB broth without chitin in the same conditions, and culture supernatant was used to assay for enzyme production and enzyme purification.

3.2 Culturing of *B.t.i.* for mosquito larvicidal toxicity test

The pure culture of *Bacillus thuringiensis* subsp. *israelensis* strains 4Q2-72 and c4Q2-72 were grown overnight at 37 °C in LB broth in a rotary shaker. The *B.t.i.* strain c4Q2-72 was a plasmid curing and nontoxic strain. The bacteria was then subcultured to NBS medium in the amount to make a 1% final concentration and incubated at 37°C in a shaker for 48 hrs. The NBS

medium is consisting of 1 mM magnesium chloride, 0.7 mM calcium chloride and 0.05 mM manganese chloride in NB broth.

4 Preparation of column chromatography and purification of chitinase enzyme

The chitinase enzyme from the broth culture of *Bacillus licheniformis* was subjected for purification. At each step of purification process, a 0.5-1.0 ml sample was taken and kept for enzyme and protein determination. The enzyme was purified by ammonium sulfate fractionation and ion exchange chromatography.

4.1 Matrix preparation

Carboxymethyl (CM) sephadex C-50 and Diethylaminoethyl (DEAE) sephadex A-50 (Pharmacia Fine Chemicals) were swollen in equilibrating buffer for 1-2 days at room temperature. CM sephadex and DEAE sephadex (the first column) were soaked in 50 mM sodium phosphate buffer pH 6.0 whereas DEAE sephadex which used for the second column was soaked in 50 mM sodium phosphate buffer containing 0.25 M NaCl and left standing to allow gel settled down. The upperphase buffer was decanted and replaced with fresh buffer several times during the swelling period. Finally, the swollen ion exchanger was kept at chromatographic operating temperature (4°C).

4.2 Packing and equilibrating the column

Firstly, the chromatographic column (2.5 cm diameter, 50 cm height) was washed several times with 0.1 M NaOH, 70 % (v/v) ethanol and steriled deionized distilled water before matrix packing. The tube was mounted vertically on a suitable stand at 4°C and the air from the column dead spaces was eliminated by flushing the end pieces with buffer. The swollen ion exchanger was mixed with buffer until it formed a fairly thick slurry. The suspension was poured into the chromatographic tube either along a glass rod or along the side of the tube to avoid bubble formation. When the height of column matrixes reached 1/4 of the tube, the column outlet was opened and the buffer was allowed to flow out from the tube. When the buffer was almost eliminated from top surface of gel in chromatographic tube, the top of column matrix was stirred and the remain swollen ion exchanger was poured until the chromatographic tube was fully filled. The inlet and outlet of the column was connected with the peristaltic pump and fraction collector respectively. At least two bed volume of starting buffer was run through the ion exchange column in order to allow the system to reach equilibration and stabilization of the ion exchange column.

4.3 Sample application and chromatographic operation

The sample was dialysed against each starting buffer consisting of 50 mM sodium phosphate buffer pH 6.0 in CM sephadex and the first DEAE sephadex column, 50 mM sodium phosphate buffer containing 0.25 M NaCl in the second DEAE sephadex column. Then, the sample in the amount 1-5 % of the bed

volume was applied to the chromatographic column by using peristaltic pump. The flow rate was set at 20 ml per 60 min and the fraction collector was operated by setting the fraction speed at 30 min per sample. Therefore, each collected tube will contain 10 ml sample. The chromatographic column inlet was connected to the washing buffer container when the sample was already sucked into the column. At least half of bed volume of washing buffer consisting of 50 mM sodium phosphate buffer pH 6.0 in CM sephadex C-50 and the first DEAE sephadex A-50 column , 50 mM sodium phosphate buffer containing 0.25 M NaCl in the second DEAE sephadex was passed through the ion exchange column in order to allow other protein to go out from the column. Since *Bacillus licheniformis* chitinolytic enzymes did not bind to CM sephadex matrix in 50 mM sodium phosphate buffer pH 6.0, therefore enzymes were eluted directly from the column by using the same buffer. Enzymes were eluted with salt gradient. The chromatographic column inlet was connected to gradient former of elution buffer. The elution buffer was consisting of 0-1 M NaCl in 50 mM sodium phosphate buffer pH 6.0 for the first DEAE sephadex A-50 column and 0.25-1 M in 50 mM sodium phosphate buffer pH 6.0 for the second DEAE sephadex A-50 column. Each fraction was measured for the protein concentration by spectrophotometry method during the chromatographic period (see 5.1). When the protein was eluted from the column, each fraction was analysed for enzyme activity by colorimetric method (see 6.1) and for purity of enzyme by activity detection method in polyacrylamide gel (see 6.2). Fractions containing pure enzyme was pooled and dialysed several times against deionized distilled water.

4.4 Matrix regeneration

In order to reactivated the matrix for future use, the used matrix was poured from chromatographic tube into the container and the buffer was decanted. An equal volume of 0.2 M NaOH was added to the matrix, stirred and left standing for 10-20 min. The NaOH solution was eliminated by Buchner suction system. The matrix was washed several times with steriled deionized distilled water until the pH of filtrate reached 7. Finally, the matrix was washed several times with steriled starting buffer and kept in the same buffer at 4°C.

4.5 Chitinase purification

4.5.1 Ammonium sulfate fractionation

A 3,090 ml supernatant from 48 hrs grown culture of *Bacillus licheniformis* was precipitated with ammonium sulfate. The solution was stirred vigorously with magnetic bar at 4°C while solid ammonium sulfate was added gradually until it reached 35% saturation. The total amount of solid ammonium sulfate added was 608.73 g (see appendix). The solution was left standing at 4°C for overnight. The solution was then centrifuged at 10,000 rpm for 30 min and the precipitate was discarded. The volume of supernatant was measured which was approximately 3,150 ml. The solid ammonium sulfate was gradually added to the supernatant until it reached 65% saturation in which 589.05 g was used. The solution was kept cold at 4 °C and continuously stirred during the addition of ammonium sulfate salt. The precipitate was allowed to form by kept standing at 4°C for overnight and then was collected by centrifugation

at 10,000 rpm for 30 min. The precipitate was then dissolved with 25 ml of cold 50 mM sodium phosphate buffer pH 6.0 and dialysed at 4 ^{o}C against cold 50 mM sodium phosphate buffer pH 6.0 with several changes of this buffer. The volume was adjusted by addition of same buffer to reach final volume of 35 ml after dialysis. The dialysis bag (Sigma) was prepared by boiling in 0.1 M EDTA for 10 min, washing with 70% (v/v) ethanol and followed by washing with several changes of steriled deionized distilled water. The enzyme preparation was further purified by ion exchange chromatography.

4.5.2 Ion exchange chromatography

The procedure of ion exchange chromatography to purify *Bacillus licheniformis* 's chitinolytic enzyme was performed by passing concentrated protein that was prepared by ammonium sulfate precipitation of supernatant through the column. The enzyme preparation was firstly passed through the carboxymethyl (CM) sephadex column by using 500 ml of 50 mM sodium phosphate buffer pH 6.0 as an equilibrating buffer, washing buffer and eluting buffer. Since the chitinase enzymes were not bound to CM sephadex gel, therefore no salt elution buffer was needed. The high chitinolytic activity fractions were pooled and the volume of pool fractions was reduced by 65 % ammonium sulfate precipitation. The precipitated protein was dissolved in 30 ml of 50 mM sodium phosphate buffer pH 6.0 and dialysed against the same buffer. The dialysed enzyme solution of approximately 35 ml was then loaded into the first DEAE sephadex chromatographic column. After the enzyme passed through the column, the gel column was washed with 50 mM sodium

phosphate buffer pH 6.0 and followed by elution with 1,000 ml of 50 mM sodium phosphate buffer pH 6.0 containing gradient concentration of NaCl from 0 to 1 M. The high chitinolytic activity fractions were pooled and the volume of pool fraction was reduced by 65 % ammonium sulfate precipitation method. The precipitated protein was dissolved in 30 ml of 50 mM sodium phosphate buffer pH 6.0 containing 0.25 M NaCl and dialysed against the same buffer. The dialysed enzyme solution was then loaded into the second DEAE sephadex chromatographic column that equilibrated with the same buffer. The column was washed by washing buffer consisting of 50 mM sodium phosphate buffer pH 6.0 containing 0.25 M NaCl. The sample was eluted by 50 mM sodium phosphate buffer pH 6.0 containing gradient concentration of NaCl from 0.25 M to 1 M. The fractions that contained pure chitinolytic enzyme, as demonstrated by SDS-PAGE and activity detection of the gel, were pooled. Finally, the volume of fraction was reduced by ammonium sulfate precipitation and then dialysis against deionized distilled water for further analysis.

5 Protein determination

5.1 Direct spectrophotometric method

This method was used for detection of protein concentration by directly measuring optical density (O.D.) value in spectrophotometer. The measurement was based on the phenolic group of tyrosine amino acid and indolic group of tryptophan amino acid in any unknown protein. They were absorbed by ultraviolet light.

The unknown sample was measured the absorbance value in quartz cuvettes at 280 nm and the buffer solvent was used as blank control.

5.2 Colorimetric assay by Bradford's method

The method of Bradford, which involved the binding of Coomassie Brilliant Blue G-250 to protein resulting in the shift absorbance maximum of the dye from 465 to 595 nm, was used in determining the amount of protein in the samples. The increasing in absorbance at 595 nm determined the protein concentration.

The protein detection by Bradford's method was done as follow. A 50 µl of either standard protein or tested protein was added into a 3 ml of Bradford's reagent, mixed by using vortex mixer and allowed to stand at room temperature for 5 min. Bovine serum albumin (BSA) at concentrations of 100, 200, 400, 600 800 and 1,000 µg/ml was used as standard protein. Deionized distilled water was used as control blank. The 1 litre of Bradford's reagent was consisting of Coomassie Brilliant Blue G-250 100 mg, 95 % (v/v) ethanol 50 ml and 85 % (w/v) phosphoric acid 100 ml, the final volume was adjusted to 1 litre with distilled water and filtered before used. The color of the reaction was stable for 3 hrs. The absorbance of BSA at different concentration at 595 nm was measured against control blank and the protein concentration was plotted against the corresponding absorbance to make a standard curve. The amount of protein in the unknown sample was determined by comparison with the BSA standard curve.

6 Enzymatic assays and detection

6.1 Quantitative assays of chitinolytic enzymes by colorimetric method

The method for chitinolytic enzyme assay was done by the detection of N-acetyl-D-glucosamine according to the method of Reissig (1955). N-acetyl-D-glucosamine was the end product obtained from digestion of chitin by certain enzyme. It was changed to intermediate compound, possibly a glucozazoline by heating with alkali solution. This intermediate compound was then reacted with ρDAB in an acid medium and purple color development was observed.

The enzyme assay was done by the following procedure. The 200 µl of enzyme solution or control blank were transferred to Eppendorf tube that containing 50 µl of substrate solution. Deionized distilled water was used as a control blank. The substrate solution was consisting of 0.52 % (w/v) colloidal chitin in 100 mM sodium acetate buffer pH 5.0. The solution was incubated at 60°C for 1 hr and centrifuged at 10,000 rpm for 10 min at 4°C. A 200 µl of supernatants either test or blank and standard N-acetyl-D-glucosamine were transferred to 13x100 test tubes that contained 40 µl of 0.8 M potassium tetraborate pH 9.1 that was adjusted pH value with 1.0 M KOH before used. The standard stock concentration of standard N-acetyl-D-glucosamine was made as 0.05, 0.10, 0.20, 0.30, 0.40, and 0.50 µmoles/ml in 20 mM sodium acetate buffer pH 5.0. The mixture solution was heated in a boiling water for exactly 3 min and immediately cooled in tap water. A 1.2 ml of ρ

DAB reagent was then added and the tubes was placed in a waterbath at 37°C immediately after mixing. The ρDAB reagent was made by dissolving 10 g of ρDAB in 100 ml of glacial acetic acid which contains 12.5 % (v/v) 10 N HCl and stored at 4°C. It was diluted with 9 volumes of glacial acetic acid shortly before used. After precisely 20 min of warming, the tubes were cooled in tap water and the absorbance value was observed and recorded without delay at 585 nm. The absorbance at wavelength 585 nm was measured against blank control. The standard curve was made by plotting between different concentration of standard N-acetyl-D-glucosamine against their absorbance at 585 nm and the enzyme activity of the unknown sample was estimated from the standard curve. One unit of chitinase activity was determined as amount of enzyme which released 1 μmoles of N-acetyl-D-glucosamine or its equivalent per minute at 60°C.

6.2 Activity detection in polyacrylamide gel (Trudel and Asselin, 1989)

6.2.1 Activity detection in native-PAGE (native poly acrylamide gel electrophoresis)

6.2.1.1 Native polyacrylamide gel preparation

The 10 % acrylamide separating gel was prepared by mixing 3 ml of solution A, 3 ml of solution B, 3 ml of distilled water and 25 μl of solution C. The solution A was consisting of 29.2 % (w/v) acrylamide, 0.8 % (w/v) N,N'-methylene bisacrylamide in

distilled water, the solution B was 1.5 M Tris-HCl buffer pH 8.8 and the solution C was freshly prepared of 10 % (w/v) ammonium persulfate. Then 6 µl of N,N,N',N'-tetramethylethylenediamine [TEMED] was added and the mixture was mixed thoroughly. The mixture was immediately poured into the slot between glass plate and alumina plate (Hoefer Scientific Instruments, USA) that previously washed with detergent, distilled water and 70 % (v/v) ethanol respectively. One ml of distilled water was gently added to cover the top layer of separating gel and allowed the acrylamide to polymerize at least 30 min. After polymerization, the surface of gel was flushed several times with distilled water and the stacking gel solution of 4.6 % acrylamide mixture which compose of 1.5 ml of solution A, 2.5 ml of solution D, 6 ml of distilled water, 25 µl of solution C and 7 µl of TEMED, was poured over the separating gel. The solution D was 0.5 M Tris-HCl buffer pH 6.8. The comb was inserted at once and allowed gel to polymerize at least 30 min. After removing the comb, the wells were flushed 3 times with distilled water to eliminating non-polymerize acrylamide, then the gel plates were set in the electrophoresis apparatus (Miniprotein II gel set, Hoefer Scientific Instruments, USA). Cold electrophoresis buffer was added until the buffer covering the wells of gel. The buffer was consisting of 0.025 M Tris-HCl buffer, 0.192 M glycine, pH 8.3. The electrophoresis was operated with constant voltage mode at 150 Volts for 30 min at 4°C for gel equilibration and stabilization the electrophoresis system before loading the sample.

6.2.1.2 Sample preparation and electrophoresis operation

The protein samples were prepared by diluting with 1/3 volume of tracing dye at room temperature. The tracing dye was consisting of 15 % (w/v) sucrose, 0.01 % (w/v) bromphenol blue. The sample mixtures were loaded carefully into electrophoresis wells without contamination between each sample. The electrophoresis apparatus was continuously operated until the tracing dye reached the gel bottom (5-6 hrs).

6.2.1.3 Activity detection of chitinase in native-PAGE

After electrophoresis was completed, the separating gel was removed from glass plate and incubated for 5 min in 150 mM sodium acetate buffer pH 5.0. The gel was then placed on glass plate and covered with substrate gel consisting of 10 % (w/v) polyacrylamide gel containing 0.01 % (w/v) glycol chitin in 100 mM sodium acetate buffer pH 5.0. The substrate gel was prepared by mixing 3 ml of solution A, 3 ml of solution B, 2.01 ml of distilled water, 25 µl of solution C, 90 µl of 1% (w/v) glycol chitin, 900 µl of 1 M sodium acetate buffer pH 5.0 and 6 µl of TEMED. The double gel layer was covered with glass plate and then kept in moisturizing chamber for 15 hrs at 37°C. The substrate gel was then soaked with freshly prepared brightener solution for 10 min. The brightener solution was made by mixing calcofluor white M2R 0.01 % (w/v) in 0.5 M Tris-HCl pH 8.9. The brightener solution was discarded and substrate gel was washed several times with distilled water at room

temperature. Substrate gel was placed on UV-transilluminator for clear zone visualization. Pictures were taken at once by type 400 color film with 1/60 second exposing time (shutter speed-60) and 1/16 aperture (f_{16}). The gel could be dehydrated and kept for future visualization.

6.2.2 Activity detection in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

The activity of *Bacillus licheniformis* chitinase enzyme could also be detected in SDS-PAGE after renaturizing of protein by using Triton X-100. The procedure was performed as follows.

6.2.2.1 Gel preparation

The 10 % acrylamide mixture was prepared by mixing 3 ml of solution A, 3 ml of solution B, 2.91 ml of distilled water, 90 µl of 1 %(w/v) glycol chitin, 25 µl of solution C and followed by the addition of 6 µl of TEMED. The solution A was consisting of 29.2 % (w/v) acrylamide, 0.8 % (w/v) N,N'-methylene bisacrylamide in distilled water, the solution B was 1.5 M Tris-HCl buffer pH 8.8, containing 0.4 % (w/v) SDS and the solution C was freshly prepared of 10 % (w/v) ammonium persulfate. The gel mixture was poured into the slot between glass plate and alumina plate. A 1 ml of distilled water was added to cover the surface layer of separating gel and the acrylamide gel was allowed to polymerize at least 30 min. After polymerization, the surface of gel was flushed several times with distilled water and the stacking gel solution of 4.6 % acrylamide mixture consisting of 1.5 ml of solution A , 2.5 ml of

solution D, 6 ml of distilled water, 25 µl of solution C and 7 µl of TEMED]was poured over the separating gel. The solution D was consisting of 0.5 M Tris-HCl buffer pH 6.8 containing 0.4 % (w/v) SDS. The comb was inserted and gel was allowed to polymerize at least 30 min. After removing the comb, the wells were flushed 3 times with distilled water to eliminating non-polymerize acrylamide. The gel plates were set in the electrophoresis apparatus. The cold electrophoresis buffer consisting of 0.025 M Tris-HCl buffer, 0.192 M glycine and 0.1 % (w/v) SDS, pH 8.3 was added into the gel chamber and the gel was electrophoresed at 150 Volts with constant voltage mode for 30 min at 4°C before loading the sample.

6.2.2.2 Sample preparation and electrophoresis operation

The protein samples and standard molecular weight protein (Rainbow marker, Amersham LIFE SCIENCE) were prepared by diluting 2 folds with 2x sample solubilizing buffer and heated in a boiling waterbath for 2 min. The sample solubilizing buffer was consisting of 125 mM Tris-HCl, pH 6.7 containing 2.5 % (w/v) SDS, 2 % (v/v) β-mercaptoethanol and 0.01 % (w/v) bromphenol blue. The diluted protein samples were carefully loaded into electrophoresis wells. The electrophoresis was performed until the tracing dye reached the bottom of the gel (5-6 hrs).

6.2.2.3 Enzymes renaturation and activity detection

In order to determine the molecular weight of enzyme and to confirm the enzyme protein band by activity staining, the denatured protein in SDS-PAGE was renatured.

The SDS protein gel was removed from glass plate and was incubated in 100 mM sodium acetate buffer pH 5.0 containing 1 % (v/v) Triton X-100. The tray containing gel was gently shake in rotary shaker for 15 hrs at 37°C and washed 3 times with distilled water. The lytic zone was visualized by soaking the separating gel with freshly prepared brightener solution for 10 min. The brightener solution was consisting of calcofluor white M2R 0.01 % (w/v) in 0.5 M Tris-HCl pH 8.9. The brightener solution was discarded and excess solution was removed by washing several times with distilled water at room temperature. Separating gel was placed on UV-fransilluminator for clear zone visualization. Pictures were taken at once or the gel was dehydrated for future visualization as described in native-PAGE procedure (see 6.2.1.3).

7 Physicochemical characterization

The physicochemical characterization of *Bacillus licheniformis* chitinolytic enzyme was monitored by enzymatic assays and detection of enzyme by colorimetric method.

7.1 Optimal temperature

A 200 µl of enzyme sample and 50 µl of 0.52 % (w/v) chitin substrate in 100 mM sodium acetate buffer pH 5.0 were incubated for 1, 3, and 6 hrs at various temperature; i.e., 30, 37, 40, 45, 50, 55, 60, 65 and 70°C. The optimal temperature was analysed from the activity of enzyme that could release different concentration of product (N-acetyl-D-glucosamine) at different temperature under same assay condition with 200 µl of 8.67 mU/ml of enzyme and incubation period of 1 hr.

7.2 Temperature stability

The Eppendorf tubes containing 200 µl of 8.67 mU/ml of enzyme were incubated at 40, 50, 60 and 70°C for various time intervals; i.e., 0, 1, 2, 3, 4, 5, and 6 hrs. At the end of each indicated time, a 50 µl of 0.52 % (w/v) chitin substrate in 100 mM sodium acetate buffer pH 5.0 was added and mixed. The mixture was incubated for 1 hr at 60°C. The stability of enzyme at various temperature was analysed from the enzymatic activity that was capable to hydrolyse chitin substrate and released different concentration of N-acetyl-D-glucosamine.

7.3 Optimal pH

The optimal pH of chitinase enzyme was determined by assaying enzyme in buffer with different pH.

A 200 µl of 8.67 mU/ml of enzyme in each of Eppendorf tubes was mixed with 50 µl of 0.52 % (w/v) chitin substrate in 250 mM Clark and Lubs buffer with varying pH from 3, 4, 5, 6, 7, 8 and

9 (see appendix). The mixtures were incubated for 1 hr at 60°C. The pH value was adjusted to 5.0 by adding 0.1 M HCl or 0.1 M NaOH solution with continuous mixing. The volume of mixtures was adjusted to 500 µl with deionized distilled water. Finally, the concentrations of product formation were measured and calculated. The activity of enzyme was then analysed and the optimal pH was determined.

7.4 pH stability

7.4.1 Stability of enzyme in various pH

The stability of enzyme in various pH ranges kept at 4°C was determined.

A 50 µl of 250 mM Clark and Lubs buffer at various pH levels; i.e., 3, 4, 5, 6, 7, 8, 9 or deionized distilled water was added to Eppendorf tubes that contain 200 µl of 8.67 mU/ml of enzyme and tubes were kept at 4°C for 0, 3 and 6 hrs. The pH in each tube was adjusted to 5.0 by adding 0.1 M HCl or 0.1 M NaOH solution with continuous mixing. The volume were adjusted to a total volume of 500 µl with deionized distilled water. The 200 µl of these enzyme solutions were mixed with 50 µl of 0.52 % (w/v) chitin substrate in 100 mM sodium acetate buffer pH 5.0 and incubated for 1 hr at 60°C. Finally, concentration of product was measured and the results were analysed.

7.4.2 Stability in pH 5.0

In order to monitor the rate of enzyme activity reduction, the enzyme was kept at pH 5.0 for different time intervals and enzyme activity was determined.

The solutions of 200 µl containing 8.67 mU/ml of enzyme and 50 µl of 250 mM Clark and Lubs buffer pH 5.0 were kept at 4°C for various time intervals; i.e., 0, 2, 4, 6, 8, 12, 18 and 24 hrs. A 200 µl of these solutions were mixed with 50 µl of 0.52 % (w/v) chitin substrate in 100 mM sodium acetate buffer pH 5.0 and incubated for 1 hr at 60°C. The enzymatic activity was measured and calculated, the pH stability was analysed.

7.5 Enzyme kinetics determination: initial velocity (v_i), Michaelis's constant (K_m) and maximum velocity (V_m)

In order to determine enzyme kinetic of chitinase enzyme, the v_i, K_m and V_m of enzyme against chitin substrate was determined.

A 200 µl of 8.67 mU/ml enzyme in each of Eppendorf tubes was mixed with 50 µl of either 0.2 or 0.5 or 1.0 or 1.25 % (w/v) chitin substrate in 100 mM sodium acetate buffer pH 5.0 and separately incubated at 60°C for various time interval; i.e., 0, 5, 10, 15, 20, 30, and 60 min. The concentration of product (N-acetyl-D-glucosamine) was analysed, the kinetic curves of substrate concentration [S] against product concentration [P] were plotted and the initial velocity (v_i) of each substrate concentration was calculated from slope of its kinetic curve. By using Lineweaver and Burk's equation, (1/[s] against $1/v_i$ plotting) and protein concentration of

enzyme used in reaction, the Michaelis's constant (K_m) and maximum velocity (V_m) of *Bacillus licheniformis* chitinolytic enzyme was measured.

7.6 Determination of molecular weight of enzyme in SDS-PAGE

The molecular weight of chitinase enzyme was determine in SDS-PAGE. The 10 % SDS-polyacrylamide gel was prepared as previously described (see 6.2.2.1) with standard molecular weight protein (Rainbow marker) as marker.

Two sets of same enzyme samples were loaded onto the same polyacrylamide gel for activity detection in gel and for Coomassie brilliant blue (CBB) staining. The gel was electrophoresed at 150 Volts for 6 hrs. However, the amount of enzymes loaded on the gel was not the same. Samples containing about 10-20 µg of protein were loaded on each well of SDS-PAGE for CBB staining. The amount of enzyme loaded on the gel for enzyme detection was about 4 times less than amount of enzyme loaded on the gel for CBB staining. After electrophoresis, gel was cut and subjected for CBB staining and enzyme detection. The activity of enzyme on gel was detected after renaturization as previously described (see 6.2.2.3). Coomassie blue staining of SDS gel was done by staining the gel in staining solution for a few hours at room temperature. The staining solution was consisting of 0.2 % (w/v) Coomassie brilliant blue G-250 in fixing solution containing 50 % (v/v) methanol and 10 % (v/v) glacial acetic acid in distilled water. Under such high concentration of methanol and glacial acetic acid, the gel size would be a bit reduced.

The excess stain was removed by soaking the gel in the fixing solution for another few hours. The fixing solution was discarded and was replaced by destaining solution containing 5 % (v/v) methanol and 7 % (v/v) glacial acetic acid in distilled water. The gel was left in the destaining solution until the gel reverted back to normal size and became clear with protein bands were clearly seen. The gel was kept in 7 % (v/v) glacial acetic acid in distilled water solution. The protein band of chitinolytic enzymes in gel was determined by comparison between protein band present in gel stained with Coomassie brilliant blue and corresponding lytic zone in enzymatic activity staining gel at the same path length mobility. The molecular weight of enzyme was calculated from standard molecular weight curve obtained from standard molecular weight proteins.

8 Mosquito larvicidal test

8.1 Large scale test

The killing activity of chitinase in *Bacillus licheniformis*, *E. coli* JM109 and *E.coli* strain that carrying a plasmid with chitinase gene from *Aeromonas hydrophila* cells was assayed against mosquito larvae. The bacterial cells were cultured in medium with and without chitin substrate supplement (see 3.1). The assay was done as follow.

Ten ml of cell culture was added into steriled cups containing 90 ml distilled water. A series of ten-fold dilution was made from the first cup to the fifth cup (from 10^{-1} to 10^{-5}). Ten 2nd instar larvae of *Aedes aegypti* were added into each cup. The tests were done in duplicated set. For the control group, distilled water was used instead

of bacterial suspensions and the experiments were done in the same manner. The cups were kept at room temperature and the number of dead larvae were recorded 2 days after exposure. The activity was recorded as 50 % lethal concentration value (LC_{50}) by using the cell concentration that calculated by colony forming unit. Bacterial cells were diluted to appropriate concentration (10^{-5} to 10^{-7}) with 0.85 % (w/v) NaCl, 100 µl of cell suspensions were spreaded on LB agar and incubated for overnight at 37°C. The bacterial colonies were counted and bacterial concentration value was calculated. The calculation of LC_{50} was done according to the method described by Reed and Muench (1938). The calculation method as shown in appendix.

8.2 Small scale test

This method was used for screening of the mosquito larvicidal property of crude and partial purify chitinolytic enzymes of *Bacillus licheniformis*. The test was performed by using enzyme with or without *B.t.i.*4Q2-72 or *B.t.i.*c4Q2-72. The test was performed in order to determine the enhancing effect of enzyme to cells in killing mosquito larvae. The mosquito larvicidal property of *B.t.i.*4Q2-72 and *B.t.i.*c4Q2-72 alone was also tested and was used as a control.

This experiment was done in the same manner as large scale test except that number of tested larvae was five instead of ten. The total volume was reduced to only 2 ml and the experiment was performed in plastic plate with 24 wells instead of plastic cup.

CHAPTER IV
RESULTS

1 Standard curve of N-acetyl-D-glucosamine for chitinolytic enzyme assay and bovine serum albumin for protein assay

Standard curve for N-acetyl-D-glucosamine (GlcNAc) was made by determining the absorbance value at 585 nm of standard N-acetyl-D-glucosamine according to the method of Reissig (1955). Two hundred microlitres of various standard concentrations of GlcNAc (0.25, 0.50, 1.00, 1.50, 2.00 and 2.50 μmoles/ml in 20 mM sodium acetate buffer pH 5.0) was reacted with a 40 μl of 0.8 M potassium tetraborate pH 9.1 by boiling for 3 min. After cooling, the mixture was reacted with 1.2 ml of ρ-dimethylamino benzaldehyde. The stock concentration of GlcNAc of 0.1 μmoles/ml should give an optical density value at 585 nm of about 0.25. The plots between different concentration of standard N-acetyl-D-glucosamine against their absorbance value at 585 nm were shown in Figure 2. Calculation of chitinolytic enzyme activity (U/ml) was made from this GlcNAc standard curve by using unit definition as amount of enzyme which could release 1 μmoles of GlcNAc or equivalent per minute at 60°C.

$$U/ml = \frac{X}{2.5} \times \frac{1}{t} \times Y \times \frac{1,000}{v}$$

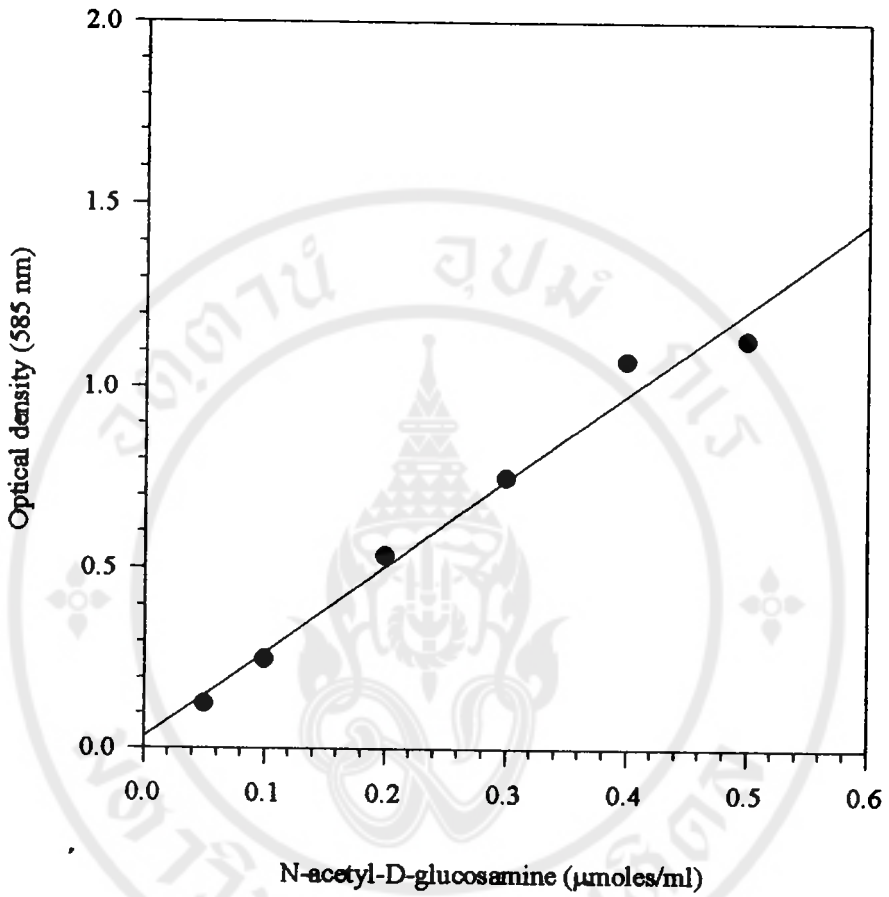


Figure 2. Correlation between final concentration of standard N-acetyl-D-glucosamine and optical density (absorbance) at 585 nm.

Where X = Absorbance value at 585 nm

t = time of reaction between enzyme and substrate

Y = dilution factor of enzyme

v = volume of enzyme or diluted enzyme in µl

The standard curve of protein determination by Bradford's colorimetric method was made by using bovine serum albumin (BSA) 50 µl at concentration of 100, 200, 400, 600, 800, 1,000 µg/ml as the standard protein. The absorbance value at 595 nm was measured and data were shown in Figure 3. The amount of protein in the unknown sample was determined by comparison with this BSA standard curve.

2 Growth curve and chitinolytic enzymes production of *Bacillus licheniformis*

To study the correlation between growth curve and enzymatic production, the enzyme productions at different time interval of growth were determined. Pure culture of *Bacillus licheniformis* was grown by shaking overnight in LB broth at 37°C. The bacteria was subcultured into 1/2 NB broth containing 0.1 % chitin in the amount to make 1% final concentration and incubated at 37°C in rotary shaker. The samples of cell culture were randomly taken at 0, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 48 and 72 hrs of culturing time. The relationships between colony forming unit and total chitinolytic enzyme activity of all samples were shown in Figure 4. From these data, it was shown that the enzyme production of *Bacillus licheniformis* in 1/2 NBC broth was gradually increased and the

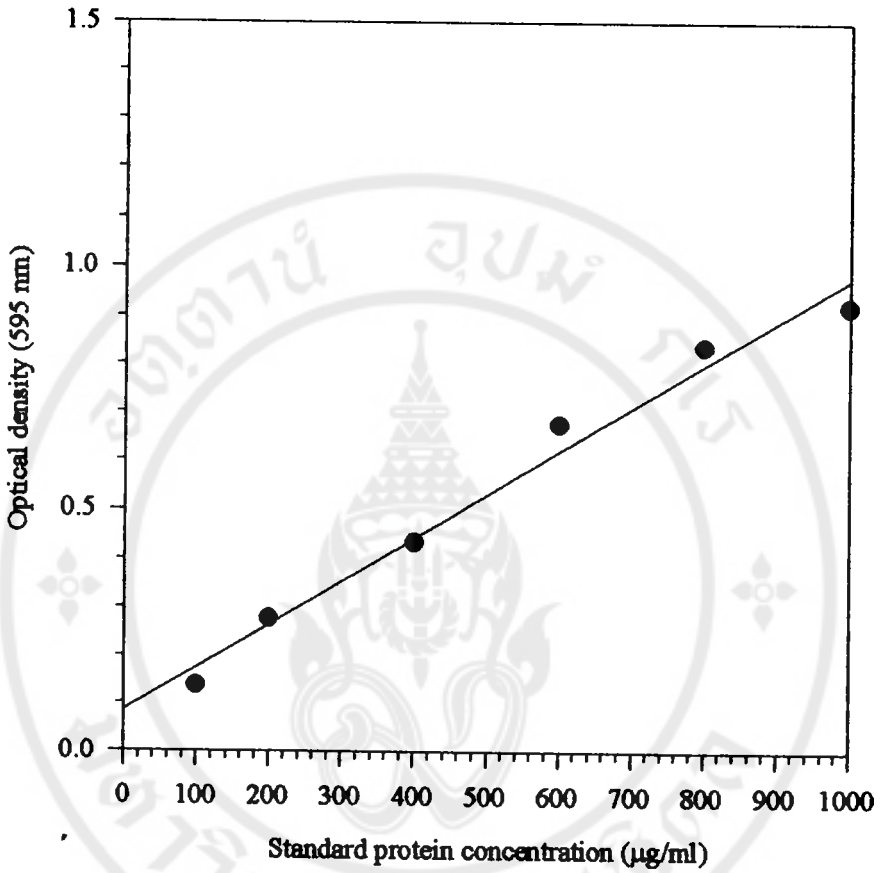


Figure 3. Relationship between standard protein (BSA) concentration and optical density (absorbance) at 595 nm.

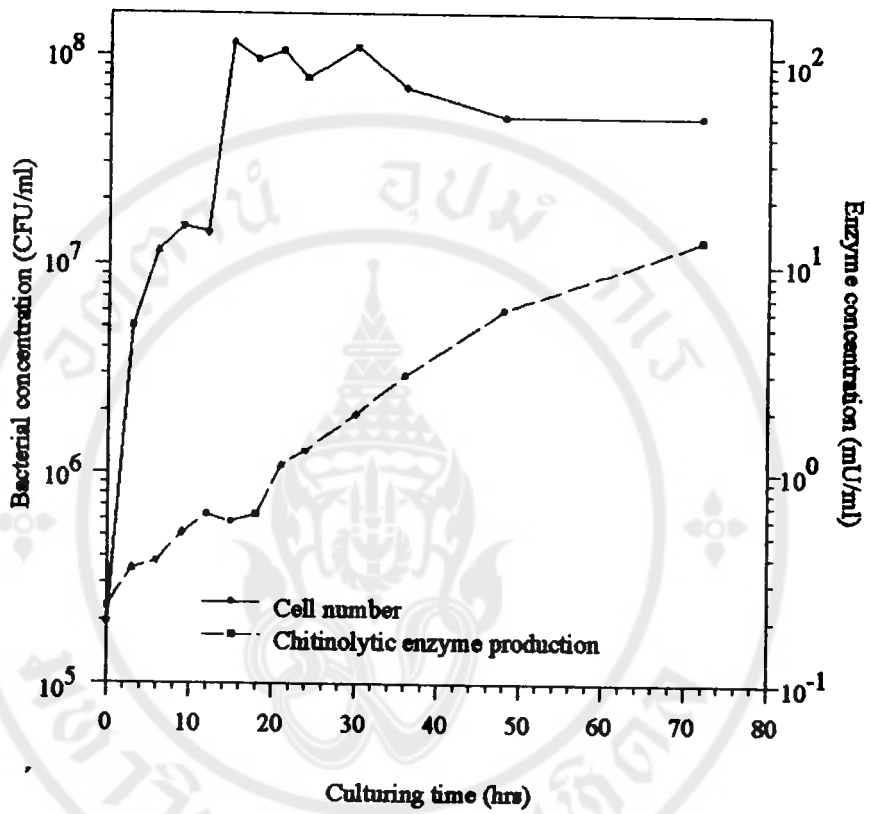


Figure 4. The relationships between cell number at different culturing time (bacterial concentration, CFU/ml) and chitinolytic enzyme production (enzyme concentration, mU/ml) of *Bacillus licheniformis*.

enzyme production continued to increase when the cells entered the stationary phase and kept increasing until the end of this phase.

The correlation between production of each isotype of chitinolytic enzymes of *Bacillus licheniformis* and it's growth stage was also studied by the detection of chitinolytic activity in SDS-PAGE. The activity was detected in SDS-PAGE gel after renaturation with 100 mM sodium acetate buffer pH 5.0 containing 1 % (v/v) Triton X-100 for 15 hrs at 37°C and the result was shown in Figure 5. Several isotypes of *Bacillus licheniformis* chitinolytic enzyme were produced and maximum activity of enzyme as detected by staining was detected at 72 hrs. The high level of enzyme activity was probably due to enzyme accumulation. From this figure, at least 3 bands of enzyme activity were detected. These bands were probably derived from the subunits of enzymes or enzymes were in the form of isozymes. Further study and characterization of this enzyme(s) was required.

3 Purification of *Bacillus licheniformis* chitinolytic enzyme

In order to characterize the enzyme, the purification of enzyme was performed by fractionation and column chromatography. Firstly, 3,090 ml of supernatant from 48 hrs grown culture of *Bacillus licheniformis* was fractionated by ammonium sulfate precipitation. The precipitate obtained from the first fraction of 0-35 % saturation which contained only trace enzyme activity was discarded and the supernatant was further subjected to 35-65 % ammonium sulfate precipitation. The precipitate protein was collected. The chitinolytic

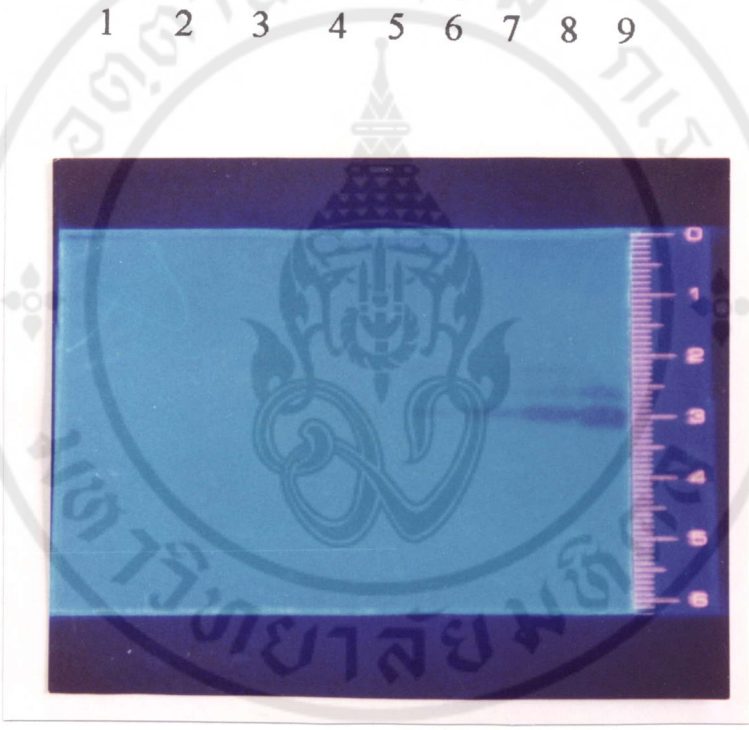


Figure 5. Chitinolytic enzyme activity of *Bacillus licheniformis* culture supernatant at 0, 3, 6, 9, 12, 24, 36, 48 and 72 hrs of culturing time (lane 1 through 9). The activity was detected in SDS -PAGE gel after renaturation with 100 mM sodium acetate buffer pH 5.0 containing 1 % (v/v) Triton X-100 for 15 hrs at 37°C. Each lane contains 15 µl of culture supernatant.

enzymes in this crude precipitate were further purified by passing this fraction through the carboxymethyl (CM) sephadex C-50 column. The chromatographic pattern of total protein (absorbance at 280 nm) and enzyme activity of fractions obtained from CM sephadex were shown in Figure 6. The fractions that gave high enzymatic concentration value (fraction 5-13) were pooled, precipitated with 65 % saturation of ammonium sulfate and dialysed against 50 mM sodium phosphate buffer pH 6.0. The proteins collected from 65 % ammonium sulfate saturation fraction and pool fraction from CM sephadex were assayed for the presence of enzymatic activity by using SDS-PAGE and activity staining for chitinolytic activity detection and the proteins bands were stained by Coomassie blue staining. Results were shown in Figure 7. The pooled fraction from CM sephadex C-50 was passed through the Diethylaminoethyl (DEAE) sephadex A-50 column. The chromatographic pattern of enzyme fractions and total protein (absorbance at 280 nm) in this column were shown in Figure 8. The top fractions from protein peaks (fractions 40, 46, 54, 61, 66 and 76) were analysed for the enzymatic purity by using chitinolytic activity detection in SDS-PAGE, the proteins was detected by Coomassie blue staining and results were shown in Figures 9 and 10 respectively. The results from activity staining as well as protein stain in both figures indicated that enzyme was not yet pured, therefore, the fractions in the second peak which contained chitinolytic enzyme activity (fraction 55-85) were pooled, precipitated with 65 % saturation of ammonium sulfate, dialysed against 50 mM sodium phosphate buffer pH 6.0 containing 0.25 M NaCl and further purified by passing the pooled

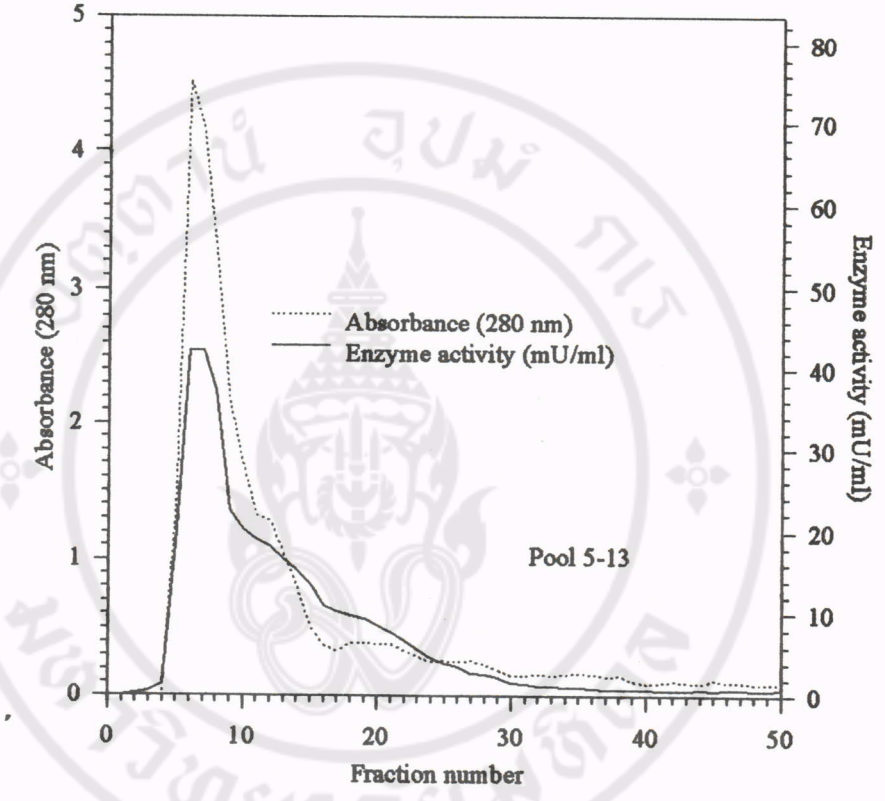


Figure 6. The chromatographic pattern of total protein (absorbance at 280 nm) and chitinolytic enzyme (enzyme activity) in CM-sephadex C-50 column.

The protein obtained from 35-65 % ammonium sulfate fractionation (crude) was passed through CM-sephadex C-50 column bufferized and eluted with 50 mM sodium phosphate buffer pH 6.0. Fraction volume of 10 ml was collected at flow rate of 20 ml/hr at 4°C. The column size used was 2.5 cm in diameter and 50 cm in height.

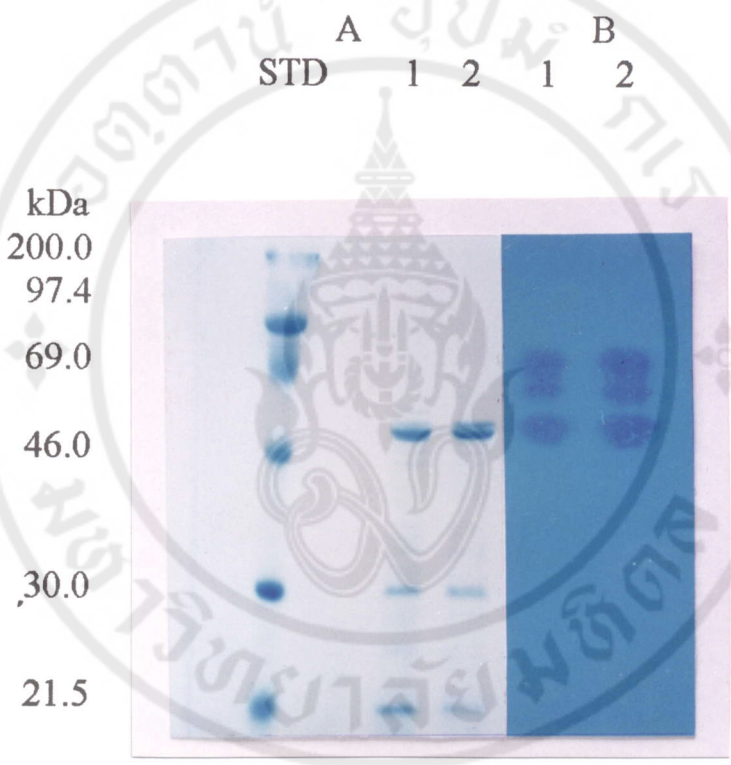


Figure 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) subsequent with Coomassie brilliant blue staining (A) and chitinolytic enzyme activity detection (B) of 48 hrs culture supernatant precipitated with 35-65 % saturated ammonium sulfate (lane 1) and pool enzymatic fractions after running through the CM sephadex C-50 column (lane 2). Each lane was loaded with about 10-20 µg of protein. STD lane represents protein standard molecular weight (Rainbow marker).

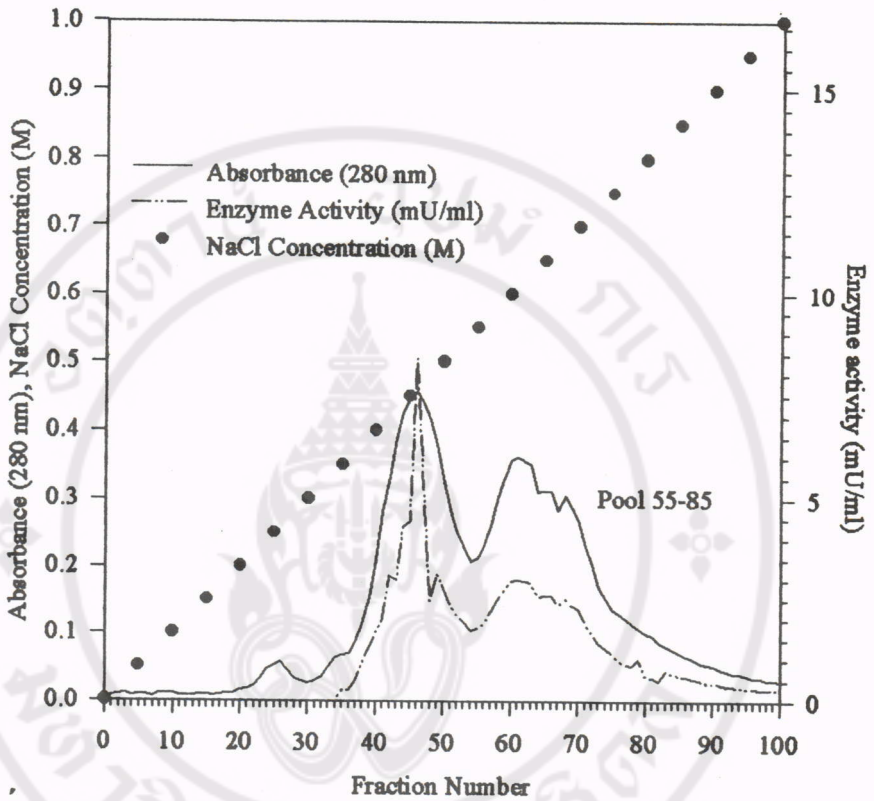


Figure 8. The chromatographic pattern of total protein (absorbance at 280 nm) and chitinolytic enzyme (enzyme activity) in the first DEAE sephadex A-50 column.

The pooled fractions from CM sephadex C-50 was run through DEAE sephadex A-50 column by using 50 mM sodium phosphate buffer pH 6.0 as equilibrating and washing buffer. A 0-1.0 M NaCl in 50 mM sodium phosphate buffer, pH 6.0 was used as eluting buffer. Fraction volume of 10 ml was collected as flow rate of 20 ml/hr at 4 °C. The column size used was 2.5 cm in diameter and 50 cm in height.

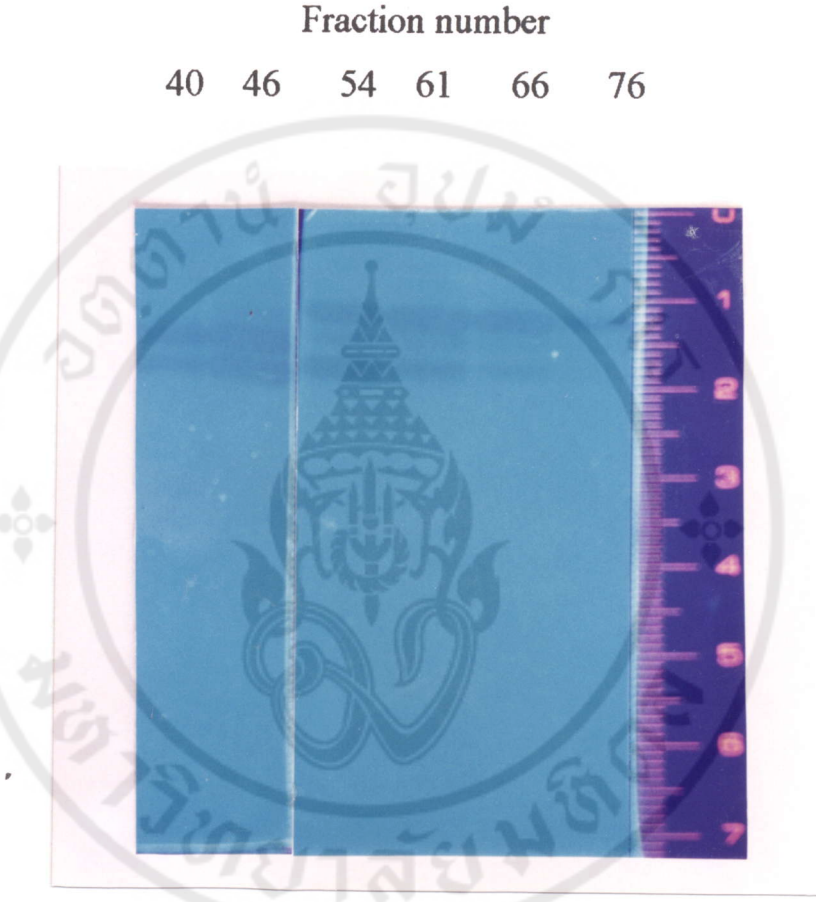


Figure 9. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and chitinolytic enzyme activity detection of the top fractions of protein peaks obtained from chromatographic pattern from the first DEAE sephadex A-50 column (see Figure 8). The number of each lane represents fraction number collected from the column. Each lane was loaded with 0.1-0.2 mU/ml of enzyme. The fractions number 55-85 were pooled for precipitation and loaded to the second DEAE sephadex A-50 column.

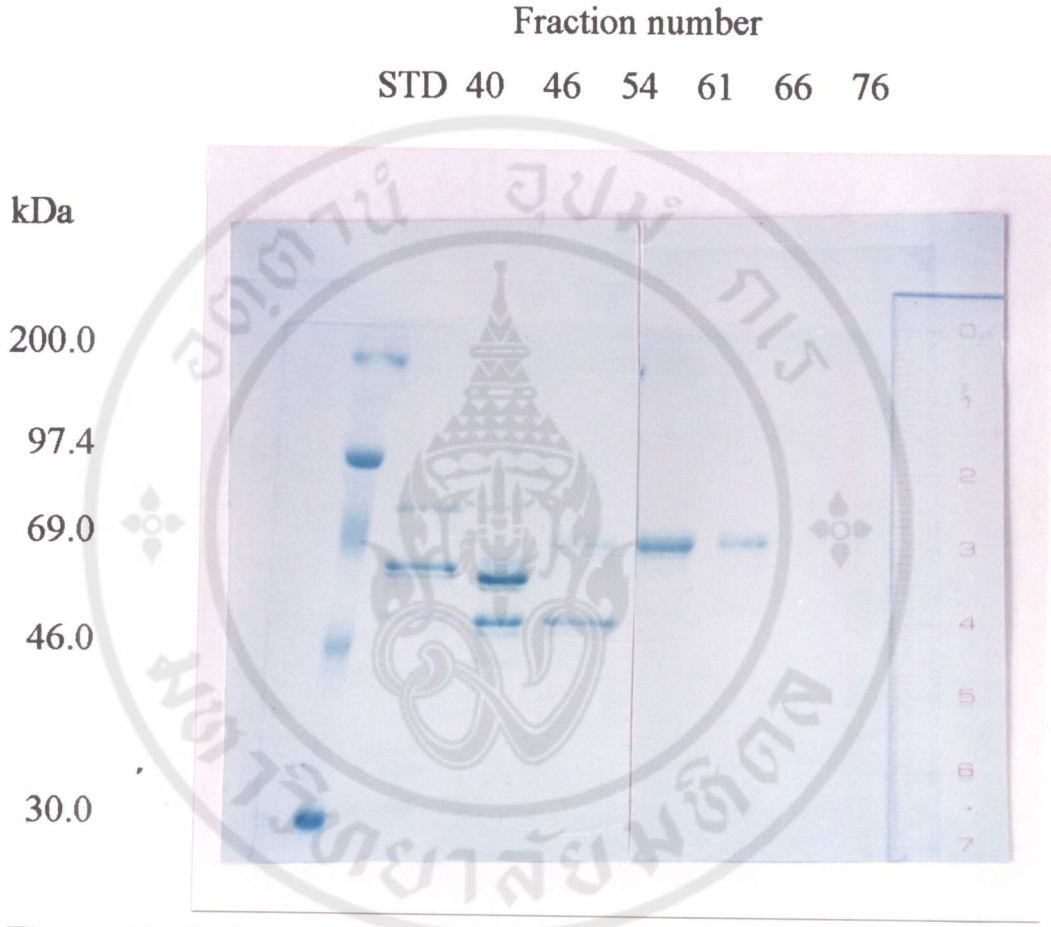


Figure 10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining of the top fractions from protein peaks obtained from chromatographic pattern from the first DEAE sephadex A-50 column (see Figure 8). The number of each lane represents fraction number collected from the column. Each lane was loaded with 0.5-1.0 mU/ml of enzyme. The STD lane represents standard molecular weight protein (Rainbow marker).

fractions through the second DEAE sephadex A-50 column. The, data of protein concentration, total protein, enzyme concentration total enzyme, percent recovery and specific activity of each fraction were shown in Table 1. From these data, some undesired proteins that contained in precipitate fraction obtained from 35 % ammonium sulfate saturation fraction were eliminated. The chromatographic pattern of total protein (absorbance at 280 nm) and chitinolytic enzyme in the second DEAE sephadex A-50 column were shown in Figure 11. Though the highest enzyme activity was in fraction number 46, but the fractions around this top fraction still contained more than one type of chitinase whereas the later fractions (58-70) displayed only one chitinase band in SDS-PAGE and activity staining gel (data not shown). Therefore, the fractions number 58-70 that contained purified of high molecular weight of *Bacillus licheniformis* chitinase enzyme were pooled, precipitated with ammonium sulfate and dialysed against deionized distilled water for future analysis. This purified enzyme was analysed for the purification factors (as shown in Table 1. The chitinolytic activity was detected by using both native-PAGE and SDS-PAGE methods and results were shown in Figure 12. The protein was detected by Coomassie blue staining and results were shown in Figure 13.

Table 1. Results of the purification of *Bacillus licheniformis* chitinolytic enzyme.

Step	Volume (ml)	Protein		Activity		Yield (% Recovery)	Specific activity (U/mg protein)	Fold
		µg/ml	Total protein (mg)	U/ml	Total activity (U)			
Super-natant 0-35 % saturation	3090	55.46	171.37	0.0118	36.462	100.00	0.213	1.0
35-65 % saturation	15	75.13	1.13	0.0115	0.1725	0.47	0.153	-
CM-sephadex	35	2794.06	97.79	0.4543	15.900	43.61	0.163	0.77
1°DEAE-sephadex	30	1616	48.48	0.4312	12.936	35.48	0.267	1.25
2°DEAE-sephadex	30	765	22.95	0.4083	12.250	33.60	0.534	2.51
	30	39.43	1.18	0.0165	0.495	1.36	0.419	1.97

A 3,090 ml supernatant of 48 hrs cells culture of *B. licheniformis* was fractionated with 35 % saturation of $(NH_4)_2SO_4$, centrifuged and the precipitate was discarded. The supernatant was continuously added with the same salt until 65 % saturation was reached. This precipitated protein was collected by centrifugation and purified by passing through the CM sephadex and 2 columns of DEAE sephadex respectively. Between each step of purification, the volume, protein and enzyme activity were determined.

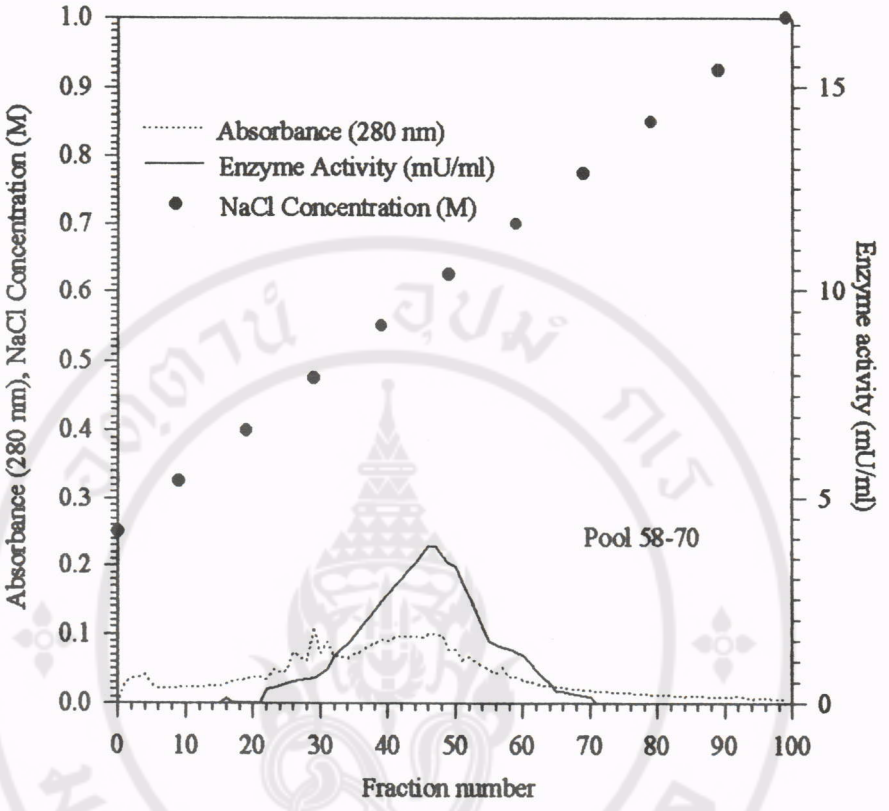


Figure 11. The chromatographic pattern of total protein (absorbance at 280 nm) and chitinolytic enzyme (enzyme activity) in the second DEAE sephadex A-50 column.

The pooled fractions of protein from the first DEAE sephadex A-50 was passed through the second DEAE sephadex A-50 column by using 0.25 M NaCl in 50 mM sodium phosphate buffer pH 6.0 as equilibrating and washing buffer. Protein was eluted from column by gradient concentration of NaCl (0.25-1.0 M) in 50 mM sodium phosphate buffer, pH 6.0. Fraction volume of 10 ml was collected as flow rate of 20 ml/hr at 4°C. The column size used was 2.5 cm in diameter and 50 cm in height.

Figure 12. The chitinolytic enzyme activity in sodium dodecyl sulfate polyacrylamide gel electrophoresis (A) and native polyacrylamide gel electrophoresis (B) of purified *Bacillus licheniformis* high molecular weight chitinase enzyme (pooled fractions 58 to 70). The number of each lane represents amount of loaded enzyme (mU).

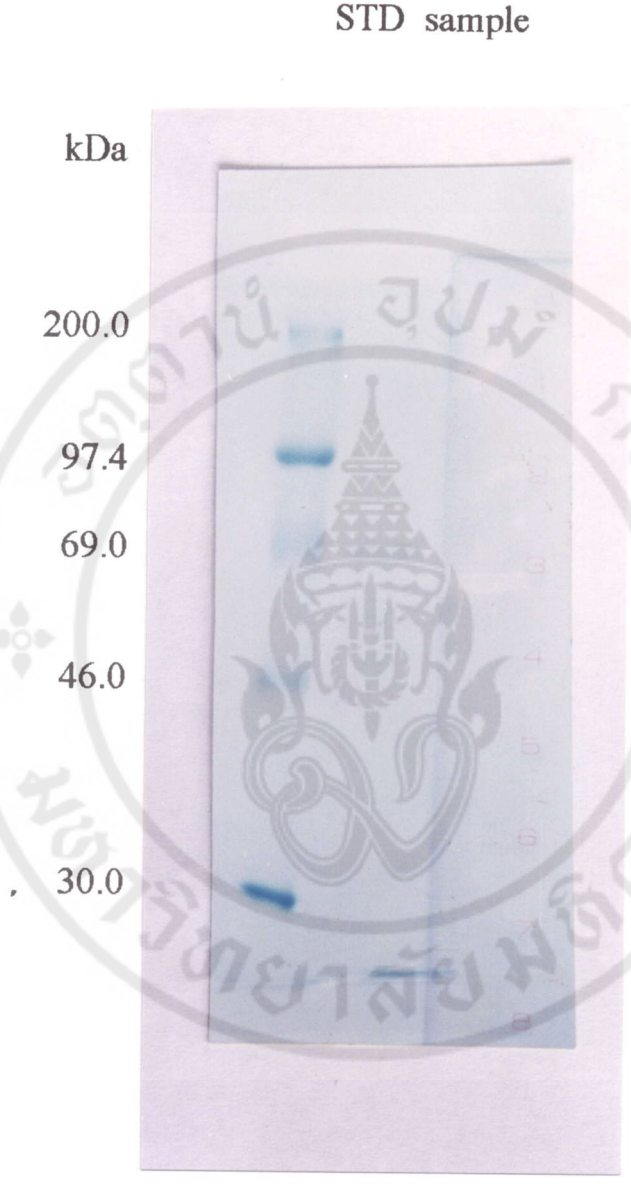


Figure 13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining of purified *Bacillus licheniformis* high molecular weight chitinase enzyme. The sample lane was loaded with 1 mU (1 μg) of enzyme from pooled fractions 58 to 70 and STD lane represents standard molecular weight protein (Rainbow marker).

4 Physicochemical characterization of purified chitinolytic enzyme from *Bacillus licheniformis*

The physicochemical characterization of *Bacillus licheniformis* chitinolytic enzyme was monitored with enzymatic colorimetric method.

The optimal temperature for enzyme reaction was determined by using 200 µl of 8.67 mU/ml enzyme and 50 µl of 0.52 %(w/v) chitin substrate in 100 mM sodium acetate buffer pH 5.0. The mixtures were incubated for 1, 3 and 6 hrs at various temperature, *i.e.*, 30, 37, 40, 45, 50, 55, 60, 65 and 70°C. Results of products released which represented enzymatic activity at various temperature were shown in Figure 14. From these data, the optimal temperatures of *Bacillus licheniformis* chitinolytic enzyme were 65, 60 and 60°C corresponded to the incubation time at 1, 3 and 6 hrs respectively. The difference of optimal temperature between 1 with those of 3 and 6 hrs incubation period might be due to enzyme inactivated upon prolonged incubation period during assay condition.

The stability of *Bacillus licheniformis* chitinolytic enzymes was analysed by incubating 200 µl (8.67 mU/ml) of enzyme alone at various temperature, *i.e.*, 40, 50, 60 and 70°C for different time intervals, *i.e.*, 0, 1, 2, 3, 4, 5, and 6 hrs. At the end of indicated times, a 50 µl of 0.52 %(w/v) chitin substrate in 100 mM sodium acetate buffer pH 5.0 was added to enzyme solution and incubated for 1 hr at 60°C. The product from the reaction was measured and results were summarized in Figure 15. The activity of chitinolytic enzyme was slightly decreased to 95 and 90 % when incubated at 40 and 50°C,

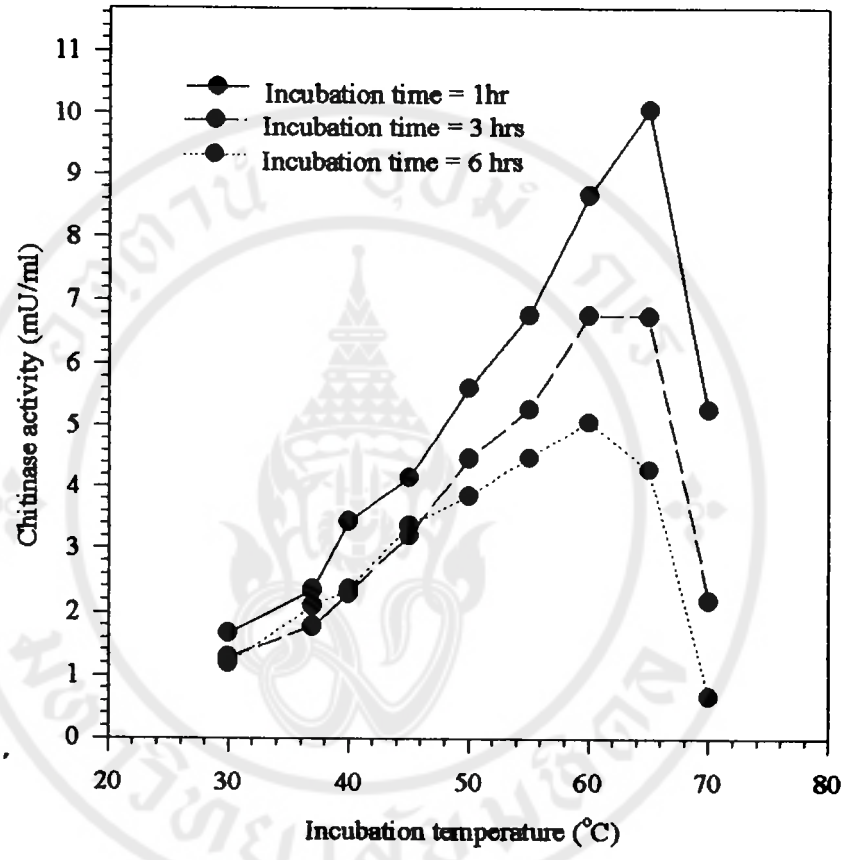


Figure 14. The activity of *Bacillus licheniformis* chitinolytic enzyme in 20 mM acetate buffer pH 5.0 and incubated with chitin substrate at various temperature, *i.e.*, 30, 37, 40, 45, 50, 55, 60, 65 and 70°C for a given time interval.

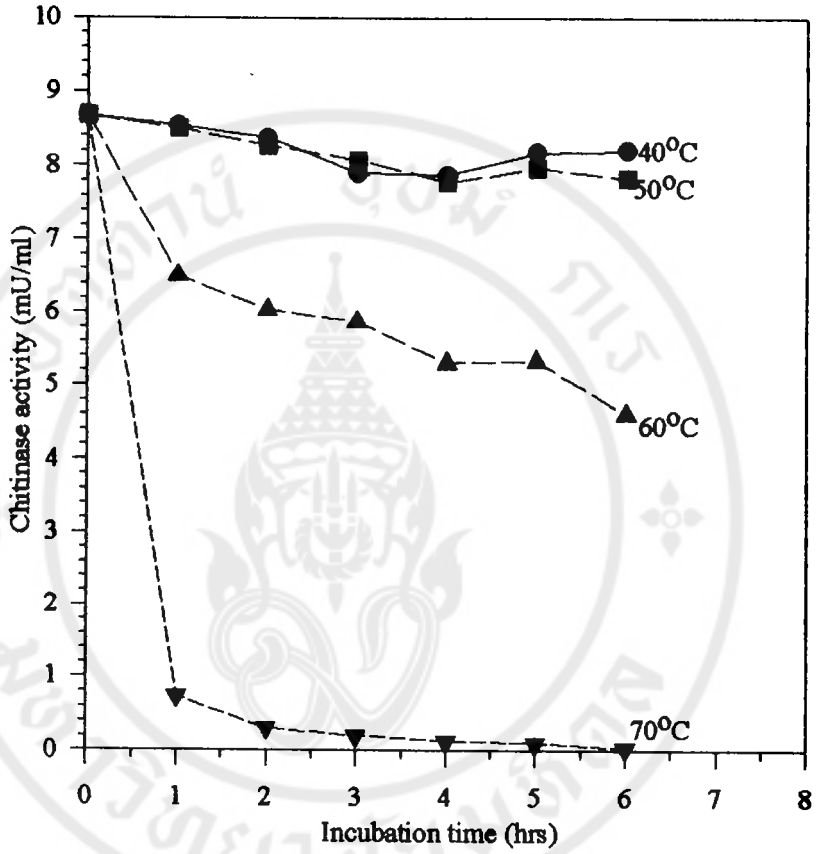


Figure 15. The stability of *Bacillus licheniformis* chitinolytic enzyme (8.67 mU/ml) kept in 20 mM acetate buffer pH 5.0 and incubated at different temperature *i.e.*, 40, 50, 60 and 70 °C for 6 hrs. The enzyme was assayed at 1 hr interval under assay condition of 60°C for 1 hr incubation period.

respectively for 6 hrs. About 53 % of enzyme activity was detected after it had been incubated at 60°C for 6 hrs. The enzyme activity was reduced 25 percent when the enzyme was kept alone at 60°C for 1 hr. The enzyme was dramatically lost its activity (>90 %) when it was kept at 70°C for 1 hr and almost completely lost activity after keeping for 6 hrs at this temperature.

The optimal pH for *Bacillus licheniformis* chitinolytic enzyme activity was determined by assaying enzymatic activity in Clark and Lubs buffer with varying pH starting from 3 to 9. A 200 µl (8.67 mU/ml) of enzyme was mixed with 50 µl of 250 mM buffer with different pH containing 0.52 % (w/v) chitin substrate to give 50 mM final concentration of buffer and incubated for 1 hr at 60°C. The pH of the mixtures were readjusted back to 5.0 with 0.1 N HCl or 0.1 N NaOH at the end of the incubation time. The volumes of mixtures were adjusted to final volume of 500 µl with steriled deionized distilled water. The product of enzyme activity was measured and calculated. Results of enzyme activity were summarized in Figure 16. The analysis of activities of *Bacillus licheniformis* chitinolytic enzyme indicated that the optimal pH was 5.0. The enzyme activity lost 96 and 88 % at pH 3.0 and 4.0, respectively whereas 86 and 84 % of activities were detected when the reactions were performed at pH 6.0 and 7.0, respectively. Therefore, the enzyme seemed to function well at pH range of 5.0 to 7.0.

The stability of enzyme against pH was also determined at various conditions. The first condition, a 50 µl of 250 mM Clark and Lubs buffer at different pH, *i.e.*, 3, 4, 5, 6, 7, 8 and 9 were mixed with 200 µl of enzyme and was kept at 4°C for various time intervals, *i.e.*,

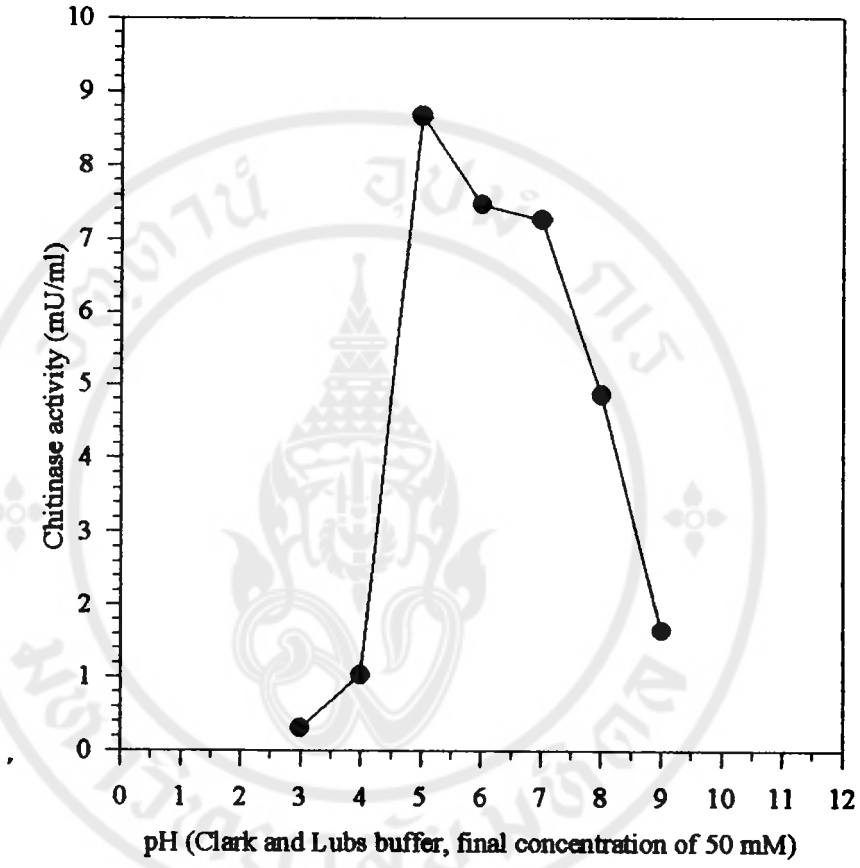


Figure 16. Activities of *Bacillus licheniformis* chitinolytic enzyme in different working pH.

0, 3 and 6 hrs. The pH value was then readjusted back to 5.0 with 0.1 N HCl or 0.1 N NaOH solution at the end of incubation. The volume of mixtures was adjusted to final volume of 500 µl with deionized distilled water. The enzymatic activity was assayed by incubating 200 µl of this enzyme solution with 50 µl of substrate solution for 1 hr at 60°C. The concentrations of product resulting from enzyme activity were measured and calculated. The enzyme kept in deionized distilled water was used as control. In the second condition, the activity of enzyme keeping in working pH 5.0 was determined. The mixtures of enzyme 200 µl and 50 µl of 250 mM Clark and Lubs buffer pH 5.0 were kept at 4°C for various time intervals, *i.e.*, 0, 2, 4, 6, 8, 12, 18 and 24 hrs. A 200 µl of these solutions were mixed with 50 µl of 0.52 % (w/v) chitin substrate in 100 mM sodium acetate buffer pH 5.0 and incubated for 1 hr at 60°C. The enzymatic activity was measured and the pH stability was analysed. Results of these two experiments were summarized in Figure 17 and Figure 18 respectively. The analysis of the chitinolytic enzyme activity of *Bacillus licheniformis* indicated that it was stable in deionized distilled water at 4°C. The activity was decreased about 30 and 40% when the enzyme was kept in pH 6.0 and 7.0, respectively at 4°C for 3 hrs. The activity of enzyme was rapidly lost when it was kept in strong acid and alkali pH. Whereas, it was found that the activity was lost for at least 38% when it was kept at pH 5.0 for 2 hrs, but the activity was gradually decreased after prolonged incubation at the same condition.

The enzyme kinetics such as initial velocity (v_i), Michaelis's constant (K_m) and maximum velocity (V_m) of *Bacillus licheniformis* chitinolytic enzyme were determined by using various substrate

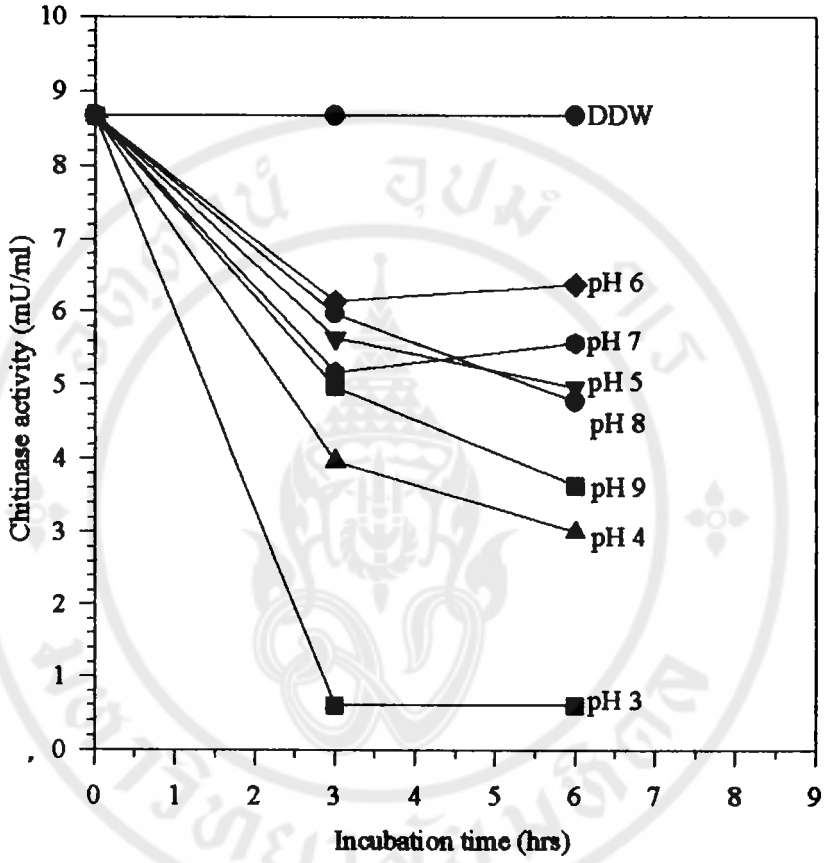


Figure 17. The stability of *Bacillus licheniformis* chitinolytic enzyme that had been kept in various working pH for 3 and 6 hrs at 4°C and then assayed at 60°C with 1 hr incubation period after the pH was brought back to pH 5.0.

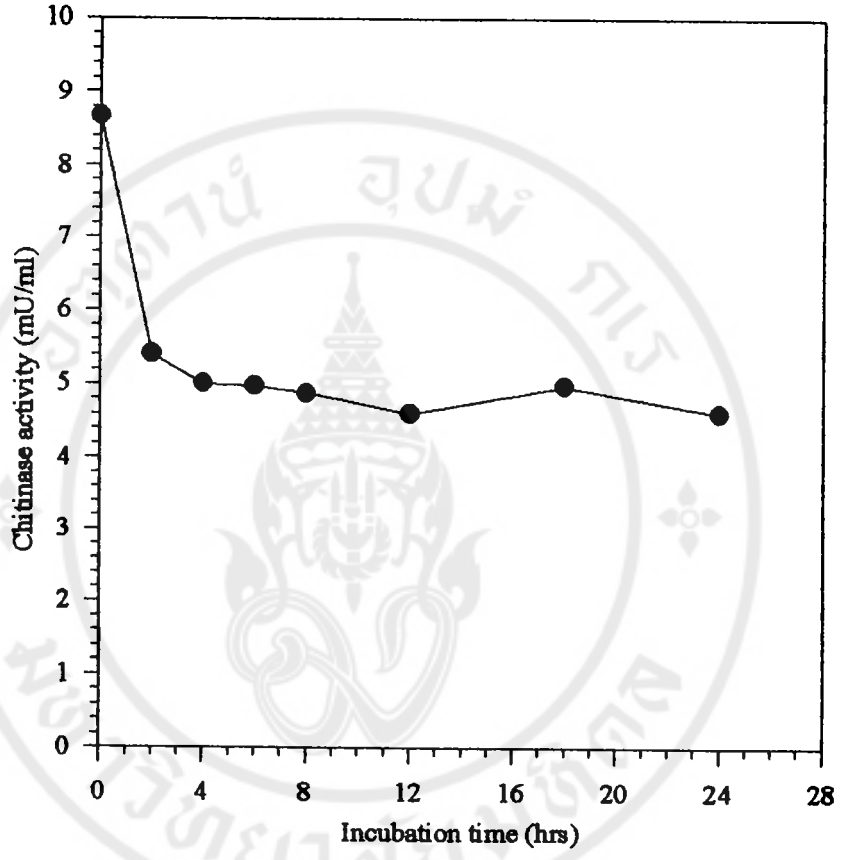


Figure 18. The activity of *Bacillus licheniformis* chitinolytic enzyme that had been kept in 50 mM Clark and Lubs buffer pH 5.0 at 4°C for various time intervals.

concentrations, *i.e.*, 0.2, 0.5, 1.0, 1.25 % (w/v) chitin substrate in 100 mM sodium acetate buffer pH 5.0, and results were summarized in Table 2, Figures 19 and 20. The initial velocity (v_i) of each substrate concentration was calculated from slope of its kinetic curve in Figure 19. By using Lineweaver and Burk's equation and protein concentration of enzyme that used in the reaction, the Michaelis's constant (K_m) and maximum velocity (V_m) of enzyme was measured. From these results, K_m was obtained from reciprocal absolute value of intersection point of 1/[S] on X-axis in Figure 20 and was calculated to be 3.33 % (w/v) while V_m was obtained from reciprocal value of intersection point of $1/[v_i]$ on Y-axis in Figure 20 and was calculated to be 30.83 mU/ml. Since the 30.83 x 10^{-3} µmoles of product was produced by enzyme solution which contained 39.43 µg of protein as measured by Bradford's method. Results of V_m indicated that the enzyme in 1 mg of protein had activity equal to 781.90 mU/ml (30.83 x 1,000/39.43). It was found that when 1 mg of enzyme in 200 µl of deionized distilled water mixed with 50 µl chitin substrate at the same concentration as K_m value [3.33 % (w/v)] in 100 mM acetate buffer pH 5.0 and incubated for 1 minute at 60°C, the amount of product (N-acetyl-D-glucosamine) released under this condition was 390.95 x 10^{-3} µmoles.

The molecular weight of *Bacillus licheniformis* chitinolytic enzyme was determined by using Coomassie brilliant blue (CBB) staining SDS-PAGE method in combination with chitinolytic activity detection of renaturing enzyme from SDS-PAGE gel. The location of chitinolytic enzymes in gel was determined by comparing between protein band present in gel stained with Coomassie brilliant blue and

Table 2. Kinetics study of *Bacillus licheniformis* chitinolytic enzyme.

Incubation time(min)	Absorbance (585 nm) of chitin substrate at various concentration (g%)			
	0.2	0.5	1.0	1.25
0	0	0	0	0
5	$2x10^{-2}$	0.01	0.014	0.022
10	$6x10^{-2}$	0.022	0.032	0.032
15	0.016	0.03	0.05	0.074
20	0.020	0.048	0.064	0.095
30	0.028	0.082	0.088	0.144
60	0.074	0.162	0.162	0.228
Initial velocity[v_i,slope,mU/ml)]	0.1027	0.2739	0.3536	0.5239
1/Initial velocity [$(mU/ml)^{-1}$]	9.73	3.65	2.83	1.91
1/Substrate concentration ($g\%^{-1}$)	5	2	1	0.8

A 200 µl of 8.67 mU/ml enzyme in Eppendorf tubes was mixed with 50 µl of 0.2, 0.5, 1.0, 1.25 % (w/v) chitin substrate in 100 mM sodium acetate buffer pH 5.0 and incubated at 60°C for 0, 5, 10, 15, 20 and 30 min. The concentration of product (N-acetyl-D-glucosamine) was analysed. The kinetic curve of substrate concentration [S] against product concentration [P] were plotted and the initial velocity (v_i) of each substrate concentration were calculated from slope of its kinetic curve. By using Lineweaver and Burk's equation, (1/[s] against $1/v_i$ plotting) and protein concentration of enzyme that used in the reaction, the Michaelis's constant (K_m) and maximum velocity (V_m) of *Bacillus licheniformis* chitinolytic enzyme was measured. The initial velocity (v_i), Michaelis's constant (K_m) and maximum velocity (V_m) of enzyme were summarized in the table.

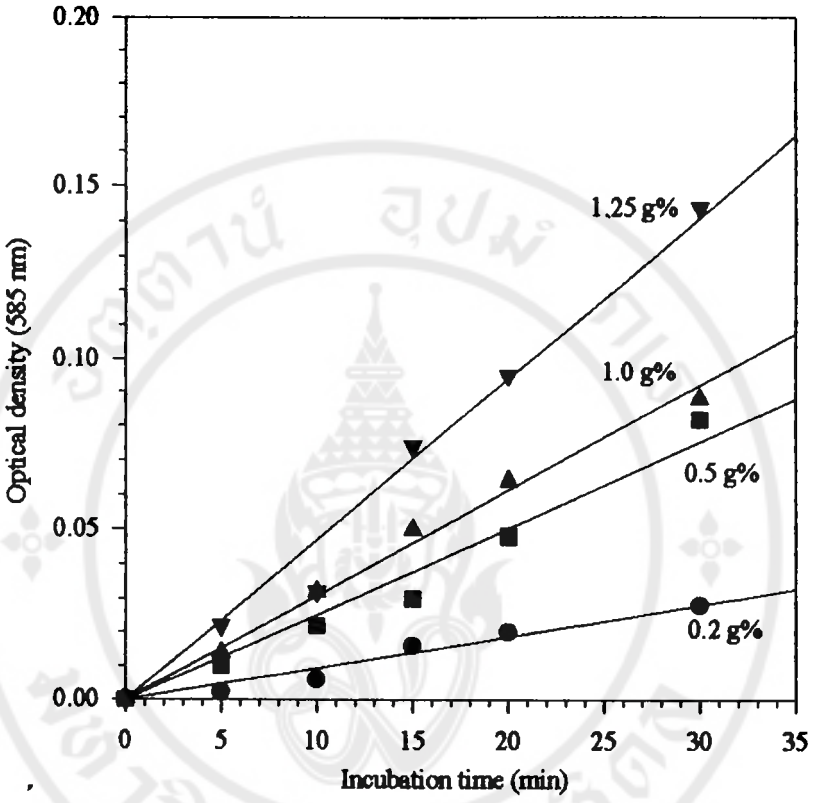


Figure 19. Kinetic curve of *Bacillus licheniformis* chitinolytic enzyme in 20 mM acetate buffer pH 5.0 containing various substrate concentration.

A 8.67 mU/ml of enzyme in the Eppendorf tubes were mixed with 0.2, 0.5, 1.0 and 1.25 % (w/v) chitin substrate in sodium acetate buffer pH 5.0 and incubated at 60°C for 0, 5, 10, 15, 20 and 30 min. The initial velocity (v_i) of each substrate concentration was calculated from it's slope.

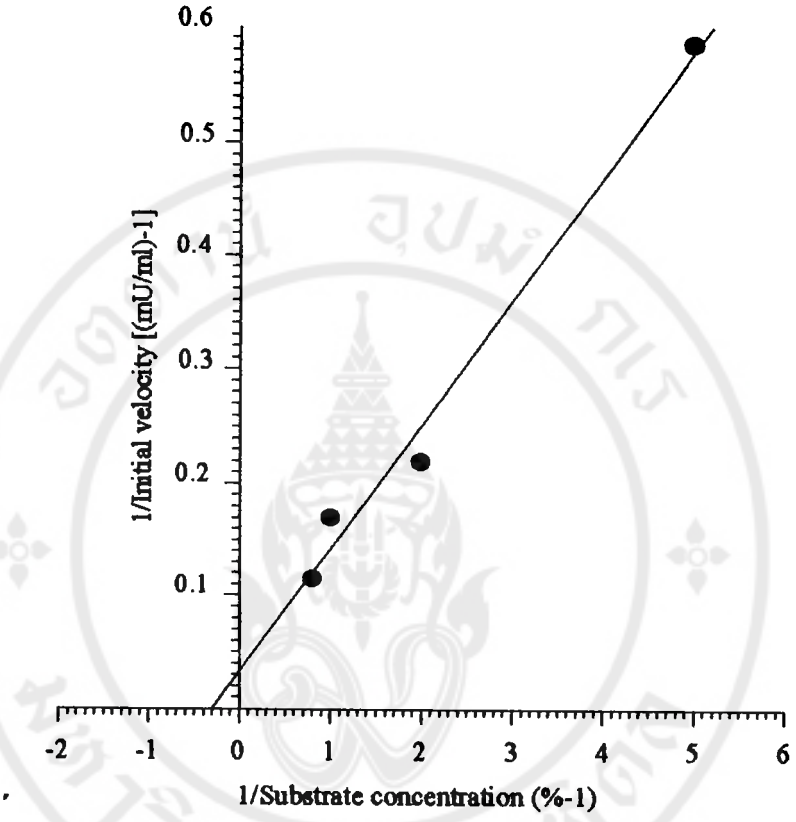


Figure 20. The Lineweaver and Burk's plot of *Bacillus licheniformis* chitinolytic enzyme.

Different substrate concentration was plotted against it's initial velocity by using Lineweaver and Burk's equation (1/[s] against $1/v_i$). The Michaelis's constant (K_m) and maximum velocity (V_m) of *Bacillus licheniformis* chitinolytic enzyme was calculated by using this curve and protein concentration value of 8.67 mU/ml of enzyme that used in the reaction.

corresponding lytic zone in enzymatic activity staining gel at the same path length mobility. Results of Coomassie brilliant blue and enzymatic activity staining gels were shown previously in Figure 12 and 13. The molecular weight of enzyme was calculated from standard molecular weight curve obtained from standard molecular weight proteins (Rainbow marker) that was shown in Figure 21 by using leading edge curve or middle line curve for comparison between front edge or middle line of the protein bands respectively. The molecular weight of *Bacillus licheniformis* chitinolytic enzyme as determined from the curve was 70.8 kDa. However, from Coomassie brilliant blue staining gel and enzymatic activity detection in gel after renaturation of *Bacillus licheniformis* culture supernatant, the additional activity bands were found nd their molecular weight were 68.1, 63.7, 53.0 and 49.5 kDa (see Figure 7). From these results, it is suggested that *Bacillus licheniformis* produces at least 5 types of chitinolytic isozymes.

5 Mosquito larvicidal test

To determine toxic property of *Bacillus licheniformis* and intracellular chitinase in *E.coli* transformant carrying a plasmid with chitinase gene from *Aeromonas hydrophila* against mosquito larvae, *Bacillus licheniformis* and *E.coli* were cultured in medium with and without chitin substrate supplement. *E.coli* JM109 was used as negative control. The cultured cells were washed several times and suspended with steriled distilled water. The cell suspension was then tested against *Aedes aegypti* larvae as described in material and

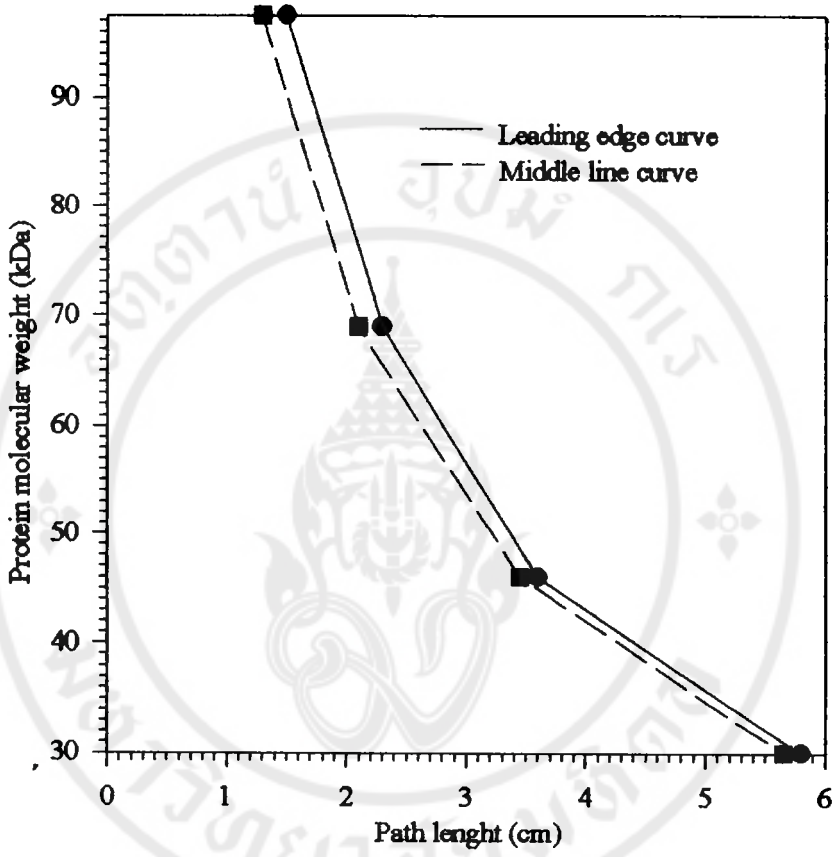


Figure 21. The standard molecular weight curve of protein (Rainbow marker).

methods. For the control group, distilled water was used instead of bacterial suspensions. The experiment was done in cups of 100 ml of water containing 10 larvae at room temperature and the number of dead larvae were recorded in 2 days after exposure. The 2 nd instar *Aedes aegypti* larvae were used. The activity was recorded as 50 % lethal concentration value (LC_{50}) by using the cell density (cells/ml of bacteria in the cup) calculated from colony forming unit on culture medium and the results were summarized in Table 3. No dead larvae were observed in all test samples. Results indicated that washed bacterial cell (without culture broth) of *Bacillus licheniformis* or intracellular chitinase of *E.coli* transformant did not contain any mosquito larvicidal activity.

The dialysed 65 % ammonium sulfate precipitated protein from culture supernatant of *Bacillus licheniformis* that had been cultured in medium with and without supplemented chitin substrate was examined for the mosquito larvicidal activity in 24 wells plastic plates containing 2 ml of enzyme suspension and 5 larvae in each well. Second instar *Aedes aegypti* larvae were used. The volume of both dialysed fractions was adjusted to 4.5 ml before used. The experiment was done at room temperature and the number of dead larvae were recorded in 2 days after exposure. Results of protein concentration (determined by Bradford's method), chitinolytic enzyme concentration (determined by Reissig's method), bacterial concentration (determined by total agar plate count) and mosquito larvicidal toxicity test of untreated and treated crude protein fractions that had been inactivated by heating at 100°C for 10 min were summarized in Table 4. It was found that the crude fractions contained about 10^3 cells/ml.

Table 3. Toxicity test of bacterial cell pellet of *Bacillus licheniformis*, *E.coli* transformant carrying a chitinase gene and *E.coli* JM109 against 2 nd instar of *Aedes aegypti* larvae.

Bacterial strain	Final cell concentration Cells/ml)	Toxicity test (Dead larvae/ total test larvae)	LC_{50} (Cells/ml)
B.licheniformis			
NB	$4.40x10^6$	0/10 0/10	$> 4.40x10^6$
NB/2+chitin	$1.62x10^7$	0/10 0/10	$> 1.62x10^7$
E.coli JM109			
NB	$1.71x10^9$	0/10 0/10	$>1.71x10^9$
NB/2+chitin	$1.47x10^9$	0/10 0/10	$>1.47x10^9$
E.coli transformant			
NB	$7.20x10^8$	0/10 0/10	$>7.20x10^8$
NB/2+chitin	$1.12x10^9$	0/10 0/10	$>1.12x10^9$

All of bacterial strains were grown overnight in LB broth at 37 °C in rotary shaker. Each overnight grown bacteria were inoculated into indicated medium and incubated at 37°C for 48 hrs. The inoculum was regulated to 1% of the total volume of the medium. Each culture was centrifuged at 8,000 rpm for 10 min and cell pellets were washed many times with distilled water. These cell pellets were tested for the mosquito larvicidal activity with a 100 ml water. The LC_{50}s were calculated from the number of dead larvae and cells density in the cup that calculated from the colony forming unit.

Table 4. The toxicity test of crude fraction of *Bacillus licheniformis* culture supernatant against 2nd instar of *Aedes aegypti* larvae.

Crude fraction obtained from medium	Bacterial concentration (Cells/ml)	Protein concentration (μg/ml)	Chitinolytic enzymes activity (mU/ml)	Toxicity test (Dead larvae/total test larvae)	
				untreated fraction	heat treated fraction
NB	$9.52x10^2$	555	1.68	0/5 0/5	0/5 0/5
NB/2+chitin	$1.80x10^3$	1205	123.5	4/5 4/5	0/5 0/5

Bacillus licheniformis was grown in LB broth for overnight at 37°C in shaker. A preculture was then inoculated into 450 ml indicated medium. The inoculum was regulated to 1% of the total volume of the medium. And then cells were grown for 48 hrs at 37°C in rotary shaker. Each culture was centrifuged at 8,000 rpm for 10 min and the cell pellet was discarded. The culture supernatant was precipitated with 65% saturation of ammonium sulfate at 4°C. The precipitate was allowed to form by kept standing at 4°C for overnight and then was collected by centrifugation for 30 min at 10,000 rpm. The precipitate was then dissolved with 3.0 ml of cold deionized distilled water and dialysed against cold deionized distilled water at 4 °C and finally the volume was adjusted to 4.5 ml with water. These NB and NB/2+chitin crude fractions were assayed for the protein concentration by Bradford's method, chitinolytic enzyme concentration by Reissig's method, and determined bacterial concentration by plate count method. The fractions were tested for mosquito toxicity directly or heating at 100°C for 10 min before assaying. The toxicity assay was performed by using 5 of 2 nd instar *Aedes aegypti* larvae in 2 ml of crude fractions suspension.

The protein in crude fraction obtained from NB/2 plus chitin (1205 μg/ml) was about 2 times higher than that of fraction from NB (555 μg/ml), whereas the enzyme activity was about 70 times (123.5 mU/ml/1.68 mU/ml). The high activity of enzyme in fraction obtained from supernatant of cells grown in NB/2 plus chitin indicated that chitinase was inducible enzyme and could be induced by chitin. The toxicity was observed only in the fraction that contained high chitinase activity and the toxicity was completely lost upon heating the fraction at 100°C for 10 min. This heat treatment was also completely inactivated the enzyme activity (data not shown). From these results, it was indicated that there were some heat labile factors in the crude fraction which responsible for larvicidal activity against mosquito larvae. The lost of chitinase activity was coincided with the lost of toxicity activity. Therefore, it is suggested that chitinase enzyme may play role in toxicity against mosquito larvae. However, results could not exclude other heat labile factor(s) that might contribute to this observation. Thus, purified chitinase enzyme is required in order to test for larvicidal activity.

The crude fraction of 65 % ammonium sulfate precipitated protein from culture supernatant of *Bacillus licheniformis* cultured in medium with supplemented chitin substrate was tested for the mosquito larvicidal activity against 2nd instar of *Aedes aegypti* larvae. The LC_{50} values was calculated according to the method described by Reed and Muench (1938) using the chitinolytic enzyme concentration (determined by Reissig's method) against 2nd instar of *Aedes aegypti* larvae. The calculation method was shown in appendix. The LC_{50} of

Bacillus licheniformis chitinolytic enzymes against 2nd instar of *Aedes aegypti* larvae was equal to 60.67 mU/ml as shown in Table 5.

The combination effect of chitinolytic enzyme in crude fraction of 65 % ammonium sulfate precipitated protein from culture supernatant of *Bacillus licheniformis* to either *B.t.i.*4Q2-72 or *B.t.i.* c4Q2-72 cells in killing the mosquito larvae was studied. The sublethal dose of enzyme activity (8.33, 16.67 and 33.33 mU/ml) was used in combination with various cell concentration of *B.t.i.*4Q2-72 to test for larvicidal activity. It was found that at least 60.67 mU/ml was required to kill 50 % of mosquito larvae. Thus, the sublethal and lethal dose of enzyme activity, *i.e.*, 16.67, 33.33 and 66.67 mU/ml were also used in combination with various cell concentrations of *B.t.i.*c4Q2-72 to test for larvicidal activity. The toxicity test was done in 24 wells plastic plates against 2 nd instar *Aedes aegypti* larvae at room temperature and the number of dead larvae were recorded in 2 days after exposure. The mosquito larvicidal activity of *B.t.i.*4Q2-72 or *B.t.i.*c4Q2-72 alone was also tested and was used as a control. Results of bacterial concentration, enzymatic concentration, toxicity test and LC_{50} value were summarized in Table 6 and 7. From Table 6, it was indicated that enzyme fraction could enhance the killing activity of *B.t.i.* 4Q2-72. The higher enzyme concentration used, the toxicity was increased. The toxicity of *B.t.i.* 4Q2-72 was increased about 1.20, 3.55 and 5.01 folds when 8.33, 16.67 and 33.33 mU/ml of the chitinolytic enzyme of *Bacillus licheniformis* was added respectively. However, result obtained from *B.t.i.*c4Q2-72 in combination with 16.67, 33.33 and 66.67 mU/ml of the chitinolytic enzyme of *Bacillus licheniformis* was shown to be different from the

Table 5. The toxicity test of *Bacillus licheniformis* chitinolytic enzymes (crude fraction) against 2nd instar of *Aedes aegypti* larvae.

Enzymes concentration (mU/ml)	Toxicity test (Dead larvae/total test larvae)	
	Untreated fraction	Heat treated fraction
133.33	5/5 5/5	0/5 0/5
116.67	5/5 5/5	0/5 0/5
100.00	5/5 4/5	0/5 0/5
83.33	5/5 3/5	0/5 0/5
66.67	3/5 4/5	0/5 0/5
50.00	2/5 1/5	0/5 0/5
33.33	1/5 0/5	0/5 0/5
16.67	0/5 0/5	0/5 0/5
0	0/5 0/5	0/5 0/5
	LC_{50} = 60.67 mU/ml	none

Crude (65 % ammonium sulfate precipitated protein) chitinolytic enzymes of *Bacillus licheniformis* was tested for the mosquito larvicidal activity and LC_{50} was calculated. Toxicity test of inactivated enzyme (heating at 100°C for 10 min) and untreated fractions were performed by using duplicated set of 5 larvae in 2 ml of enzyme suspension. The various enzyme concentrations were diluted by serial dilution method and expressed as the final enzyme concentration in the cup was indicated in the table.

Table 6. The toxicity test of *B.t.i.*4Q2-72 alone or *B.t.i.*4Q2-72 plus enzyme crude fraction obtained from *Bacillus licheniformis* against 2nd instar *Aedes aegypti* larvae.

*B.t.i.*4Q2-72 (Cells/ml)	Toxicity test (Dead larvae/ total test larvae)			
	Cells only	Cells+8.33 mU/ml	Cells+16.67 mU/ml	Cells+33.33 mU/ml
$5.90x10^5$	5/5 5/5	5/5 5/5	5/5 5/5	5/5 5/5
$1.18x10^5$	5/5 5/5	5/5 5/5	5/5 5/5	5/5 5/5
$2.36x10^4$	5/5 5/5	5/5 5/5	5/5 5/5	5/5 5/5
$4.72x10^3$	2/5 1/5	3/5 1/5	3/5 3/5	4/5 3/5
$9.44x10^2$	0/5 0/5	0/5 0/5	1/5 3/5	3/5 2/5
$1.89x10^2$	0/5 0/5	0/5 0/5	0/5 0/5	0/5 0/5
LC_{50} (cells/ml)	$7.41x10^3$	$6.17x10^3$	$2.09x10^3$	$1.48x10^3$
Toxicity increase (folds)	1	1.20	3.55	5.01

The *B.t.i.*4Q2-72 was grown in LB broth at 37°C in a rotary shaker for overnight. A preculture was then subcultured to NBS medium (NB broth that contain 1 mM magnesium chloride, 0.7 mM calcium chloride and 0.05 mM manganese chloride) and then were grown at 37°C in a shaker for 48 hrs. The inoculum was regulated to 1% of the total volume of the medium. The mosquito larvicidal activity of this cell suspension alone or in combination with *Bacillus licheniformis* chitinolytic enzymes (crude) were tested against 2nd instar *Aedes aegypti* larvae (5 larvae in 2 ml of tested suspension), LC_{50} values were calculated by using cell concentration (CFU/ml) of *B.t.i.*4Q2-72 against dead larvae. The final enzyme concentrations in the cup were indicated in the table.

Table 7. The toxicity test of *B.t.i.*c4Q2-72 alone or *B.t.i.*c4Q2- 72 plus enzyme crude fraction obtained from *Bacillus licheniformis* against 2nd instar *Aedes aegypti* larvae.

*B.t.i.*c4Q2-72 (Cells/ml)	Toxicity test (Dead larvae/ total test larvae)			
	Cells only	Cells+16.67 mU/ml	Cells+33.33 mU/ml	Cells+66.67 mU/ml
$3.72x10^5$	0/5 0/5	0/5 0/5	1/5 0/5	2/5 0/5
$7.44x10^4$	0/5 0/5	0/5 1/5	1/5 0/5	2/5 0/5
$1.49x10^4$	0/5 0/5	0/5 0/5	1/5 1/5	1/5 3/5
$2.98x10^3$	0/5 0/5	0/5 0/5	1/5 2/5	1/5 2/5
$5.95x10^2$	0/5 0/5	1/5 1/5	1/5 0/5	4/5 5/5
$1.19x10^2$	0/5 0/5	2/5 0/5	0/5 1/5	5/5 5/5

The *B.t.i.*c4Q2-72 was grown in LB broth at 37°C in a rotary shaker for overnight. A preculture was then subcultured to NBS medium (NB broth that contain 1 mM magnesium chloride, 0.7 mM calcium chloride and 0.05 mM manganese chloride) and then were grown at 37°C in a shaker for 48 hrs. The inoculum was regulated to 1% of the total volume of the medium. The mosquito larvicidal activity of this cell suspension alone or in combination with *Bacillus licheniformis* chitinolytic enzymes (crude) were tested against 2nd instar *Aedes aegypti* larvae (5 larvae in 2 ml of tested suspension), LC_{50} values were calculated by using cell concentration (CFU/ml) of *B.t.i.*c4Q2-72 against dead larvae. The final enzyme concentrations in the cup were indicated in the table.

results of *B.t.i.*4Q2-72. The fraction that containing higher enzyme activity seemed to have some killing activity as indicated by the number of dead larvae but the number was much lower than that performed in *B.t.i.*4Q2-72. Since *B.t.i.*c4Q2-72 is a plasmid cured strain of *B.t.i.*4Q2-72 which could not produces crystal toxin, therefore it is suggested that the enzyme together with cells producing toxin should contained higher activity than cells without toxin. The chitinase at sublethal concentration could help the nontoxic cell in killing mosquito larvae. Therefore, crude fraction of 65 % ammonium sulfate precipitated protein from culture supernatant of *Bacillus licheniformis* can enhance the mosquito larvicidal property of *B.t.i.*4 Q2-72. However, the result of toxicity test of crude fraction combined with *B.t.i.*c4Q2-72 cells indicated that the dead of 2nd instar of *Aedes aegypti* larvae might be resulted from the effect of *Bacillus licheniformis* chitinolytic enzyme alone.

In order to test the effect of purified chitinase activity in killing mosquito larvae, the purified enzyme was used in toxicity assay. It was shown that the *Bacillus licheniformis* produces at least 5 types of chitinolytic isozymes (see Figure 7) but only chitinase with molecular weight of 70.8 kDa was selected for the purification. It was used for toxicity test and the result was shown in Table 8. The experiment was done in 24 wells plastic plate against 2 nd instar *Aedes aegypti* larvae at room temperature and the number of dead larvae were recorded in 2 days after exposure. It was found that the LC_{50} of 70.8 kDa chitinolytic enzyme of *Bacillus licheniformis* against 2nd

Table 8. The toxicity test of purified *Bacillus licheniformis* chitinolytic enzyme (70.8 kDa) against 2nd instar *Aedes aegypti* larvae.

Test condition (Enzyme concentration)	Toxicity test (Dead larvae/total test larvae)	
	1st set	2nd set
133.33 mU/ml	5/5	5/5
66.67 mU/ml	2/5	3/5
33.33 mU/ml	0/5	0/5
16.67 mU/ml	0/5	0/5
8.33 mU/ml	0/5	0/5
LC_{50} = 66.67 mU/ml		

Crude (65 % ammonium sulfate precipitated protein) chitinolytic enzymes of *Bacillus licheniformis* was purified by CM-sephadex and two columns of DEAE-sephadex. The enzyme was dissolved and dialysed against deionized distilled water. This purified chitinolytic enzymes (70.8 kDa) was tested for the mosquito larvicidal activity using 24 wells plastic plate. The experiment was done against 5 of 2 nd instar *Aedes aegypti* larvae in 2 ml of enzyme suspension at room temperature and the number of dead larvae were recorded in 2 days after exposure. The final enzyme concentrations in the cup were indicated in the table.

instar of *Aedes aegypti* larvae was equal to 66.67 mU/ml. Since the fraction contained no cells, therefore, the killing activity should resulted from the enzyme activity. From this results it is indicated that a 70.8 kDa chitinolytic enzyme of *Bacillus licheniformis* has the toxicity activity against *Aedes aegypti* larvae.

CHAPTER V
DISCUSSION

Among the members of biological agent for mosquito larvae control, *Bacillus thuringiensis* subsp. *israelensis* (*B.t.i.*) has been one of the most extensively studied. The main reasons which led to such intensive research activities are the interest in the potential for the importance of the δ-endotoxin for biological control of mosquito larvae, safety for human and non target living in laboratory trials, field trials or real using, more variety of δ-endotoxin genes for toxicity increasing by genetic engineering and genetic manipulation (Lacey and Undeen, 1986). At present, *Bacillus thuringiensis* subsp. *israelensis* is represented a tiny fraction of the biopesticide market, which in turn is still only a small money sharing group of the annual worldwide pesticide market (Porter *et al.*, 1993). The field application of *B.t.i.* is still facing many limitations. They are UV inactivation in strong sunlight, cannot survive long in highly polluted water, rapid settling of spores even under normal field conditions, high costs of production, non sufficient toxicity and narrow host species range compared with the chemical mosquitocidal agents (Mulligan *et al.*, 1980; Mulla, 1990; Porter *et al.*, 1993). Many groups of scientist have been attempted to reduce these limitations by many methods such as expression of *B.t.i.* toxin genes in the novel hosts including the development of toxin genes by using the stronger promoters, optimizing the strength of ribosome binding sites (Vellanoweth and Rabinowitz, 1992), replacement with the more

efficiency of bacterial enhancer and terminators sequences (Gentz *et al.*, 1981; Wong and Chang, 1986), improving the formulation of the products and apply in the integrate pest management system with the other agents such as protozoan (*Tetrahymena pyriformis*) or teflubenzuron (Manasherob *et al.*, 1994; Chui *et al.*, 1995).

Chitinase enzymes have been used as the adjuvants for insecticidal activity of *Bacillus thuringiensis* and baculovirus against the spruce budworm and gypsy moth respectively (Smirnoff, 1971; Smirnoff, 1973; Morris, 1976; Shapiro, 1987). The chitinase enzymes will disrupt the chitinous layer of the host midgut and allow the toxins to exert their activities. The objective of this studied was aimed to investigate the effect of chitinolytic enzymes of *Bacillus licheniformis* to enhance the toxicity of *Bacillus thuringiensis* subsp. *israelensis* for mosquito larvicidal activity. The physicochemical properties and mosquito larvicidal activity of *Bacillus licheniformis* chitinolytic enzymes were also studied.

The production of *Bacillus licheniformis* chitinolytic enzymes in medium supplemented with chitin substrate (1/2 NBC broth) at 37 °C was gradually increased from log phase till the cells reached the late stationary phase. Since, the nutrient broth (NB) was deprived of nutritions for *Bacillus licheniformis* at 14 hrs culturing time grown at 37°C (Figure 4), thus chitin substrate was used as carbon source and might induce chitinolytic enzymes production. The culture supernatant from cells that had been cultured for 48 hrs at 37°C in the medium supplemented with chitin substrate was used as the source for enzymatic purification. In the contrary, the bacteria grown in medium without chitin produce a little amount of chitinase enzyme. The

optimum concentration of the ammonium sulfate (percent saturation) for chitinolytic enzymes precipitation of culture supernatant was determined. The results showed that *Bacillus licheniformis* chitinolytic enzymes were precipitated by ammonium sulfate at concentration ranges from 30 to 70 percent (data not shown). So that, the ammonium sulfate concentration ranges between 35 to 65 percent saturation was chosen to precipitate chitinolytic enzymes of *Bacillus licheniformis* to avoid non desired protein in adjacent concentration (30-35 and 65-70 percent saturation) for further studies.

In order to characterize and assay for mosquito larvicidal activity of the purified enzyme, the chitinolytic enzyme of *Bacillus licheniformis* was purified by precipitation with ammonium sulfate in previous discussion and fractionating with ion exchange chromatography with CM- and two times of DEAE-sephadex column respectively (Table 1). The purification results showed that fraction obtained from the 35-65 % saturation of ammonium sulfate yielded lower enzymatic specific activity than that of the supernatant source. This may cause by the lost of enzyme activity upon salt precipitation. The 35-65 % saturation fraction was chosen as the source for purification experiment, since *Bacillus licheniformis* chitinolytic enzymes were mostly precipitated with ammonium sulfate at these concentration ranges. However, the purification results showed that this 35-65 % saturation fraction yielded chitinolytic enzymes activity only half of total activity when comparing with the supernatant source. This is probably due to some chitinolytic enzymes are lost when fractionating with 0-35 % and 65-100 % saturation and also lost during the protein precipitation and centrifugation steps in 35-65 %

saturation for protein precipitation. In the ion exchange chromatography with CM-sephadex C-50 column experiment, some undesired proteins were eliminated which was indicated by the increasing of enzymatic specific activity in this fraction. This protein fraction was passed through the DEAE sephadex A-50 column after run though the CM-sephadex C-50 column by using gradient concentration of sodium chloride ranging from 0 to 1 M in washing buffer as eluting buffer. The chromatographic pattern of enzyme fractions shows only two peaks. But, the results from activity staining as well as protein stain from SDS-PAGE method of various fraction samples indicated that enzyme was not pured whereas the fractions number 55 to 85 in the second peak showed the highest molecular weight, 70.8 kDa of chitinolytic enzyme and indicated that the enzyme was almost pured (Figure 8-10). Therefore, all fraction members in the second peak of chitinolytic enzyme activity in the first DEAE A-50 chromatographic pattern were pooled and further purified by passing again through DEAE sephadex A-50 column ion exchange chromatography. However, the gradient concentration of sodium chloride ranging from 0.25 to 1 M in washing buffer was used as the eluting buffer instead of 0 to 1 M to increase the separation efficiency. The chromatographic pattern of enzyme activity derived from this second DEAE sephadex A-50 column (Figure 11) and the results from protein stain from SDS-PAGE methods (Figure 13) as well as activity staining for chitinolytic activity detection from native-PAGE, SDS-PAGE methods (Figure 12) indicated that the last fractions of the peak contained the highest molecular weight 70.8 kDa of *Bacillus licheniformis* chitinolytic enzyme. These fractions (58 to 70) were

pooled, precipitated with 65 percent saturation of ammonium sulfate and dialysed against deionized distilled water for characterization of the physicochemical properties and assay for the mosquito larvicidal activity. However, it was found that the yield of this purified. 70.8 kDa chitinolytic enzyme was only 1.36 % when compared with supernatant source. This was probably due to the other chitinolytic enzymes that contributed for the majority of the amount of chitinolytic enzymes in the supernatant source were eliminated with the purification steps. However, the enzymatic specific activity of this purified fraction was decreased when compared with the previous fraction (1°DEAE sephadex fraction). The purified enzyme in the 2 °DEAE sephadex fraction might contain lower enzymatic activity than the other chitinolytic enzymes that contained in the previous fraction and these enzymes were eliminated with the 2°DEAE sephadex ion exchange chromatography. For the confirmation of this hypothesis, further study of enzymatic activity of these undesired chitinolytic enzymes is needed.

It is very useful to determine the type of the *Bacillus licheniformis* chitinolytic enzymes whether it is endochitinase, exochitinase, β-N-acetylglucosaminidases or chitobiase. In this studies, the determination was not done because both experiments for chitinolytic activity assay and detection that were used in this study could not determine the type of tested chitinolytic activity (Domard and Vasseur, 1991; Tronsmo and Harman, 1993). So that, in this study, only the term chitinase enzyme or chitinolytic enzyme were used as the name of *Bacillus licheniformis* enzyme that can degrade chitin into smaller polymer.

The characterization of physicochemical properties of *Bacillus licheniformis* 70.8 kDa chitinolytic enzyme was done by studying the chitinolytic function in various pH, temperature, the analysis of the stability of chitinolytic function in various pH, temperature and the determination of the enzyme kinetics parameters such as Michaelis's constant (K_m) and maximum velocity (V_m) of this enzyme. These characterization will lead to the application of this enzyme against mosquito larvae. It was found that the optimal pH of this enzyme was 5.0 (Figure 16) and the activity of this enzyme were lost 88, 14 and 16 % in solution that had pH level of 4.0, 6.0 and 7.0 respectively (Figure 17). However this chitinolytic activity was lost for at least 38% when the enzyme was kept in optimal pH condition at 4°C for 2 hrs and was gradually decreased after prolonged incubation at the same condition. This phenomenon was occurred because some factor (s) in Clark and Lubs buffer system that used in this study probably interrupted or decreased the activity of this enzyme. This hypothesis was supported by the fact that the activity of this *Bacillus licheniformis* chitinolytic enzyme was stabled in deionized distilled water at 4°C. The chitinolytic activity was rapidly lost when it was kept in strong acid and alkali pH. The optimal temperatures of 70.8 kDa chitinolytic enzyme of *Bacillus licheniformis* were 65, 60 and 60 °C when it was incubated at 1, 3 and 6 hrs respectively (Figure 14). The difference in optimal temperature when 1, 3 and 6 hrs incubation times were used is probably due to the inactivation of enzyme upon the prolonged incubation period. This observation is supported by results from the study on temperature stability of this enzyme. The results of temperature stability showed that the activity of chitinolytic

enzyme was decreased about 47 % after it had been incubated at 60°C for 6 hrs and about 75 % of enzyme activity was detected when the enzyme was kept alone at 60°C for 1 hr. Moreover, the enzymatic activity was dramatically lost when it was kept at 70°C for 1 hr and the chitinolytic activity of 70.8 kDa chitinolytic enzyme of *Bacillus licheniformis* was slightly decreased when incubated at lower temperature (40 and 50°C). It was found that the enzyme kinetics parameters such as initial velocity (v_i), Michaelis's constant (K_m) and maximum velocity (V_m) of *Bacillus licheniformis* chitinolytic enzyme were 3.33 % (w/v) and 781.90 mU/ml/mg respectively (Figure 20). However, these enzyme kinetics results might not completely accurate since these assays use insoluble chitin (colloidal chitin) as substrate for the enzymatic activity detection. In the non-homogeneous suspension, the tested enzyme in Eppendorf tubes did not completely adhere, interact and digest the substrate, so that the K_m and V_m results obtained may be higher and lower value respectively than the real results.

The results of mosquito larvicidal activity between the dialysed products derived from 35-65 % saturation of ammonium sulfate precipitated protein from culture supernatant of *Bacillus licheniformis* that had been cultured in medium supplement with and without chitin substrate against *Aedes aegypti* larvae demonstrated that some factor (s) that could be induced by chitin are responsible for this activity. However, these mosquito larvicidal factor(s) might be contributed by possibly contaminated ammonium sulfate, thus, the activity testing of heated precipitated fraction was required in order to investigate this problem. The larvicidal activity resulting from heated precipitated

protein fraction showed that the activity was completely lost upon heating at 100°C for 10 min (Table 5). Therefore, it was indicated that mosquito larvae were killed by some heat labile factor(s) and this factor(s) was not the contaminated ammonium sulfate. Nevertheless, the *Bacillus licheniformis* cells that contaminated in this dialysed product of 35-65 % ammonium sulfate precipitated protein (crude fraction) might be responsible for this mosquito larvicidal activity. So that, the mosquito larvicidal activity of washed bacterial cells (without culture broth) of *Bacillus licheniformis* was done in order to investigate this problem. The larvicidal activity results showed that *Bacillus licheniformis* cells did not contain any mosquito larvicidal activity. From these data, it was indicated that some factor(s) that contained in crude fraction of cultured supernatant was responsible for the mosquito larvicidal activity. In addition, the protein in crude fraction obtained from supernatant of cells cultured in medium with chitin was about 2 times higher than that in fraction obtained from cultured in medium without chitin whereas the enzyme activity was about 70 times higher. The toxicity was observed only in the fraction that contained high chitinase activity. The heat treatment was also completely inactivated the chitinolytic activity and finally the lost of chitinolytic activity was coincided with the lost of toxicity activity. From these data, it was suggested that inducible chitinolytic(s) enzyme that could be induced by chitin and completely lost its activity upon heating at 100°C for 10 min might play a role in toxicity against mosquito larvae. The LC_{50} of mosquito larvicidal activity against 2nd instar of *Aedes aegypti* larvae of *Bacillus licheniformis* chitinolytic enzymes in this crude fraction (35-65 % ammonium sulfate

precipitated protein) from culture supernatant cultured in medium supplemented with chitin substrate was 60.67 mU/ml. However, these results cannot exclude other heat labile factor(s) that induced by chitin and coincided with chitinolytic enzymes which may contribute to this observation. Thus, the mosquito larvicidal toxicity of purified chitinolytic enzyme is required in order to answer this question. The mosquito larvicidal activity of purified of 70.8 kDa chitinolytic enzyme of *Bacillus licheniformis* against 2nd instar of *Aedes aegypti* larvae showed that the LC_{50} of this enzyme was equal to 66.67 mU/ml. Though, this purified fraction contained mainly this chitinolytic enzyme, it still contaminated with a little amount of other chitinolytic enzyme and protein but non of *Bacillus licheniformis* or other contaminated bacteria. Therefore, the killing activity might resulted from the 70.8 kDa chitinolytic enzyme of *Bacillus licheniformis* activity. The mechanism of this enzyme in killing the mosquito larvae was further investigated by studying the microscopic details of peritrophic membrane after already treated with this chitinolytic enzyme. The mosquito larvicidal activity of intracellular chitinase was also studied using the mosquito toxicity of washed cell (without culture broth) of *E.coli* transformant carrying a plasmid with chitinase gene from *Aeromonas hydrophila* against *Aedes aegypti* larvae. This *E.coli* transformant perhaps contained different type and amount of chitinolytic activity. It was found that this *E.coli* transformant did not contain any mosquito larvicidal activity.

The mosquito larvicidal toxicity results of crude fraction protein from culture supernatant of *Bacillus licheniformis* combination with *B.t.i.*4Q2-72 was indicated that enzyme fraction could enhance the

killing activity of *B.t.i.* 4Q2-72. This phenomenon was supported the hypothesis that chitinolytic enzyme of *Bacillus licheniformis* enhanced the mosquito larvicidal activity of *Bacillus thuringiensis* subsp. *israelensis* by disrupting and destroying the chitinous layer of peritrophic membrane in the mosquito larvae midgut, where the *B.t.i.* toxin proteins were proteolytically activated, binding and destroying to the epithelial cell membranes. Nevertheless, further study of this mosquito larvicidal activity enhancement between *B.t.i.* toxin proteins and chitinolytic enzyme of *Bacillus licheniformis* was required in order to understand the mechanism of this observation. Whereas, the results obtained from *B.t.i.*c4Q2-72 in combination with various concentrations (both sublethal and lethal dose) of the chitinolytic enzyme in crude fraction of 35-65 % ammonium sulfate precipitated protein from culture supernatant *Bacillus licheniformis* was shown to be different from the results of *B.t.i.*4Q2-72 (Table 6 and Table 7). The test condition that *B.t.i.*c4Q2-72 containing higher enzyme activity seemed to have some killing activity as indicated by the number of dead larvae but the toxicity was still lower than the dead larvae caused by *B.t.i.*4Q2-72 alone or in combination with chitinase enzyme. Since *B.t.i.*c4Q2-72 is a plasmid cured strain of *B.t.i.*4Q2-72 which does not produce crystal toxin, therefore it is suggested that the enzyme together with cells producing toxin should contain higher activity than cells without toxin. The chitinase at sublethal concentration could help the nontoxic cell in killing mosquito larvae (Table 7). Therefore, crude fraction of 35-65 % ammonium sulfate precipitated protein from culture supernatant of *Bacillus licheniformis* which contained only sublethal dose of the enzyme can enhance only

the mosquito larvicidal property of *B.t.i.*4Q2-72 (Table 6). Thus, the results of toxicity test of crude fraction combined with *B.t.i.*c4Q2-72 cells indicated that the death of mosquito larvae in these condition might be contributed by the effect of *Bacillus licheniformis* chitinolytic enzyme alone. The LC_{50} value of crude enzyme (Table 5) and purified enzyme (Table 8) against mosquito larvae was about the same (60.67 and 66.67 mU/ml, respectively). This result suggested that all of chitinase in crude fraction contributed to mosquito larvicidal toxicity. If only the purified 70.8 kDa chitinolytic enzyme involved in larval toxicity, the LC_{50} value (mU/ml) of it should be at much lower concentration.

The application of 70.8 kDa chitinolytic enzyme of *Bacillus licheniformis* for controlling of mosquito is of interesting. The chitinolytic activity of this enzyme was found to be stable in deionized distilled water and slightly decreased when incubated at lower temperature (40 and 50°C) and slightly decreased when incubated at room temperature. However, the field application of *Bacillus licheniformis* chitinolytic enzymes (both chitinolytic enzymes in the crude fraction and purified 70.8 kDa chitinolytic enzyme) for mosquito control with or without the combination of *Bacillus thuringiensis* subsp. *israelensis* may not be practical. Since, the LC_{50} of mosquito larvicidal activity of *Bacillus licheniformis* chitinolytic enzymes in the crude fraction and the enzymatic concentration for mosquito larvicidal activity of purified 70.8 kDa chitinolytic enzyme of *Bacillus licheniformis* showed that the high amount of these enzymes were required to kill the mosquito larvae. In addition, the requirement of high amount of chitinolytic enzyme suggested that some tested

chitinolytic enzyme were not directly interacted with peritrophic membrane in mosquito larvae midgut and lost by diluting with the tested distilled water that surrounding the tested mosquito larvae. So that, the gene(s) cloning of *Bacillus licheniformis* chitinolytic enzyme and the transformation of these cloned gene(s) to *Bacillus thuringiensis* subsp. *israelensis* was required to solve these problems.

CHAPTER VI
SUMMARY

This *Bacillus licheniformis* strain produced at least 5 type of chitinolytic enzymes and their molecular weight were 70.8, 68.1, 63.7, 53.0 and 49.5 kDa, respectively. These enzymes were produced when *Bacillus licheniformis* cells entered the stationary phase in medium supplemented with crude chitin. The 70.8 kDa chitinolytic enzymes was purified by fractionation with ammonium sulfate and passed through column chromatography with CM- and 2 columns of DEAE-sephadex matrix respectively. This purified enzyme was used to study the physicochemical properties such as optimal temperature, temperature stability, optimal pH, pH stability, and enzyme kinetic factors [initial velocity (v_i), Michaelis's constant (K_m) and maximum velocity (V_m)]. The results showed that the optimal temperature and pH of 70.8 kDa chitinolytic enzyme of *Bacillus licheniformis* were 60 °C and pH 5.0 respectively. The activity of this enzyme was decreased when incubated at high temperature (60°C) and dramatically lost when incubated at higher temperature (≥70°C) or prolong incubation at high temperature. The analysis of the activity of *Bacillus licheniformis* 70.8 kDa chitinolytic enzyme indicated that it was stabled in deionized distilled water at 4°C and this activity was rapidly lost when it was kept in strong acid and alkali pH. The enzyme kinetics studying of this enzyme showed that Michaelis's constant (K_m) and maximum velocity (V_m) value were 3.33 % (w/v) and 781.90 mU/ml/mg of enzyme respectively. Crude chitinolytic enzymes

prepared by ammonium sulfate precipitation of culture supernatant proteins supplemented with crude chitin played a role in toxicity against mosquito larvae. The LC_{50} of this crude chitinolytic enzymes against 2nd instar of *Aedes aegypti* larvae was 60.67 mU/ml. These enzymes enhanced larvicidal effect when assayed for the toxicity against mosquito larvae in combination with *Bacillus thuringiensis* subsp. *israelensis* strain 4Q2-72. This result is probably due to disruption of peritrophic matrix of mosquito larval gut which contained chitin by the enzyme resulting in greater penetration of *B.t.i.* into the mosquito haemolymph and subsequently septicemia. The purified 70.8 kDa chitinolytic enzyme of this *Bacillus licheniformis* contained the mosquito larvicidal activity and the LC_{50} of this enzyme against 2nd instar of *Aedes aegypti* larvae was equal to 66.67 mU/ml. Boiling of enzyme fraction at 100°C for 10 min completely abolished the toxic property.

Hence, the chitinolytic enzymes of *Bacillus licheniformis* have a potential to be used as the novel choice of the mosquito larval control method when used alone or better used in combination with *B.t.i.*4Q2-72. However, the improvement of the toxicity by genetic engineering of chitinolytic enzymes genes and formulation of the product were required.

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APPENDIX

1. The amount of solid ammonium sulfate to be added to a solution to give the desired final saturation at 0°C

Initial conc. (%) of ammonium sulfate	Final concentration of ammonium sulfate, % saturation at 0°C										
	30	35	40	45	50	55	60	65	70	75	80
	g solid ammonium sulfate to add to 100 ml of solution										
0	16.6	19.7	22.9	26.2	29.5	33.1	36.6	40.4	44.2	48.3	52.3
5	13.9	16.8	20.0	23.2	26.6	30.0	33.6	37.3	41.1	45.0	49.1
10	11.1	14.1	17.1	20.3	23.6	27.0	30.5	34.2	37.9	41.8	45.8
15	8.3	11.3	14.3	17.4	20.7	24.0	27.5	31.0	34.8	38.6	42.6
20	5.6	8.4	11.5	14.5	17.7	21.0	24.4	28.0	31.6	35.4	39.2
25	2.7	5.7	8.5	11.7	14.8	18.2	21.4	24.8	28.4	32.1	36.0
30	0	2.8	5.7	8.7	11.9	15.0	18.4	21.7	25.3	28.9	32.8
35		0	2.8	5.8	8.8	12.0	15.3	18.7	22.1	25.8	29.5
40			0	2.9	5.9	9.0	12.2	15.5	19.0	22.5	26.2
45				0	2.9	6.0	9.1	12.5	15.8	19.3	22.9
50					0	3.0	6.1	9.3	12.7	16.1	19.7
55						0	3.0	6.2	9.4	12.9	16.3
60							0	3.1	6.3	9.6	13.1

Initial conc. (%) of ammonium sulfate	Final concentration of ammonium sulfate, % saturation at 0°C										
	30	35	40	45	50	55	60	65	70	75	80
	g solid ammonium sulfate to add to 100 ml of solution										
65								0	3.1	6.4	9.8
70									0	3.2	6.6
75										0	3.2
80											0

2. Clark and Lubs buffer system preparation

Buffer pH (0.05 M)	Solution 0.1 M (x ml)
3.0	KH phthalate 50 ml, HCl 22.3 ml
4.0	KH phthalate 50 ml, HCl 0.1 ml
5.0	KH phthalate 50 ml, NaOH 22.6 ml
6.0	KH_2PO_4 50 ml, NaOH 5.6 ml
7.0	KH_2PO_4 50 ml, NaOH 29.1 ml
8.0	KH_2PO_4 50 ml, NaOH 46.1 ml
9.0	$KCl + H_3BO_3$ 50 ml, NaOH 20.8 ml

Each pH was diluted to 100 ml with distill water

3. Calculation of LC_{50} (Reed and Muench, 1938)

Bacterial dilution	Conc. (cell/ml)	Mortality ratio[1]	Died	Survived	Accumulated value Died[2]	Survived[3]	Mortality Ratio[4]	Percent
10^{-3}	$5.1x10^4$	10/10	10	0	27 ↑	0 ↓	27/27	100.00
10^{-4}	$5.1x10^3$	9.5/10	9.5	0.5	17 ↑	0.5 ↓	17/17.5	97.14
10^{-5}	$5.1x10^2$	6.5/10	6.5	3.5	7.5 ↑	4 ↓	7.5/11.5	65.22
10^{-6}	$5.1x10^1$	1/10	1	9	1 ↑	13 ↓	1/14	7.14
10^{-7}	5.1	0/10	0	10	0 ↑	23 ↓	0/23	0

(1) The data represented the average of duplicate experiment.

(2) Accumulated values of dead larvae are obtained by adding in the up-direction indicated by the arrow.

(3) Accumulated values of survived larvae are obtained by adding in the down-direction indicated by the arrow.

(4) Mortality ratio represents the accumulated number of dead larvae (2) over the accumulated total number (2+3) in each dilution.

Proportionate distance

$$= \frac{\text{\% mortality at dilution next above 50 \% - 50 \%}}{\text{\% mortality at dilution next above 50 \% - mortality at dilution next below 50 \%}}$$

$$= \frac{65.22-50}{65.22-7.14} = 0.26$$

Negative logarithm of LC_{50} and end point titer

= negative logarithm of the dilution above the 50 % mortality plus the proportionate distance factor (corrected for dilution series used)

= -2.71 + (0.26 x log 10)

= -2.45

LC_{50} titer = $10^{2.45}$

LC_{50}' = 2.8×10^2 cells/ml