# IDENTIFICATION AND CHARACTERIZATION OF CHITINOLYTIC BACTERIA FROM BEETLE LARVAE GUT AND SEAWATER SAMPLES

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**FACULTY OF SCIENCE**
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**KUALA LUMPUR**

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**IDENTIFICATION AND CHARACTERIZATION OF CHITINOLYTIC BACTERIA FROM BEETLE LARVAE GUT AND SEAWATER SAMPLES**

## ABSTRACT

An abundant amount of chitin could be found in the world and most of them are disposed as waste and cause a major problem in industry. Chitin flakes are mainly derived from shellfish such as crab and shrimp that could be converted into useful product that can benefit human and reduce problems for environment. Several steps including isolation of chitinolytic bacteria, purification of chitinase and the performance of the purified chitinase in various conditions were done throughout this study. Chitinolytic bacteria produce enzymes that help in chitin degradation and it can be isolated from several sources. In this study, the chitinase producing bacteria were locally isolated from rhinoceros beetle (*Oryctes rhinoceros)* larvae gut found in mushroom compost, and from seawater, collected at Beras Basah Island, Langkawi. Two samples were selected from these two sources and labelled as RBLG1 and BBL1. Both samples were identified using 16s rDNA sequencing. Both identified strains have a strong homology to *Bacillus cereus*. Chitinases that produced from both strains then undergo purification process using ammonium sulphate precipitation, ion exchange chromatography and gel filtration process. The purified chitinase from RBLG1 shows the homogeneity 1.9 fold while homogeneity of purified chitinase from BBL1 was 2.78 fold after gel filtration process. The purified chitinases were identified and analyzed using SDS-PAGE and the molecular mass from both strains was approximately 40 kDa. Then, several characterization studies such as effect of various temperatures, pHs, substrate concentrations and also metal ion were done using purified enzymes. Enzyme from RBLG1 shows highest chitinase activity at pH 4 (0.0330 U/mL) and 60°C (0.0354 U/mL), while chitinase from BBL1 shows highest enzymatic activity at pH 6 (0.0360

U/mL) and 45°C (0.0367 U/mL). The maximum activities for both strain RBLG and BBL1 were 0.0341 U/ml and 0.0346 U/ml respectively at 1.5% substrate concentration at 37°C. For metal ion test, chitinase from RBLG1 shows lowest enzymatic activity when chitinase incorporated with $Zn^{+}$, while chitinase from BBL1 shows lowest enzymatic activity when chitinase incorporated with $K^{+}$. Lastly, study on kinetic performance for chitinase using Michaelis-Menten equation from RBLG1 shows $K_m$ and $V_{max}$ values were 0.5093 g/L and 0.0066 [P] (g/L/min) respectively meanwhile for chitinase from BBL1, the $K_m$ and $V_{max}$ were 0.3887 g/L and 0.0080 [P] (g/L/min) respectively.

**Keywords**: chitinolytic bacteria, chitinase, protein purification, SDS-PAGE, enzyme kinetic

**PENGENALPASTIAN DAN PERINCIAN BAKTERIA KITINOLITIK DARIPADA SAMPEL USUS LARVA KUMBANG DAN AIR LAUT**

## ABSTRAK

Sejumlah besar kitin boleh didapati di dunia dan kebanyakannya dilupuskan sebagai sisa dan menyebabkan masalah utama dalam industri. Serpihan kitin terutamanya berasal dari hidupan laut yang bercengkerang contohnya ketam dan udang dapat diubah menjadi produk yang lebih bermanfaat kepada manusia dan mengurangkan masalah kepada persekitaran. Beberapa langkah termasuk pengasingan bakteria kitinolitik, pembersihan enzim kitinolitik dan analisis prestasi enzim yang telah ditulenkan dalam beberapa keadaan berbeza telah dilakukan sepanjang kajian ini. Bakteria kitinolitik menghasilkan enzim yang dapat membantu dalam degradasi kitin dan ia boleh diasingkan daripada beberapa sumber. Bakteria yang dapat menghasilkan enzim kitinolitik ini telah diasingkan dari perut larva kumbang tanduk (*Oryctes rhinoceros)* yang ditemui di dalam kompos cendawan, dan dari air laut yang telah diambil di Pulau Beras Basah, Langkawi. Dua strain bakteria dipilih daripada kedua-dua sumber tersebut dan dilabel sebagai RBLG1 dan BBL1. Kedua-dua jenis bakteria ini dikenal pasti menggunakan urutan 16s rDNA. Enzim kitinolitik yang dihasilkan dari kedua-dua jenis bakteria ini kemudian menjalani proses penulenan menggunakan pemendakan amonium sulfat, kromatografi pertukaran ion dan penapisan gel. Enzim kitinolitik yang dibersihkan dari RBLG1 menunjukkan homogenity 1.9 kali ganda manakala homogeniti enzim kitinolitik yang dibersihkan dari BBL1 adalah 2.78 kali ganda selepas proses penapisan gel. Enzim-enzim yang ditulenkan telah dikenal pasti dan dianalisa menggunakan SDS-PAGE dan jisim molekul bagi kedua-dua enzim ialah pada saiz 40 kDa. Kemudian, beberapa kajian seperti kesan enzim pada suhu, pH, kepekatan substrat dan juga ion logam dilakukan dengan menggunakan enzim yang telah dibersihkan.

Enzim dari RBLG1 menunjukkan aktiviti kitinolitik tertinggi pada pH 4 (0.0330 U/mL) dan 60 ° C (0.0354 U/mL), manakala enzim dari BBL1 menunjukkan aktiviti kitinolitik tertinggi pada pH 6 (0.0360 U/mL) dan 45 ° C (0.0367 U/mL). Aktiviti maksimum untuk kedua-dua strain RBLG1 dan BBL1 masing-masing adalah 0.0341 U / mL dan 0.0346 U / mL pada kepekatan substrat sebanyak 1.5% pada suhu 37 ° C. Untuk ujian ion logam, enzim kitinolitik dari RBLG1 menunjukkan aktiviti enzim yang paling rendah apabila digabungkan dengan $Zn^{+}$, manakala enzim kitinolitik dari BBL1 menunjukkan aktiviti enzim yang paling rendah apabila kitinolitik enzim digabungkan dengan $K^{+}$. Kajian prestasi kinetik terhadap enzim menggunakan prinsip *Michaelis-Menten* daripada RBLG1 menunjukkan nilai $K_m$ dan $V_{max}$ masing –masing sebanyak 0.5093 g/L and 0.0066 [P] (g/L/min) manakala bagi enzim daripada BBL1, nilai $K_m$ and $V_{max}$ ialah masing-masing sebanyak 0.3887 g/L dan 0.0080 [P] (g/L/min).

**Kata kunci**: bakteria kitinolitik, enzim kitinolitik, penulenan protein, SDS-PAGE, kinetik enzim

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | | |
|---|---|---|
| BSA | : | Bovine serum albumin |
| $C_2H_3NaO_2$ | : | Sodium acetate |
| $C_2H_3NaO_2$ | : | Sodium acetate |
| $C_2H_5NO_2$ | : | Glycine |
| $CH_3COOH$ | : | Acetic acid |
| CO | : | Carbon monoxide |
| *E* | : | Enzyme |
| ES | : | Enzyme substrate complex |
| GlcNAc | : | N-acetyl-D-glucosamine |
| $H_2$ | : | Hydrogen |
| $K_m$ | : | Substrate concentration at half-maximal velocity |
| $Na_2HPO_4$ | : | Dibasic sodium phosphate |
| NaCl | : | Sodium chloride |
| $NaH_2PO_4$ | : | Monobasic sodium phosphate |
| NaOH | : | Sodium hydroxide |
| *P* | : | Product |
| PEG | : | Polyethylene glycol |
| *S* | : | Substrate |
| SMC | : | Spent mushroom compost |
| U | : | Unit |
| V | : | Reaction velocity |
| $V_{max}$ | : | Maximal velocity |

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# CHAPTER 1: INTRODUCTION

## 1.1 Background Problem

Chitin is the most abundant biomaterial after cellulose and exists as a biomass constituent of organisms such as cell wall of fungi, exoskeleton of crustaceans and insects. Approximately, about 75% of the total weight of shellfish, such as shrimp, crabs and krill are consist of materials considered as waste, of which chitin comprises 20–58% of the dry weight of it (Kuddus & Ahmad, 2013). Annually, around 6 million to 8 million tonnes of waste crab, shrimp and lobster shells are produced globally and Southeast Asia produces 1.5 million tons of described waste. These wastes are often disposed in dumping area or in the sea in developing country (Yan & Chen, 2015). Biomass wastes such as from the seafood industries are produced in large quantities with little or no cost and utilizing them present an attractive alternative as a feedstock for chemical industries. The utilization of microorganisms such as fungi as bioconverter of lignocellulosic materials into secondary feedstock such as chitin presents an opportunity to circumvent the bottlenecks in utilization of lignocellulosic materials. The high energies requirements of conventional pre-treatments such high temperatures and pressures prohibits the utilization of these materials. When these wastes are properly utilized, they can be used as raw materials for the production of biochemical feedstock in the industries. The utilization of various enzymes such as cellulases, chitinases and peroxidases would enable viable productions of fermentable sugars from the converted biomass feedstock. The produced biochemical feedstock could be readily converted to chemical of industrial importance via biological transformation using biocatalyst or chemical reactions. Biochemical conversion of chitin would change these biopolymers into monomeric sugars and the usage of biocatalyst to facilitate the change can be considered as sustainable technology (Zhou et al., 2013).

Chitin is biopolymer that practically can undergo chemical and mechanical modifications and widely useful in many applications, functions and properties while chitinases are the enzymes used for chitin degradation into monomeric components of N-acetyl-D-glucosamine (Pillai et al., 2009). Chitinases can be extracted and purified from broad range of organisms including bacteria (*Bacillus, Aeromonas, Vibrio, Serratia,* and *Pseudomonas*), fungi (*Aspergillus* and *Trichoderma*), higher plants, crustaceans, insect, invertebrates and some vertebrates. Chitinases that have been found in different organisms possess different roles in nature for example as bio control of fungal pathogens and modification of the organisms structural constituent chitin (Karunya, 2011). Chitinolytic bacteria can be isolated from different sources and environments. In this study, as most of the seafood wastes were dumped on the sea, seawater sample was used to isolate chitinolytic bacteria. A coconut beetle (*Oryctes rhinoceros*) larvae found in spent mushroom compost was used to isolate chitinolytic bacteria from the larvae‟s gut. Spent mushroom compost consists of mushroom‟s mycelium and the coconut beetle larvae could possibly own enzymes that can utilize the mycelium as they live in the compost.

In order to study the effectiveness of chitinases on chitin degradation, purified chitinases were used. The purification steps will separate the protein and non-protein in a mixture based on their protein sizes, binding affinity and biological activity to get the desired protein. There are many ways can be used in protein purification and many factors affecting the level of purified protein. In this experiment, several purification steps have been used including centrifugation, ammonium sulphate, ion-exchange chromatography and gel separation.

Characterization of the purified protein has been done in this experiment. Investigation on biochemical properties on purified chitinase are crucial in order to find

new efficient chitinases with novel characteristics that can be used in commercial process to increase industrial demands (Cheba et al., 2016). Chitinases from *Bacillus* sp. were purified and optimized in this experiment might have potential in converting chitin from agriculture and marine wastes into single cell protein, ethanol and fertilizers (Karunya, 2011). The characterization of chitinases on several characteristics such as effect of pH, effect of temperature and effect of substrate concentration on chitinase production have been done using chitin from shrimp shells.

Study of kinetic performance of enzymes will provide researcher a better understanding on how an enzyme works. The task of enzyme kinetics is the systemic analysis of such process, involving a study of the dependence of reaction rates on substrate concentration, pH, temperature, ionic strength and other relevant variables (Engel, 2013). Shrimp shells used in this experiment were undergo pretreatment to get colloidal chitin and used as a substrate for enzyme assays. Different concentration of colloidal chitin will give different results of rate of reaction. This study will show and calculating enzyme kinetic using different concentration of substrate by applying Michaelis-Menten model.

## 1.2 Objectives

- To screen and isolate the chitinolytic bacteria from two different sources.
- To conduct purification of chitinases from isolated bacteria.
- To analyze the characteristics and kinetics performance of the purified chitinases.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Chitin

Chitin is a polysaccharide that consist of β-1, 4-linked *N*-acetylglucosamine (GlcNAc). It is the second most abundant biopolymer after cellulose found on earth. Besides having unique structure, this natural amino polysaccharide also has multidimensional properties, highly sophisticated functions and wide ranging application industries, with approximately $10^{10}$-$10^{12}$ of its production every year (Al Sagheer et al., 2009; Pillai et al., 2009). Chitin can be widely found in the marine and terrestrial environments and has major contribution in structural component of insects, crustacean, cell walls of most fungi as well as cell wall of algae (Kuddus & Ahmad, 2013).

Chitin and its derivatives can be used in biomedical applications particularly in cartilage tissue engineering (Liu et al., 2017), wound healing (Minagawa et al., 2007), drug delivery and nerve generation due to its antifungal activity and highly biocompatible quality (Yang, 2011). The conclusive elements of outstanding biocompatibility and commendable biodegradability with ecological safety and low toxicity with multifaceted biological activities such as antimicrobial activities and low immunogenicity have distributed adequate options for further development (Pillai et al., 2009). Products from chitin degradation also can be used in environment and agriculture activities, food technology and cosmetics (Priya et al., 2011).

Chitin is chemically similar to cellulose except for one of the hydroxyl groups of each glucoside residue that replaced by an acetylated or deacetylated amino group as shown in Figure 2.1 (Jalal et al., 2012).

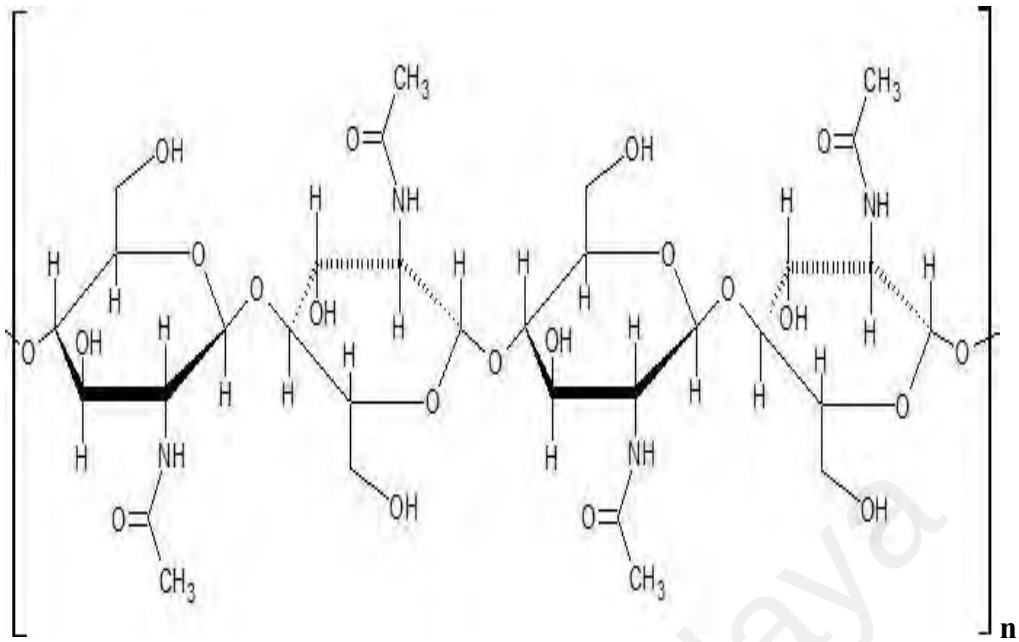


**Figure 2.1** Chitin structure.

Therefore, cellulose and chitin are important and structurally related polysaccharide that provide structural integrity and protection to plants and animals respectively. In nature, chitin appear as ordered crystalline micro fibrils forming structural component in the exoskeleton of arthropods or in cell wall of fungi and yeast meanwhile in crustaceans, chitin is appear as fibrous material embedded in six stranded protein helix (Pillai et al., 2009).

Chitinoclastic is a process where chitin is degraded, whereas chitinolytic is a process where chitin degradation involves the initial hydrolysis of the β-1,4-glycosidic bond. (Beier & Bertilsson, 2014). Chitin is not necessarily break down into its polymeric structure, instead, it can be broken down into chitosan or possibly even cellulose-like forms, if it is further undergo deamination as shown in Figure 2.2. Chitinases and chitosanases efficiency is controlled by the degree of deacetylation of the polymeric substrate since both have overlap in substrate specificity. Cellulases also can possess considerable chitosan-cleaving activity (Beier & Bertilsson, 2014).

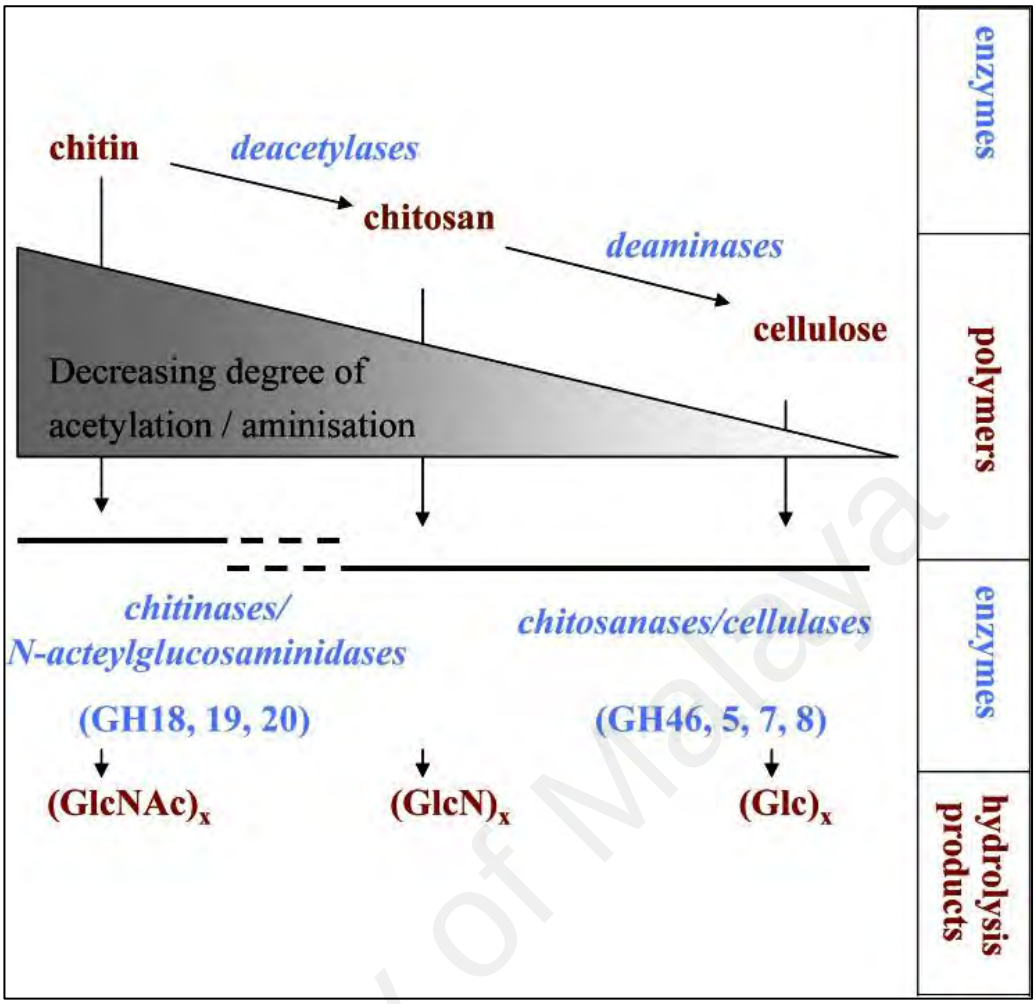



**Figure 2.2** Processes involved in chitin degradation (Beier & Bertilsson, 2014).

### 2.1.1 Chitin Potential as Second Generation Fermentation Feedstock

First generation fermentation feedstocks were primarily produced from food crops such as sugar crops, oil seeds and cereals. The production of the feedstock, for example corn ethanol in US, sugarcane ethanol in Brazil and other biofuels have been increased between 2000 and 2008 to meet the total global demand which more than quadrupled. These biofuels are more environmentally friendly because it significantly reduce greenhouse gas emission compared to fossil fuels (Sims et al., 2010).

The first generation ethanol plants utilize either sugar or starch which predominantly comes from sugarcane and corn/sorghum respectively. There are a few steps in

producing biofuels, including pretreatment, hydrolysis, fermentation, distillation, centrifugation and evaporation. The accumulated knowledge on all these steps is widely gathered from decades of industrial production and there are very few uncertainties involved in the process, raw materials, and the market. Hence, the production of the first generation comes with relatively low risk even though it was low rate return (Lennartsson et al. , 2014). Despite the importance of these biofuels to the environment, there are some limitations that force the production come under close scrutiny (Sims et al., 2010). The issues in general relate to (Bioenergy, 2008):

- food security and contribution to food shortage and higher food prices;
- government subsidies often required in production and processing cost;
- carbon debt created and leads to the net positive greenhouse gas (GHG) production;
- land use change on deforestation and habitat loss; and
- other environmental impacts for example competition for water supplies and also fertilizer run –off that cause pollution

Increase in public and political concern regarding on barriers in production of first generation biofuels has stimulated interest in developing second generation biofuels. Second generation biofuels are sugars made up from lignocellulosic material, for example agriculture waste (straw, sawdust, sugarcane baggase) by using advanced technological processes (Antizar & Gomez, 2008). Approximately, around 7 to 8 billion tons of lignocellulosic biomass is available for human exploitation every year. Technically, second generation biofuels have no issue with feedstock supply, but the process are currently limited by technical and economic challenges (Lennartsson et al., 2014).

There are two ways in producing biofuels from lignocellulosic materials which are through biochemical and thermochemical processes. In biochemical way, cellulose and

hemicellulose component are converted to sugars during fermentation process to produce ethanol by using enzymes and other microorganisms. Meanwhile in thermochemical, production of synthesis gas ($CO + H_2$) from a wide range of long carbon chain biofuels for example diesel or aviation fuel can be reformed by using pyrolysis/gasification technologies (International Energy Agency (IEA), 2008).

There are several variations and alternatives pathways that are still under evaluation in research laboratory and pilot plants other than two processes stated before. Both technologies show no clear commercial or technical advantage after many years research and development and remain unproven at fully commercial scale and have significant technical and environmental barriers to be overcome (Sims et al., 2010). Since chitin and is the second most abundant biopolymer after cellulose, it can also be renewable carbon sources for a variety of biochemical products as shown in Figure 2.3 (Yan & Fong, 2015).

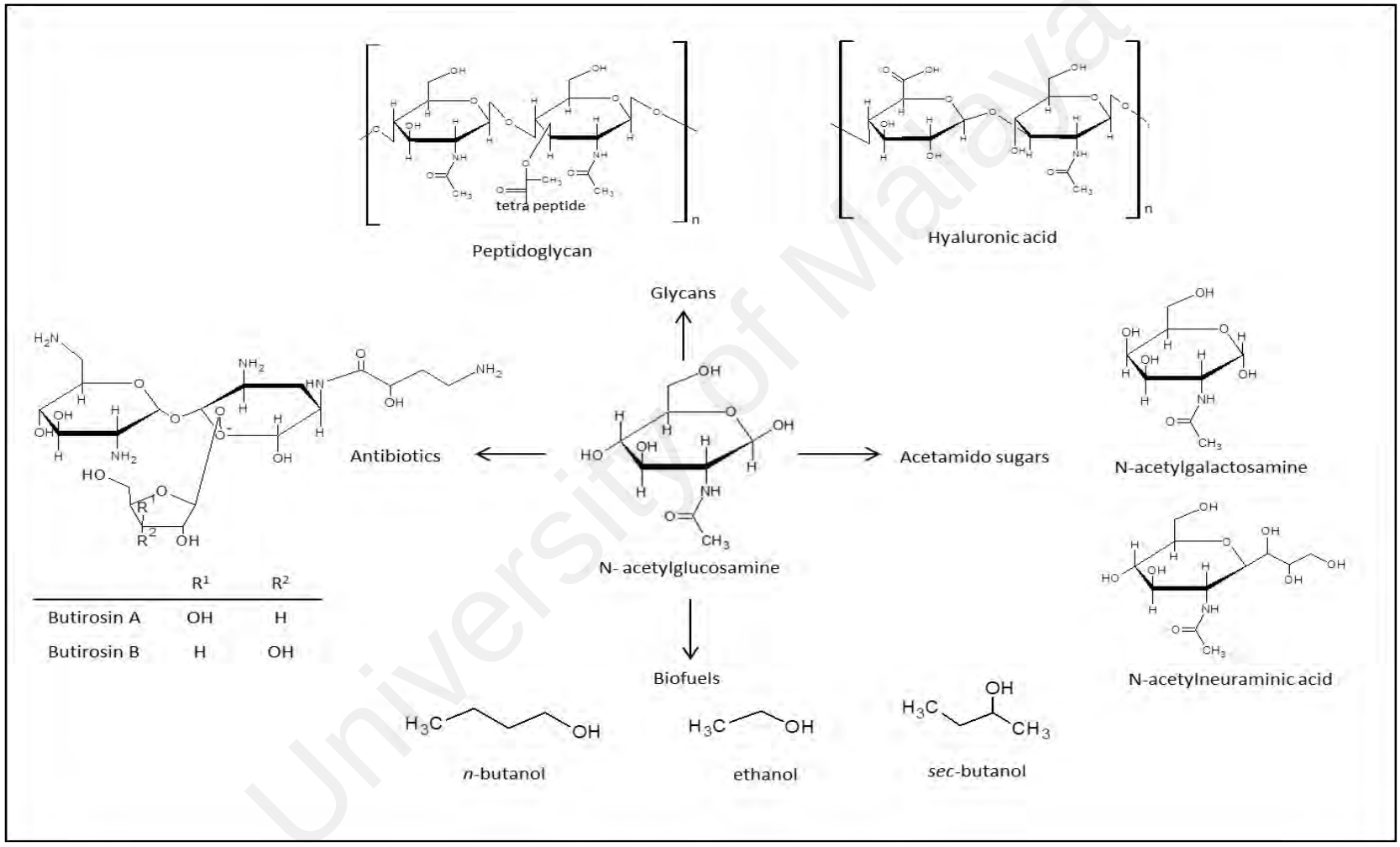



**Figure 2.3** Potential metabolic target from *N*-acetylglucosamine.

## 2.2 Enzyme Chitinases

Chitinases are essential enzymes as they contributing in a diverse application despite their slow-reacting characteristic. The application of chitinases in the bio control of plant pathogenic fungi, production of chito-oligosaccharides and mycolytic enzyme preparation are initiating a vast of interest towards chitinases (Matroodi et al., 2013). Chitinolytic enzymes are glycosyl hydrolases with the sizes ranging from 20 kDa to about 90 kDa (Hamid et al., 2013). Chitinases are classified according to the manner in which they cleave chitin chains (Lee et al., 2009).

Chitinases can be classified into three categories which are endochitinases, exochitinases, and N-acetylglucosaminases according to the manner in which they cleave chitin chains (Lee et al., 2007). Endochitinases randomly cleave β- 1, 4-glycosidic bonds of chitin then forming the dimer diacetyl-chitobiose and soluble low molecular mass multimers of GlcNAc such as chitotriose, and chitotetraose. Exochitinases that cleave the chain from non-reducing end of the chitin micro fibril to form diacetyl-chitobiose are called chitobiosidase, meanwhile, N-acetylglucosaminases cleaving the oligomeric products of endochitinases and chitobiosidase and producing monomers of GlcNAc (Hamid et al., 2013; Lee et al., 2007). Chitinases are commonly categorized into family 18 and 19 and 20 glycoside hydrolases (GH) (Beier & Bertilsson, 2014).

Chitinases have been intensively studied by researchers in the last decades and the production of chitinases can be divided into several methods including two basic techniques, which are production by the natural resource or by genetically modified

organism followed by purification method (Dušková et al., 2011). Different applications of chitinases produce from different organisms depending on their own physiology and use (Bhattacharya et al., 2007).

Serine/threonine-rich glycosylated domain is one of the characteristics of chitinases structure has been found accompanied by a different multi-domain structures including catalytic domains and both a cysteine-rich chitin-binding domain. Fungal and bacterial chitinases have similarity in all these catalytic domains because they show the same characteristics. The 8 cysteine within the chitin binding domain in plant proteins are greatly conserved as well as plants own chitin-binding proteins (CBPs) that have a cysteine-rich chitin-binding domain without chitinase activity. Chitin-binding domain between plants and bacteria are differing as plants contain mainly 8 conserved cysteine residue while bacteria have mainly tryptophan residue that is conserved within their chitin-binding domain. The binding of a non-catalytic chitin-binding protein to chitin are important in identification of the role of chitinases (Hamid et al., 2013).

### 2.3 Bacterial Chitinase

Bacterial chitinase have a size ranging from ~20-60 kDa, that almost same with plant chitinases that have range between ~20-40 kDa and smaller than insect chitinases (~40-80 kDa). They are active in a vast range of temperature and pH based on the source of bacteria and from which they have been isolated. For example, thermo stable chitinases isolated from *S. thermoviolaceus* and endochitinase from *S. violaceusniger* have an optimum temperature of ~80°C and 28°C, respectively. The example for pH, *Stenotrophomonas maltophilia* C3 has an optimum pH around 4.5 to 5.0. Other than temperature and pH, chitinases also have a broad range of isoelectric points (pI ~4.5-8.5) (Kim et al., 2017).

Bacterial chitinases have the chitin-binding domain that can be located either in the N-amino terminal or in the carboxyl terminal domain of the enzyme. Bacterial chitinases that have been isolated and sequenced are commonly categorized into family 18 glycoside hydrolases (Beier & Bertilsson, 2014). Based on amino acid sequence similarity, almost all bacterial chitinases are comes from family 18, however some *Streptomyces* chitinases are from family 19 while β-*N*-acetylhexosaminidases from bacteria are classified in family 20 (Yan & Fong, 2015). The differences between family for chitinases are principally due to different amino acid sequence similarity, three dimensional structure and catalytic mechanism (Paulsen et al., 2016).

In the past few years, researchers took more interest on the development and widespread use of culture independent molecular methods in microbial ecology in order to enable further understanding on microbial processes in controlling chitin degradation. The aim is to get a better understanding on how microbial chitinases works in more complex natural environment and diverse microbial community. Figure 2.4 shows how the methodological advanced combined with the significance of chitin as a critical link between the carbon and nitrogen cycle. The link between carbon and nitrogen cycle with chitin degradation has stimulates interest in the quantitative importance of chitin turnover in marine systems (Beier & Bertilsson, 2014; Souza et al., 2011).

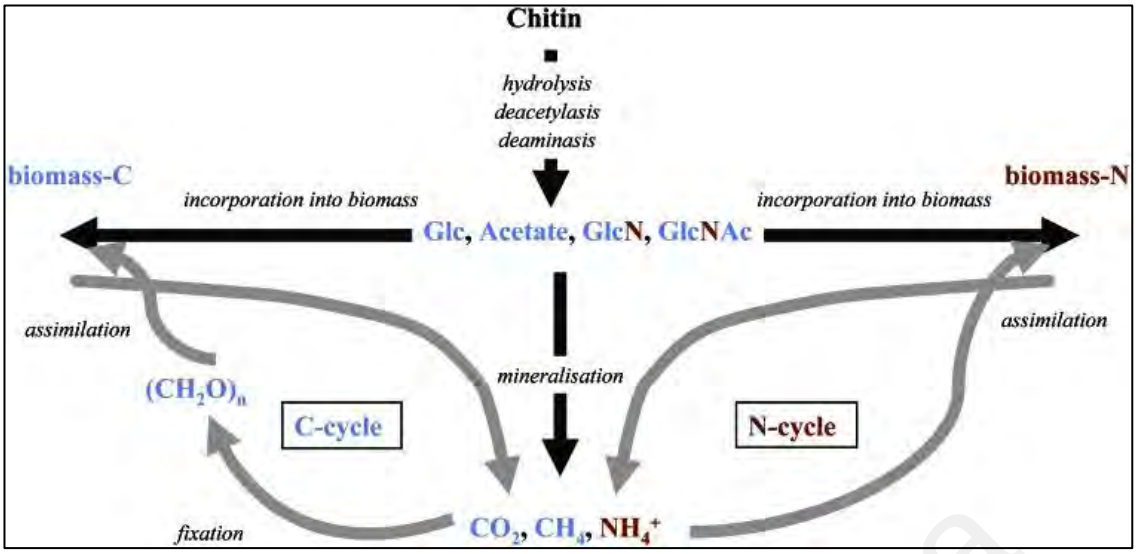



**Figure 2.4** Fate of possible chitin degradation intermediates and degradation products at the interface of the global N and C-cycles (Beier & Bertilsson, 2014).

A recent study on the diverse mechanisms involved in chitinolysis and the ecological consequences of this process for bacteria are needed. The bacteria metabolism and growth are performs as central role in most ecosystem-scale and biogeochemical cycles as the bacterial chitin degradation takes place in all major ecosystem. Hence, it is crucial to master knowledge in bacterial chitin degradation rather than all other organisms involved in chitin degradation. Evolutions in molecular methods have develops interest on how the participation and interactions of specific microbial populations and community compositions effect the process. Nonetheless, the activity of non-bacterial or non-chitinolytic chitin degrader should be monitored and studied as they would influence bacterial chitin degradation (Beier & Bertilsson, 2014).

## 2.4 Importance of Bacterial Chitinase

The basic role of bacterial chitinases is mainly for chitin degradation as energy sources (Hamid et al., 2013). Chitinases give a numerous advantages to human which they have been used in several applications and industries. Chitinases are used as a biocontrol agent against fungal attack in agriculture field since they have been identified as one of the pathogenesis-related proteins (PR proteins). Considering that chitin forms

a major constituent of the the fungal cell wall, chitin are likely susceptible to various chitinases (Bhattacharya et al., 2007; Kim et al., 2017). Moreover, chitinases have the potential use of as food and seed preservative agents and for engineering plants for resistance to phytopathogenis fungi for example, *S. Marcescens* use as a bio control agent against *Sclerotium rolfsii* by using its culture filtrate (Hamid et al., 2013).

In the recent study, bacterial chitinases are discovering to be a potential insecticide, and various chitinases have been engineered into *Bacillus thuringiensis* with the aim to increase the insecticidal activity. *S. Marcescens* provide chitinases that can be used as bio control for larvae of the wax moth, *Galleria mellonela*. To accelerated protoplast generation, chitinase extracted from *Burkholderia gladioli* CHB101 was showing protoplast-forming activity against fungal mycelia, while chitinase expressed by *Streptomyces* was found to be active in generation of protoplasts from *Aspergillus oryzae* and *Fusarium solani*. These bacteria are indirectly applied in industry that helps in strain improvement and development of new strains that are economically viable. Bacterial chitinases are also used in bioconversion in marine waste treatment including all the shellfish waste. (Bhattacharya et al., 2007).

## 2.5 Sources of Bacterial Chitinases

### 2.5.1 *Oryctes rhinoceros*

*Oryctes rhinoceros* also known as Asiatic rhinoceros beetle, coconut rhinoceros beetle or coconut palm rhinoceros beetle can be widely found in Asia, between India and Indonesia and has spread to Yemen, Reunion, and Hawaii. Throughout this distribution, the coconut rhinoceros beetle is most closely associated with its preferred host plant, *Cocos nucifera* L., the coconut palm (Hinckley, 1973). It is a pest species appear throughout many tropical region of the world. Rhinoceros beetle belongs to Scarabaeidae family and the subfamily of Dynastinae. The larvae of rhinoceros beetle as shown in Figure 2.5 are known as grub and it is yellowish-white in color and may grow

to about 60-100 mm long. Prepupa has quite similar appearance with grub, except that it is smaller than the final grub instar and if being disturbed, it will shake it body actively. The rhinoceros beetle pupa is yellowish-brown in color and the length of the horn-shaped protuberances may indicate the sex of the adult. The adults have notable horn on head, commonly dark brown to black and shiny, 35-50 mm long and 20-23 mm wide. The males have round, shiny terminal abdominal segment while female has a relatively hairier „tail" (Bedford, 2015).

**Figure 2.5** Rhinoceros beetle larvae.

The selection of habitats between the immature and the adult rhinoceros beetle has a distinct divergent which immature beetles are choose to breed in a dead standing coconut palm that already affected by disease, pest or lightning. Other materials that provide suitable breeding environment for immature beetle are compost material such as sawdust heaps, rotting logs, decaying vegetable, bridges made of coconut trunk, dead pandanus, sugar cane baggase, rice straw and also humus rich soil. The adults occupy most of their life time on fresh plant eventually return to decomposing sites during mating and breeding. Several studies show increase in rhinoceros beetle population is

mainly when the ground cover of more than 70cm, decomposing tree trunk with 77% moisture content, soil pH lower than 4.2 and high rainfall (Manjeri et al., 2014).

#### 2.5.1.1 Life Cycle of *Oryctes rhinoceros*

The life cycle for rhinoceros beetle consist of four stages including egg, larva, pupa and adult with the duration in each cycles are variable depending on climatic conditions, nutrition and humidity of the places which the development process took place. Commonly, rhinoceros beetle can produce more than one generation a year because the time taken for the complete development is between four to nine months. The beginning stage, adult female lay egg inside the dead palms, decaying plants material, soil with organic matter content and wooden structures (Manjeri et al., 2014).

Following hatching process, the first instar grubs feast on the chorions and start feeding on the burrow frass. The instar grub can move freely inside the soft logs or heap during development process and it can grow into third instar grub in six week period under such favorable conditions. Because of the larger grubs are appearing to avoid one another and are spaced out within the site, the larval density in different sites were studied. Pupation process occurs when the late third instar grub bore into a firm portion of log from the partly decomposed log. The grub form a pseudo-cocoon of compacted fiber or frass then it goes into non-feeding prepupal period for approximately one week right after the pupal chamber has been formed (Hinckley, 1973). After prepupal stage, pupating stage takes place within a pupal chamber made from the food substrate and the time taken for this stage is about 17-30 days. Adults may live up to 6 months or more (Bedford, 2015). The life cycle of the beetle is shown in the Figure 2.6.

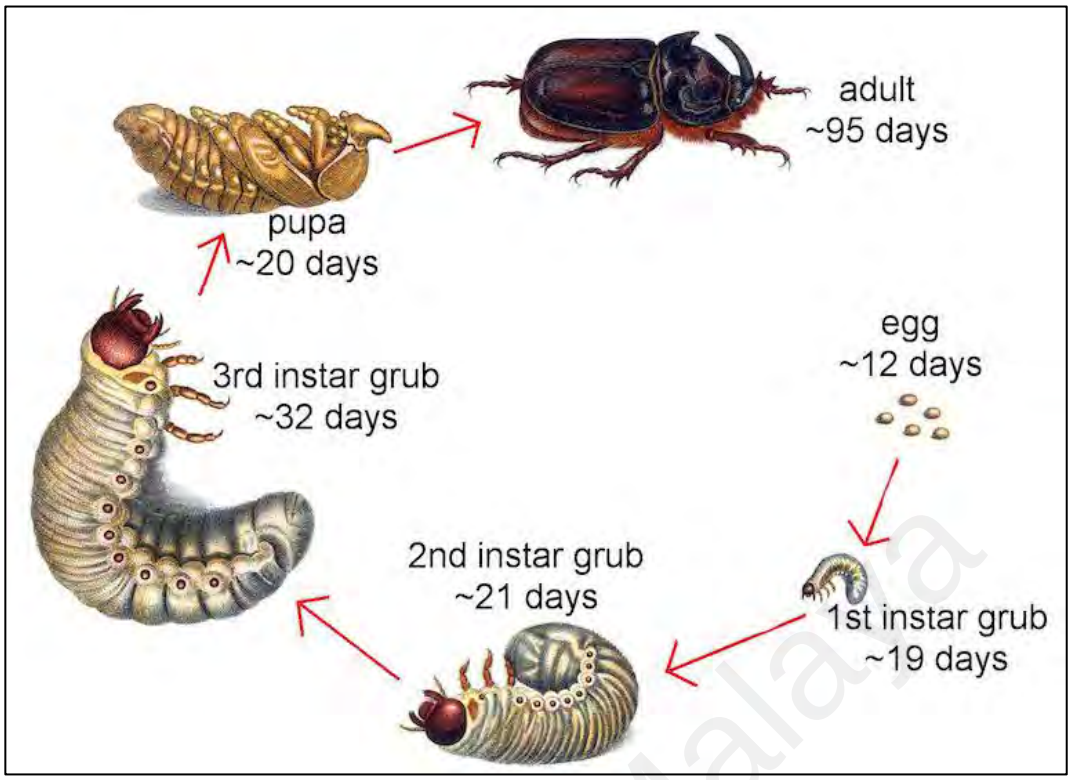



**Figure 2.6** Life cycle of *Oryctes rhinoceros* (Plant Heroes, 2012).

#### 2.5.1.2 Impact of *Oryctes rhinoceros* Towards Environment

Coconut rhinoceros beetle is a serious pest of oil palm plantation in Malaysia. About 15% of the leaf area can be lost in area of extreme damage and causes decline in the plantation production up to 25% (Moslim et al., 2006). The mated females lay eggs in different part of palm tree which are between the hairy root, all along the stem at the leaf axils and at the base of cut branches. Larvae burrow into the targeted places and the larval feeding causes vast damage to the respiratory root (Soltani et al., 2008). The adult feeds on the sap and causes destruction on the still-furled fronds after it burrow into the crown or heart of the coconut palm, or into the base of the group of unopened fronds of young oil palms. The effect of the attacks kill the growing points leading in the death of the coconut palm (Bedford, 2013).

In Malaysia, after a few years of plantation of oil palm, there are several reports on the attack of this pest on local plantation in the west and east coast of Peninsular

Malaysia. The west coast was most affected by the beetle‟s attack because the coconuts were cultivated on the land during earlier usage. As the rhinoceros beetles are nocturnal, the feeding and mating take place at night resulting initial attacks go unnoticed (Manjeri et al., 2014).

### 2.5.2 Marine Bacterial Chitinase

Seeing that chitin is the basic component for exoskeleton in shellfish and marine bacteria that contain chitinases can be isolated in marine environment. The shrimps composed 50-70% of non-edible part including head, tail and shell out of total volume of raw materials (Suresh, 2012). Chitin provides carbon and nitrogen sources in the oceans and in the twentieth century, research on the ecological importance of chitin in the marine environment has been recognized. Since carbon and nitrogen are usually limited in marine ecosystem, one of the finding shows that carbon and nitrogen would disappear from the ocean in relatively short time if chitin did not return in a biologically useful form. Since the global production of chitin mainly comes from marine environment, the oceans represent an extremely promising and largely unexplored environment for the discovery of new microorganisms and bioactive compounds for biotechnology and sustainable development. Marine microorganisms are remarkably essential for sustaining oceanic life and carry out many important functions for other living organisms to survive (Souza et al., 2011).

Marine microorganisms are remarkably essential for sustaining oceanic life and carry out many important functions for other living organisms to survive. Other study reported that isolated bacteria from marine environment consist of antibacterial compound because chitin will influenced the production of secondary metabolite including antibacterial compounds (Paulsen et al., 2016). Bioconversion of marine bio-wastes using bacterial chitinases extracted from marine is possible because as the

produced chitin will be degraded equally by marine bacterial chitinases without left substantial accumulation of chitin in ocean sediments (Suresh, 2012).

## 2.6 Chitinase Purification

A crude enzymes, that can be extracted from disrupted liver, yeast and bacterial cell contains mixture of thousands of different enzymes (Burgess & Deutscher, 2009). Enzyme purification process is a must in order to study characterization of the specific enzyme. Protein can be purified according to their solubility, size, weight, charge and also binding affinity. The common major parameter used to distinguish two molecules is the molecular weight. All the conventional physical separation methods are depends on the different of molecular size (Determann, 2012). Nowadays, a broad range of chromatographic column packing materials used in protein purification are widely available for example gel filtration, ion exchange, reversed phase packing, hydrophobic interaction adsorbents and affinity chromatography adsorbents (Janson, 2012).

Adsorption chromatography relies on the interactions of different types between solute molecules and ligands immobilized on a chromatography matrix, such as in separating macromolecules, the interaction between charged solute molecules and oppositely charged moieties covalently linked to a chromatography matrix. Separation and purification of protein, polypeptides, nucleic acids, polynucleotides and other biomolecules are often used this ion exchange as chromatographic techniques. Ion exchange (Khan, 2012). Protein will be separated based on their surface ionic charge when the protein purification take place using resin which modified with either positively-charged or negatively-charged chemical group (Tan & Yiap, 2009).

Purification of chitinase consists of several steps including centrifugation, precipitation using ammonium salt, dialysis, ion exchange chromatography and size exclusion chromatography. The details of each step including how it works and the

suitability of pH, temperature and chemicals used were briefly explained in the next paragraph.

### 2.6.1 Centrifugation, Precipitation and Dialysis

Centrifuging is an essential procedure in separating protein precipitation in laboratory scale, meanwhile in industrial scale, filter system is often used (Scopes, 2013). Basic significance of characteristic differences between molecules is sedimentation velocity and space filling. Ultracentrifugation might be possible to identify the molecular weight of sensitive macromolecules, but it cannot be an appropriate tool in preparative need. Nonetheless a mixture of macromolecules may be centrifuged in a density gradient and the mixture will be isolated to a zones with specific densities depending on the macromolecules" weight (Determann, 2012).

Protein can be precipitated from crude extract by adding salts, organic solvents, organic polymer or by varying the pH or temperature of the solution. Ammonium sulphate, sodium sulphate, ethanol, acetone and polyethylene glycol are the most common precipitating agents being used in experiment. An anti chaotropic ions is the most efficient salting out agents which can increase the hydrophobic effect in the solution and stimulating protein aggregation by connecting the association of hydrophobic surfaces. Unlike anti chaotropic ions that act as efficient water molecule binders, chaotropic ions will decrease the hydrophobic effect and thus help protein to maintain in solution. Ethanol is one of the polar organic solvent that has been used in protein precipitation and this polar organic solvent will decrease water activity in the protein solution by replacing water with organic solvents. During protein precipitation process, low temperature is strongly suggested to use to avoid protein denaturation. Organic polymers such as polyethylene glycol (PEG) also can be used in fractionating a protein solution. It is inflammable, does not have charged, not poisonous, inexpensive,

easily handled and low concentration is required compared to organic solvents. The disadvantages of PEG are it is viscous in higher concentration and not easily removed from protein. By adjusting and varying pH and temperature, protein precipitation also can occur because proteins are less soluble at their isoelectric points (Janson, 2012).

In dialysis, semi-permeable membranes was used and the concept of this membrane are closer to the principal of a sieve at molecular level (Determann, 2012). Dialysis should be done process after salting out protein. Protein concentration and dialysis are closely related processes in protein purification works. Dialysis process can be carried out using dialysis tubing that available in a variety of size and materials (Scopes, 2013).

### 2.6.2 Ion Exchange Chromatography

To carry out ion exchange experiments, five main phases should be done as shown in Figure 2.7. Equilibration of ion exchanger in terms of pH and ionic strength in the first stage will allows the binding of the desired solute molecules. Simple anion or cation, for example chloride or sodium performs as exchangeable counter-ions are incorporated with exchanger group. The solute molecules carrying appropriate charge displace counter-ions and bind reversibly to the gel so that unbound substances can be washed out from the exchanger bed using starting buffer in second phase. By changing to elution conditions in the third phase, it will cause solute molecules unfavorable for ionic bonding so the substances are removed from the column. The process works by increasing the ionic strength of the eluting buffer or changing its pH. An increase in the salt concentration gradient makes the solute molecules released from the column in the order of their strengths of binding, the most weakly bound substances being eluted first. The substances that not eluted in the third phase will be removed from the column in the fourth phase and the column will re-equilibrated at the starting conditions in the fifth phase for the next purification (Khan, 2012).

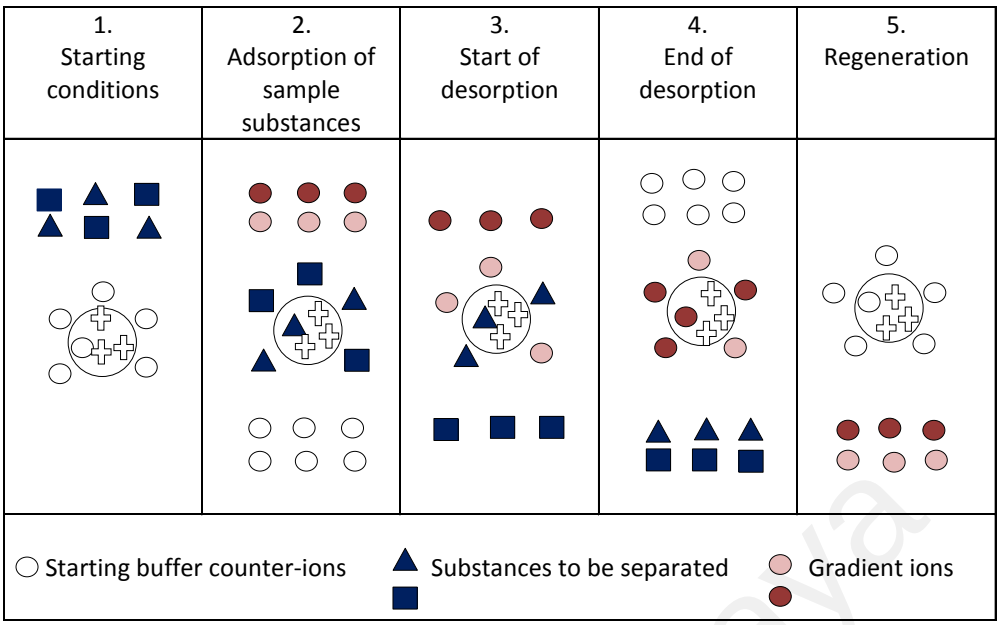



**Figure 2.7** The principle of ion exchange chromatography (salt gradient elution).

### 2.6.3 Size Exclusion Chromatography

Gel filtration chromatography also known as size exclusion chromatography, separates proteins according to molecular sizes and shape and the molecules do not bind to the chromatography medium. The principal of this chromatography is the large molecules passes through the column faster than the small molecules, since small molecules enter all the tiny holes of the matrix and takes more time to run out of the column. The large molecules will pass through the matrix in the column without entering the holes. There are three main types of separating molecules which are total exclusion, selective permeation and total permeation limit. Total exclusion takes place when the large molecules cannot enter the pores and elution of molecules is faster (Tan & Yiap, 2009). The gel should have a suitable pore size and it must be carefully controlled to avoid any problems during separation process. The characteristic that gel forming agent should be controlled are the absence of ionizing groups and in a given solvent, low affinity to the substances to be separated. The problem regarding affinity of

the solute to the gel in organic solvent is able to control by choosing a proper eluent. The faster diffusion equilibrium to be established, the smaller the gel particle used in the column. Since the low flow resistance is determined by the bead-shaped particle, the gel particles itself must not be too soft to avoid deformation in the column and interfere with reasonable flow rate (Determann, 2012).

There are four stages in size exclusion chromatography as shown in Figure 2.8. In the first stage, the sample is shown immediately after introduced on the head of the column. The flow rate is fixed and setting up pressure gradient for the liquid mobile phase moves across the column. In the second stage, the pressure gradient makes the sample molecules pass into the column containing porous particle as a stationary phase with controlled pore size. As the molecules flow through the column, the smaller molecules are able to penetrate the pores, but the larger molecules are too large to be accommodated and remain in the interstitial space in the third stage. The larger molecules flow rapidly throughout the column because they cannot reside the pores for any period of time, while smaller molecules are temporarily retain in pore and slowly flowing down the column until enter another pores. The fourth stage shows the two molecular sizes molecules are separated into two distinct chromatographic bands (Wu, 2003).

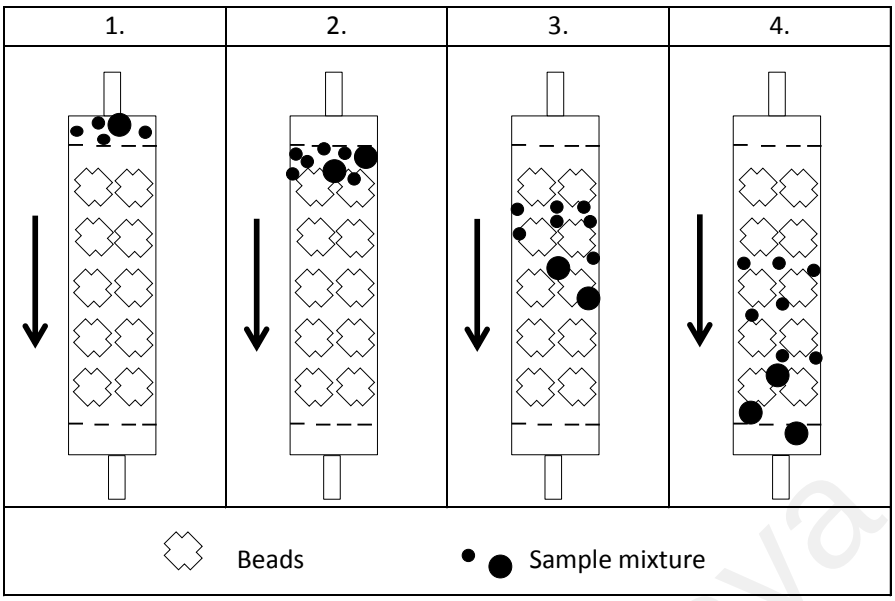



**Figure 2.8** Size exclusion chromatography of two sizes molecules: (1) sample mixture before entering column; (2) sample mixture upon the head of column; (3) size separation begins; and (4) complete resolution.

## 2.7 Michaelis-Menten Equation

Studies of performance of an enzyme kinetic were used to identify the reaction rate of enzymes. Reaction rate of enzymes has been studied since 19th century by researchers and it became fundamental tool to analyze the chemical mechanism of catalyst. Michaelis-Menten equation is the fundamental equation in enzyme kinetics that describe about characterizing enzymatic rate. It was developed one hundred years ago by Leonor Michaelis and Maud Leonora Menten (Xie, 2013). The equation described when the rate of substrate unbinding increase, the rate of enzymatic turnover will decrease (Reuveni et al., 2014). However, it is never examine thoroughly by experiment and also by modern theoretical tools.

The equation below shows the Michaelis-Menten equation. Consider the enzyme-catalyzed conversion of substrate (S) to product (P).

$$E + S \underset{k_1}{\overset{k_1}{\rightleftharpoons}} E.S \xrightarrow{k_{cat}} E + P$$

Michaelis-Menten assumed that the enzyme and substrate are at equilibrium during catalysis and the rate of product formation does not disturb this equilibrium. The equilibrium is expressed by the dissociation constant for the ES complex.

$$K_s = [E][S]/ [ES]$$

The modern Michaelis-Menten equation relates the velocity of an enzyme-catalyzed reaction to two kinetic constants ($V_{max}$ and $K_m$) where $K_m$ is related to $K_1$ as noted later. The equation is expressed as follows:

$$v = V_{max}[S]/ (K_m + [S])$$

where v represents the reaction velocity, $V_{max}$ is the maximal velocity, $K_m$ is the substrate concentration at half-maximal velocity and [S] is the substrate concentration (Roskoski, 2014). Figure 2.9 shows the Michaelis-Menten saturation curve for an enzyme.

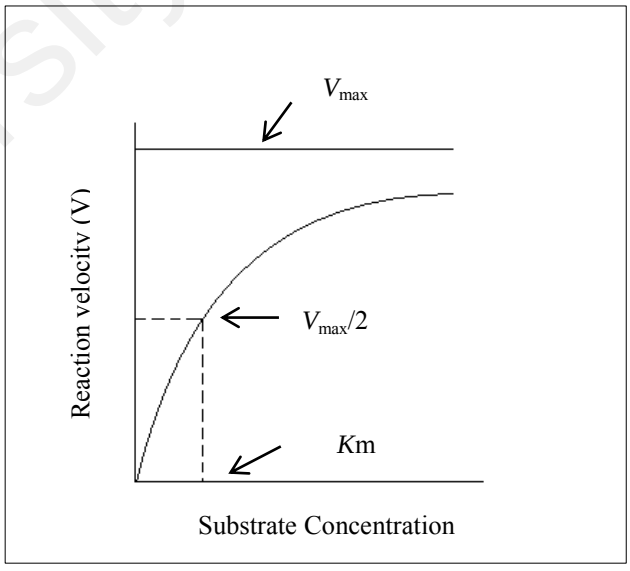



**Figure 2.9** Enzyme reaction velocity as a function of substrate concentration according to the Michaelis-Menten equation. $K_m$ is the concentration at which enzymatic velocity reaches half of its saturation value, $V_{max}$.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 Preparation of Culture Medium

Chitinolytic bacteria were isolated from several sources which are from rhinoceros beetle larvae gut and marine coastal water. Enrichment media used in this experiment consist of chitin as a substrate. Two stages were carried out during development of the culture medium for chitinolytic bacteria; preparation of substrate and preparation of enrichment media (Souza et al., 2009);

### 3.1.1 Preparation of Colloidal Chitin

Chitin from shrimp shells flakes purchased from Sigma Aldrich was grinded and sorted out according to size using 250 μm sieves. Five grams of chitin powder was added slowly into 60 ml of 37% hydrochloric acid (Merck, UK). The mixture was left overnight at room temperature with vigorous stirring. Then, it was added into 200 mL of ice cold 95% ethanol (Systerm, Malaysia) and was left overnight at room temperature. The mixture was stirred vigorously during the process. It was centrifuge at 5000 rpm, 4°C for 20 minutes. Supernatant was removed, and the precipitate was transferred to glass funnel with filter paper. The precipitate was washed with sterile distilled water until neutral (pH 7.0). Then, it was removed from filter paper, weighted and stored in the dark at 4°C (Priya et al., 2011). This method was shown in Appendix A (Figure A.1).

### 3.1.2 Preparation of Enrichment Medium

Enrichment medium contained; colloidal chitin -10 g, $Na_2HPO_4$ (Systerm, Malaysia) -6 g, $KH_2PO_4$ (Systerm, Malaysia) -3 g, $NH_4CL$ (Systerm, Malaysia) -1 g, NaCl (Systerm, Malaysia) –0.5 g, Yeast extract (Difco, USA) -0.05 g, Agar (Difco, USA) – 15 g, in 1 litre distilled water. Enrichment medium was prepared and pH was set to pH 7. The enrichment media were labeled as colloidal chitin agar (CCA) and colloidal chitin broth (CCB).

## 3.2 Isolation of Chitin Degrading Bacteria

Sample from rhinoceros beetle larva gut collected from mushroom compost in Biotechnology Research Centre Glami Lemi, Negeri Sembilan and from marine coastal water collected from Beras Basah Island, Langkawi were incubated in 100 ml CCB for 1-7 days at 37°C with constant shaking at 150 rpm. By using spread plate method, samples were cultured onto CCA. The cultured plates were incubated at 27 ± 2°C for 1-7 days. Serial dilution was done before spread plate in the experiment is to overcome the overpopulated of microorganisms on the CCA plate. It was done by adding 1 ml of sample into 9 ml of saline solution and the step was repeated for 5 to 7 times. The serial dilution and spread plate method was shown in Figure 3.2. The figure shows a ten-fold dilution on a 1 ml scale as it was consist of 1 ml of bacteria culture and 9 ml of saline solution. The mixed solution and bacteria culture was serially transferred and lastly, the bacteria were diluted enough to count on spread plate.

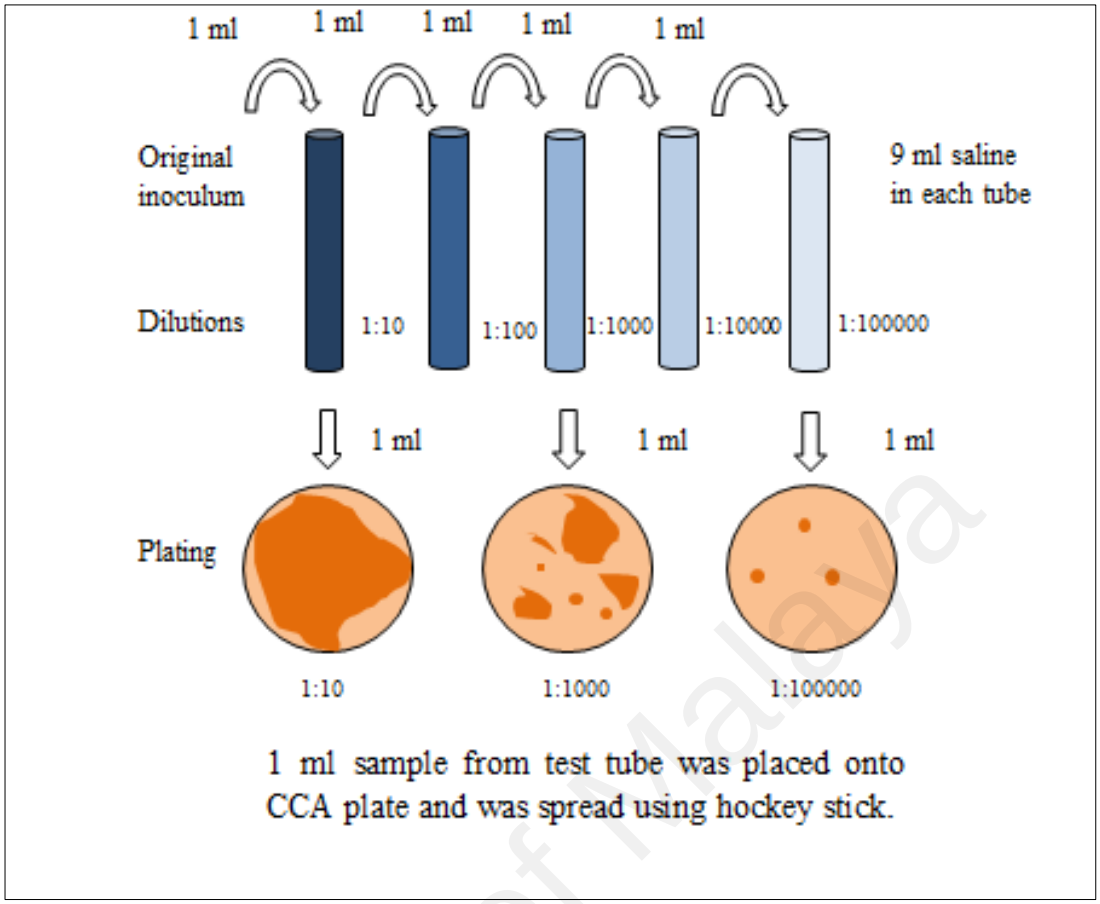



**Figure 3.1** Serial dilution and streak plate method.

### 3.3 Screening of Chitin Degrading Bacteria Strain

Clear zones that appear around the bacterial colonies represent extracellular chitinase activity from chitin degrading bacteria. The selected strains were purified using serial streaking method on CCA plate. The pure bacterial colony obtained from the plates was then preserved (Anuradha & Revathi, 2013).

#### 3.3.1 Gram's Staining

The pure strains were underwent Gram‟s staining and observed under light microscope (DM 750 M, Leica, Germany). The Gram‟s staining method was explained in Appendix B.

### 3.3.2 Catalase Production

The single pure colony from a CCA plate was streaked on a glass slide and a droplet of 3% hydrogen peroxide (Sigma-Aldrich, UK) was dropped on the cell. The bubble formation was observed.

### 3.3.3 Preservation of Chitinolytic Bacteria

The pure colony obtained by serial streaking was cultured into CCB and incubated at 37°C, 150 rpm. After 7 days of incubation period, the culture was centrifuged at 4°C, 20 minutes and 5000 rpm. Then, the pellet was preserved in 30% glycerol (Systerm, Malaysia) and it was stored at -20°C.

## 3.4 Identification of Chitin Degrading Bacteria

The isolated strains RBLG1 and BBL1 were identified using 16S rDNA sequencing. The pure bacterial colonies of RBLG1 and BBL1 on the CCA plate were sent to Apical Scientific Sdn. Bhd., for further identification. The simplified method for this 16S rDNA was provided by the company. The bacterial 16S rDNA, full length 1.5kb, was amplified using universal primers 27F and 1492R. The total reaction volume of 25 µl contained gDNA purified using in-house extraction method, 0.3 pmol of each primer, deoxynucletides triphosphates (dNTPs, 400 µM each), 0.5 U DNA polymerase, supplied PCR buffer and water. The PCR was performed as follow, 1 cycle (94°C for 2 min) for initial denaturation; 25 cycles (98°C for 10 sec; 53°C for 30 sec; 68°C for 1 min) for annealing and extension of the amplified DNA. The PCR products were purified by standard method and directly sequenced with primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Searching for sequence similarity was conducted using NCBI BLAST system and the nearest relatives of the 16S rDNA were identified using GenBank database.

### 3.5 Identification of Bacterial Growth Curve

The RBLG1 and BBL1 were incubated in CCB medium for three days (72 hours) at 37°C and at pH 7. Every 6 hours, 1 ml of bacterial culture were collected from both samples and enzyme assay for both strains were done and recorded.

### 3.6 Chitinase Production and Extraction

The preserved pellet in the glycerol was inoculated into enrichment media (pH 7) containing colloidal chitin -10 g, $Na_2HPO_4$ (Systerm, Malaysia) -6 g, $KH_2PO_4$ (Systerm, Malaysia) -3 g, $NH_4CL$ (Systerm, Malaysia) -1 g, NaCl (Systerm, Malaysia) –0.5 g, Yeast extract (Difco, USA) - 0.05 g. It was incubated at 37°C for 48 hours. Cultures were harvested by centrifuged at 12000x g for 20 minutes at 4°C. The supernatant obtained was collected and was used as crude enzymes sources (Suryanto et al., 2012).

### 3.7 Purification of Chitinase

Chitinase purification can be done using ion exchange chromatography and also gel filtration. In ion exchange chromatography, Sephacryl S-100 (Sigma-Aldrich, UK) was used meanwhile in gel filtration; DEAE Sepharose CL-6B (Sigma-Aldrich, UK) was used. The Sephacryl S-100 and DEAE Sepharose CL-6B were used as a stationary phase in the column. The stationary phase will be fixed in the column and act as a filter. Methods for both chromatographies were further explained. Summarizations of both chromatographies were shown in Appendix E.

#### 3.7.1 Size Exclusion Chromatography Using Sephacryl S-100 Chromatography

Ammonium sulphate (608 g/L) was added to the crude enzyme and the mixture was stored at 4°C overnight. The precipitate formed was collected by centrifugation at 4°C for 20 min at 12000 x -g. The precipitate was dissolved in 0.1M sodium phosphate buffer (pH 7) and dialysed against the buffer for two days. Three ml dialysate was loaded into a sephacryl S-100 gel filtration column. The column was equilibrated and

eluted with 50 mM sodium phosphate buffer (pH 7) (Liang et al., 2014). The fractions with high elution peak were collected and chitinase activities were assayed. The fraction containing highest chitinase activity was collected and concentrated by adding amoonium sulphate (608 g/L) (Wang et al., 2015). The mixture was left overnight and the precipitate formed was collected by centrifugation and dissolved in 5 ml of 50 mM sodium phosphate buffer (pH 7) then dialysed against the buffer for 48 hours (Liang et al., 2014).

### 3.7.2 Ion Exchange Chromatography Using DEAE-Sepharose CL-6B Chromatography

DEAE-sepharose CL-6B purchased from Sigma Aldrich was packed into column chromatography. Three ml of of the resultant enzyme solution from ion exchange chromatography was loaded onto DEAE-Sepharose CL-6B column. The column was equilibrated with 50 mM sodium phosphate buffer (pH 7). The enzyme solution was washed and eluted with the 0-1 M NaCl linear gradient in the same buffer. Ten ml of 0.2 M, 0.4 M, 0.6 M, 0.8 M, and 1.0 M of NaCl in 50 mM sodium phosphate buffer were used as eluent buffer solutions. The fractions were collected and the chitinase activity was measured. The fraction with highest chitinase activity was chosen and concentrated using ammonium sulphate (608 g/L). The precipitate formed was collected by centrifugation and dissolved in 2 ml of 50 mM sodium phosphate buffer (pH 7) (Liang et al., 2014). Then, it was dialysed against the same buffer for 48 hour. The collected protein (dialysate) was stored and used in characterization.

## 3.8 Protein Determination

Protein concentration can be measured using Bradford Assay. Bradford reagent was prepared using Coomasie Blue G 250 purchased from Sigma Aldrich. Firstly, 100 mg Coomasie Blue G 250 was dissolved into 50 ml of 95% ethanol. Then, 100 ml of 85% phosphoric acid was added and followed by addition of distilled water until

become 1 litre. To measure protein concentration, 100 ml of sample was added with 5 ml of Bradford reagent and left for 5 to 10 minutes before absorbance measurement at 595 nm. (Anuradha & Revathi, 2013).

### 3.8.1 Preparation of Protein Standard Calibration Curve

Bovine serum albumin was used as a standard solution and the absorbance was also measured at 595 nm using spectrophotometer (Jasco V 730). BSA standards were prepared at different concentration (g $L^{-1}$); 0.01, 0.02, 0.03, 0.04, and 0.05. Bradford assay was used and absorbance readings were taken triplicate. BSA standard calibration curve is shown in Appendix C.

### 3.8.2 Specific Activity Calculation

The yield of enzyme per protein content can be calculated in U/mg as shown in Equation 3.1;

$$\text{Specific Activity (U/mg)} = \frac{\text{Total enzyme activity (U/ml)}}{\text{Concentration of protein (mg/ml)}} \quad \ldots\ldots \textbf{(Equation 3. 1)}$$

## 3.9 Chitinase Assay

Chitinase activity can be calculated by measuring reducing sugar released from colloidal chitin. Dinitrosalicylic acid (DNS) method by Miller (1959) was used (Appendix D). Colloidal chitin (0.5%) was added in 0.5 ml phosphate buffer (pH 5.5) and was used as substrate. The mixture was incubated at 37°C for 30 minutes (Liang et al., 2014). Three ml DNS reagent was added into the mixture and it was heated at 100°C for 10 minutes. Then, 1 ml of Rochelle‟s salt solution was added to the mixture. The mixture was centrifuge at 1000 rpm for 5 min. The supernatant were collected and the absorbance was measured at 530 nm. Under assay condition, one unit (U) of chitinase activity is defined as the amount of enzyme that is required to release 1µmol of *N*-acetylglucosamine per minute from 0.5% of colloidal chitin solution (Sudhakar & Nagarajan, 2010).

### 3.9.1 Preparation of *N*-acetylglucosamine Standard Calibration

*N*-acetylglucosamine was prepared at different concentration (g $L^{-1}$); 0.02, 0.04, 0.06, 0.08 and 0.1 and were used as standards. DNS assay was used and the absorbance readings were taken triplicate for each concentration at 530 nm. Standard calibration graph was plotted; absorbance against *N*-acetylglucosamine concentration and shown in Appendix C.

### 3.9.2 Reducing Sugar Calculation and Enzyme Activity Calculation

Equation 3.2 shows the calculation of reducing sugar meanwhile Equation 3.3 shows the calculation of chitinase activity;

$$\text{Reducing sugar (µmol)} = \frac{\text{OD x 1000}}{\text{m X RMM}} \quad \text{(Equation 3. 2)}$$

Where:
OD = Absorbance of the sample
m = Slope of glucose standard curve
RMM = Relative molecular mass
RMM of N-acetylglucosamine = 221.209 g/mol

$$\text{Chitinase Activity (U/mL)} = \frac{\text{Reducing sugar (µmole)}}{\text{Volume of sample (ml) x 30 mins}} \quad \text{(Equation 3. 3)}$$

## 3.10 Characterization of the Purified Enzyme Using SDS PAGE

The molecular mass of the purified chitinase can be determined using SDS-Polyacrylamide gel electrophoresis using 12 % gel according to the method of Laemmi (1970). Table 3.1 shows the resolving and stacking gel ingredient. Resolving gel was prepared as in the table and the mixture was swirled gently for a few seconds directly after adding 10% APS and TEMED and immediately poured into the assembled glass plate. It was left for about 30 to 40 minutes to make sure the resolving gel was solidified. Stacking gel was prepared and added to the assembled glass plate and gel

comb was inserted on the top of it. It was left 20 to 30 minutes for the completed polymerisation. The experiment on handling both gels should be done in a careful manner to avoid bubble formation inside the gels. The gel casting was set up after polymerization completed. The samples were thawed and 5 µL of each samples were put into 20 µL sample buffer in a micro centrifuge tubes then they were heat up at 95°C for 4 minutes. Running buffer was put inside the gel casting until full and it was placed inside tank. The tank also was filled up with running buffer until reached the certain level in the tank. 2 µL protein marker (Novogen) was loaded meanwhile for sample, 20 µL sample was loaded into well. The tank was connected to power supply and the electrophoresis was run at 120 V for about 50 to 60 minutes to complete gel separation. Then, gel protein was stained using Coomasie Blue staining method overnight and de-stains with distilled water (Ikeda et al., 2009). List of stock solutions used and staining solution were shown in Appendix F.

**Table 3.1** Resolving and stacking gel preparation.

| Components | Resolving gel | Stacking gel |
|---|---|---|
| Distilled water | 3.2 mL | 3.0 mL |
| 30% acrylamide | 4.0 mL | 0.67 mL |
| Buffer Tris-HCl (1.5M, pH 8.8) | 2.6 mL | - |
| Buffer Tris-HCl (0.5M, pH 6.8) | - | 1.25 mL |
| 10% SDS | 100 µL | 50 µL |
| 10% APS | 100 µL | 50 µL |
| TEMED | 10 µL | 5 µL |

* APS and TEMED were added shortly before use.

### 3.11 Effect of pH and Temperature on Chitinase Activity

Chitinases were assayed at different pH ranging from pH 3 to pH 9. Enzyme activity test was carried out with different buffer system (50 mM): glycine-HCl buffer (pH 3), acetate buffer (pH 4 and pH 5), phosphate buffer (pH 6, pH 7 and pH 8) and glycine-NaOH buffer (pH 9 and pH 10). Preparation for all buffers was shown in Appendix G. The reaction mixture containing purified enzyme and colloidal chitin was incubated at different pH, 37°C for 60 minutes and the chitinase activity was measure using standard assay (Annamalai et al., 2010). The enzyme stability was measured after it was dialysed against different buffer for 60 minutes at 37°C, chitinase activity was determined.

Activity measurements were performed at temperature 10°C-80°C. The reaction mixture containing 1 mL of enzyme, 0.5 mL colloidal chitin in 50 mM phosphate buffer pH 7 was incubated at different temperature; 25°C, 37°C, 45°C, 50°C, 60°C, 70°C and 80°C for 30 minutes and the chitinase activity was measured using standard assay. Thermsostability of enzyme was measured by adding 1 ml purified enzyme in 50 mM phosphate buffer pH 7 and incubated for 30 minutes at different temperature and chitinase activity was assayed (Senol et al., 2014).

### 3.12 Effect of Metal Ion on Chitinase Activity

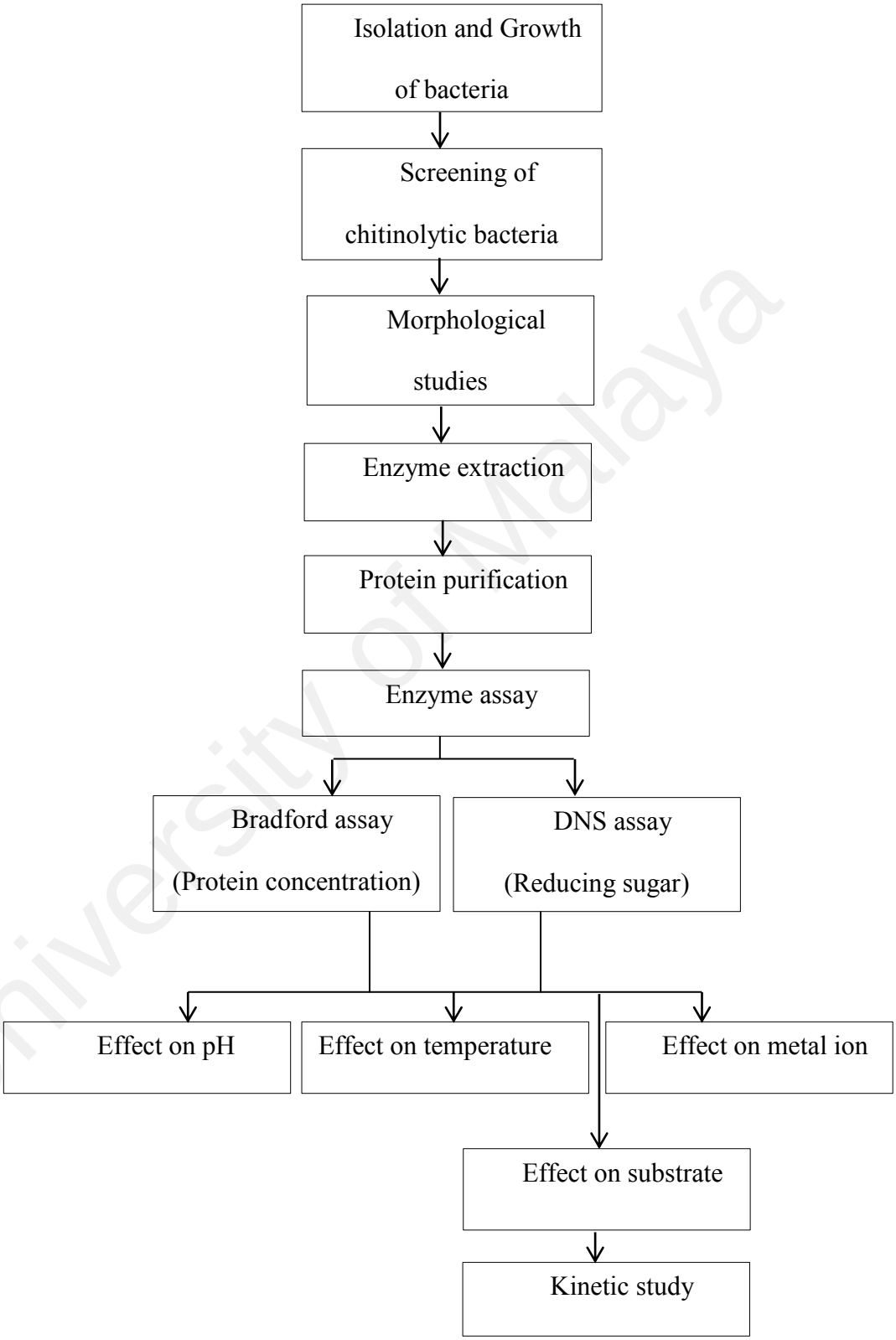
The effect of metal on enzyme activity was determined by incubating the purified enzyme with different metal (5mM concentration) at room temperature for 1 hour. The chitinase activity then carried out using standard assay.

### 3.13 Determination of $K_m$ and $V_{max}$

To study the $K_m$ and $V_{max}$ of purified chtitinase, the enzymes were incubated with colloidal chitin at various concentrations from 0.5 to 1.5 mM. The colloidal chitin was

mixed with 50 µl enzyme in 0.2 M phosphate buffer and incubated at 50°C for 20 minutes the (Han et al., 2008). Reducing sugar was measured every 5 minutes using standard DNS assay and data were recorded. The Polymath6.0 was used in this kinetic study.

**Flow chart illustrates the experimental design process**



# CHAPTER 4: RESULTS AND DISCUSSION

## 4.1 Identification of the Chitinase Producing Strain

### 4.1.1 Morphological Study for Bacterial Colony

Two strains from rhinoceros beetle larvae gut were isolated and labeled as RBLG1 and RBLG2 while from marine, three strains were isolated and labeled as BBL1, BBL2 and BBL3. Two out of five strains that shows the highest chitinase activity were selected, one from larvae gut, RBLG1 and another one from marine, BBL1. The strain from RBLG1 was identified as Gram positive bacillus, rod shape, with cream-like colony and grows in aerobic conditions meanwhile strain from BBL1 was identified as Gram positive bacillus, rod shape, and cream-like colony and also grows aerobically as summarized in Table 4.1. From Table 4.1, there are no significance differences between RBLG1 and BBL1 strain. Basically almost all the characteristics for both strains show the same character except for the elevation and margin. Figures 4.1 and 4.2 respectively show the chitinolytic bacterial colonies on colloidal chitin agar plates and Gram staining of the pure strain under microscope at 100x magnification. The chitinolytic bacterial colonies were found to have a clear zone around them as they utilize the colloidal chitin as their substrate.

**Table 4.1** Morphological characteristics of RBLG1 and BBL1.

| Characteristics | RBLG1 | BBL1 |
|---|---|---|
| *Morphological characteristics* | | |
| Shape | Bacilli | Bacilli |
| Gram stain | positive | positive |
| *Culture characteristics* | | |
| Shape | Irregular with hair like outgrowths | Irregular with hair like outgrowths |
| Elevation | Raised | Flat |
| Margin | Undulate | Lobate |
| Colour | Opaque with rough surface | Opaque with rough surface |
| *Physiological characteristics* | | |
| Growth temperature | 37°C | 37°C |
| Growth pH | pH 7 | pH 7 |
| Catalase test | Positive | Positive |
| Growth in anaerobic condition | Negative | Negative |

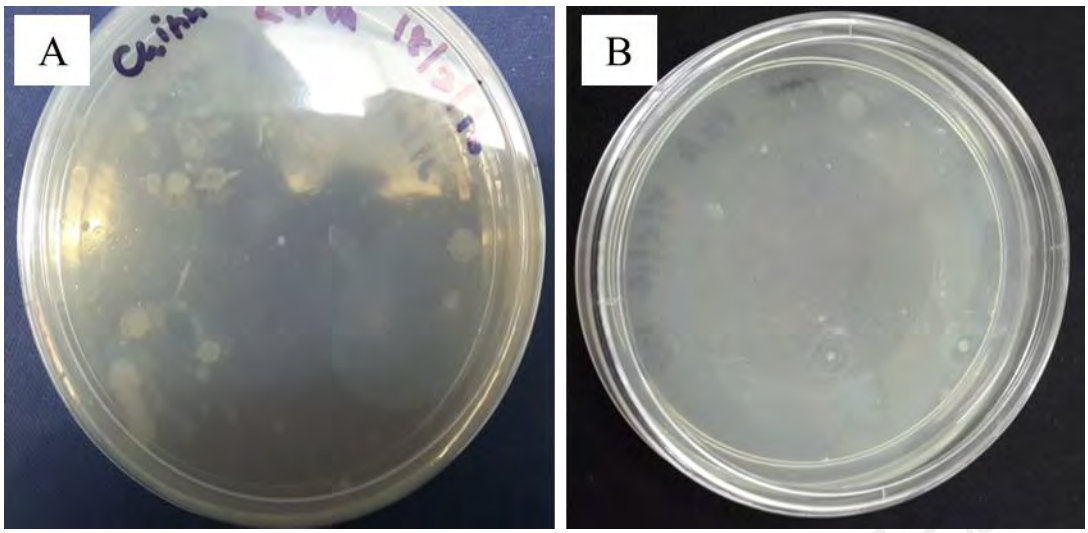



**Figure 4.1** (A) Bacterial colonies from rhinoceros beetle larvae gut on CCA plate; (B) Bacterial colonies from marine coastal water on CCA plate. Both figures show some bacterial colony with clear zone around them.

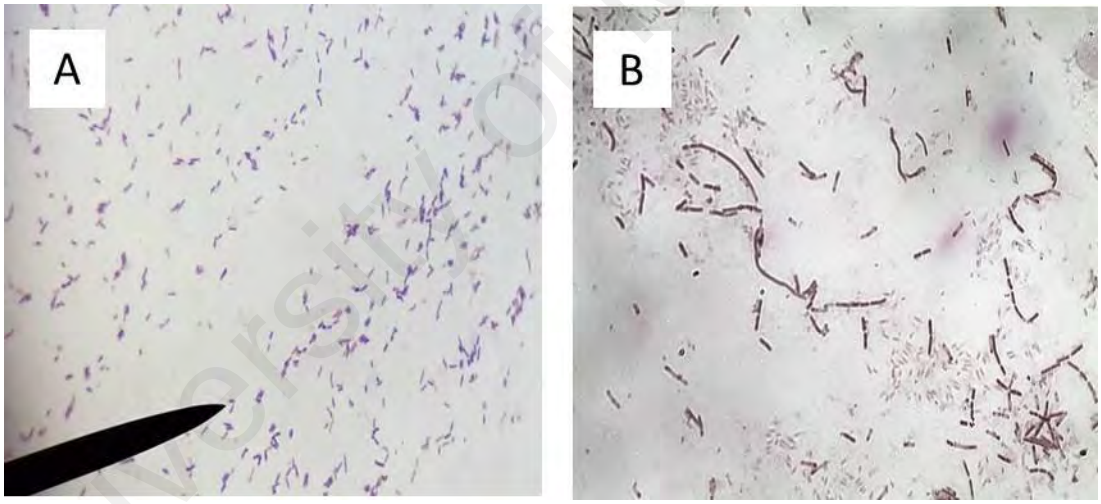



**Figure 4.2** (A) Staining of RBLG1 and ; (B) Gram stain of BBL1 at 100x magnification. The figures show bacterial morpholoy under light microscope. Both RBLG1 and BBL1 show gram positive and rod shaped bacteria.

### 4.1.2 16S rDNA Sequencing

RBLG1 strain was identified 99 % similar as *Bacillus cereus* meanwhile BBL1 strain was 100% similar as *Bacillus cereus* using 16S rDNA sequencing. The report of PCR, BLASTN and phylogenetic tree for RBLG1 and BBL1 were shown in Figure 4.3, Figure 4.4 and Figure 4.5 respectively. Figure 4.3 shows that the amplifications bands from RBLG1 and BBL1 consist of the same length which is 2500 bp. BLASTN report for both strains show high max score and lower E-value which indicates a good BLASTN were achieved for both strains. When the E-value is lower, less likely the similarities happened by chances or random similarity. RBLG1 was labelled as „Chi" in the phylogenetic tree in Figure 4.4 (B), while BBL1 labelled as „BBL1 in Figure 4.5 (B). As there were no dendogram results that showing Evolutionary Distance for both strains, RBLG1 and BBL1 were considered as *Bacillus* sp.

Several studies on chitinase producing microorganisms claimed that other *Bacillus* species or strains could break down chitin such as *B. cereus* TKU030 (Liang et al., 2014), *B. subtilis* TV-125 (Senol et al., 2014), *B. cereus* TKU028 (Liang et al., 2014), *B. licheniformis* SK-1 (Kudan & Pichyangkura, 2009), *B. licheniformis* MKU3 (Radha & Gunasekaran, 2009), and *B. subtilis* JN032305 (Shivakumar et al. , 2014).

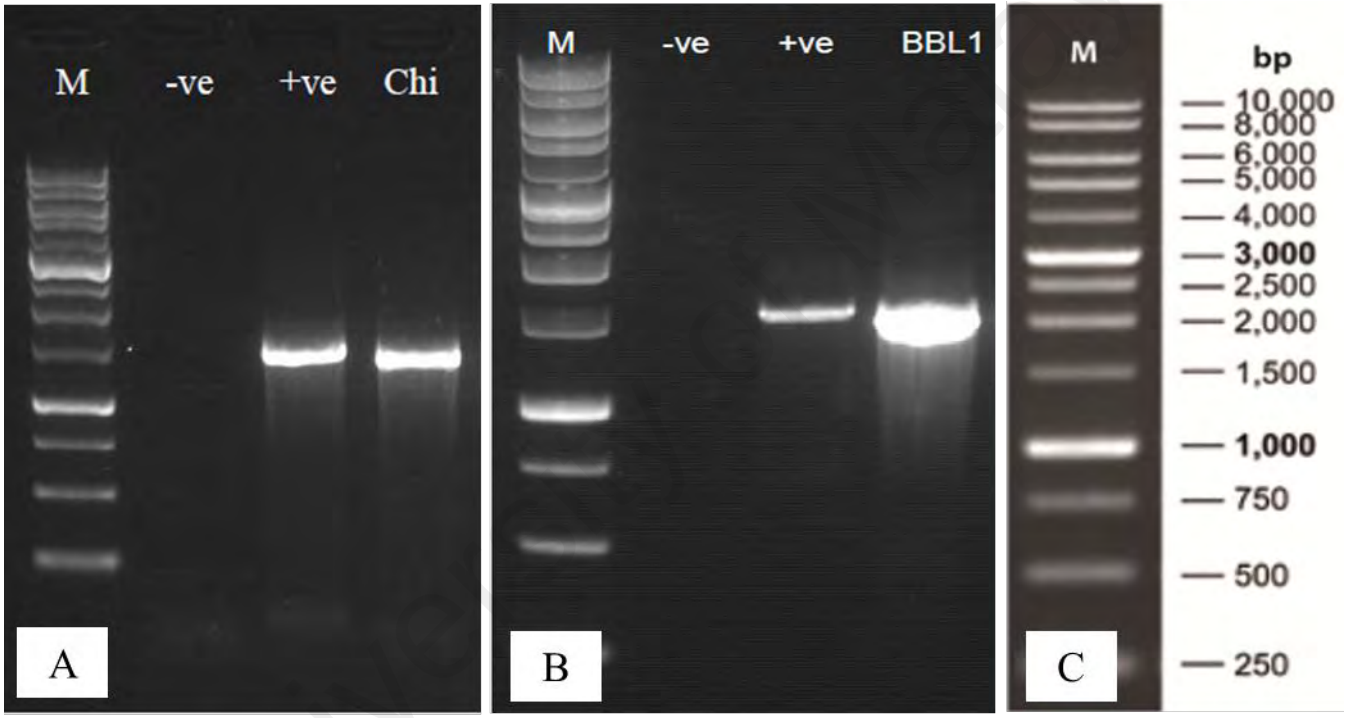


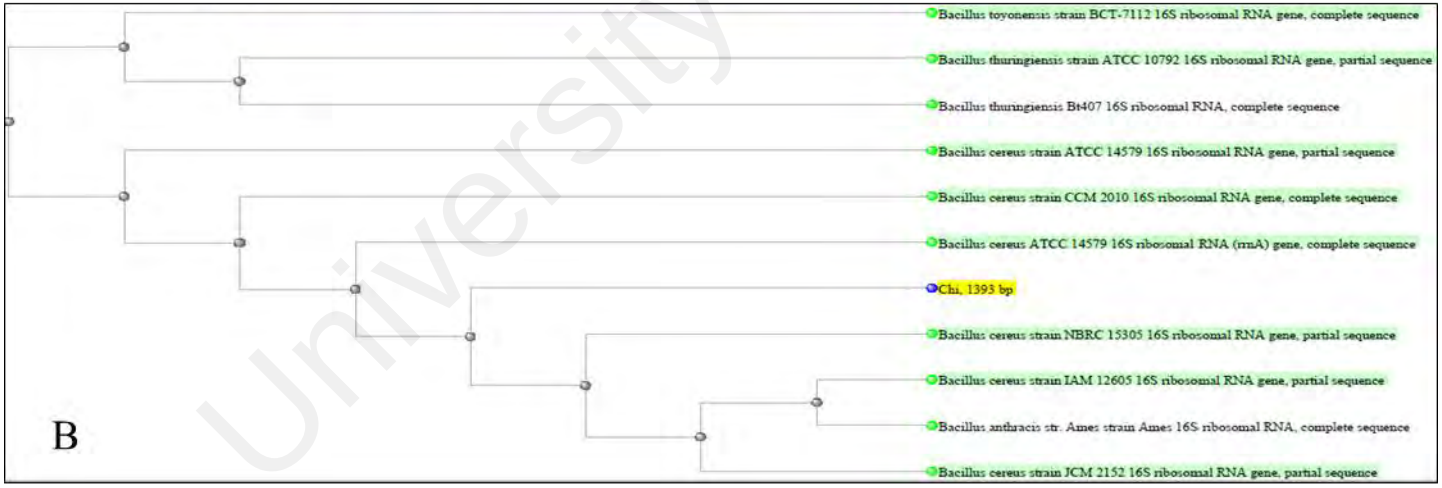

**Figure 4.3** (A) Gel electrophoresis for RBLG1 strain labelled as „Chi"; (B) Gel electrophoresis for BBL1 strain labelled as „BBL1"; (C) DNA ladder. Figure (A) and (B) show PCR amplification of 16S rDNA gene of agarose gel electrophoresis and (C) shows DNA marker which acts as indicator for the length of both RBLG1 and BBL1.

| Sample Name | Result Links |
|---|---|
| Chi | |

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|
| Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence | 2497 | 2497 | 100% | 0.0 | 99% | NR_074540.1 |
| Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence | 2497 | 2497 | 100% | 0.0 | 99% | NR_115714.1 |
| Bacillus cereus strain ATCC 14579 16S ribosomal RNA gene, partial sequence | 2497 | 2497 | 100% | 0.0 | 99% | NR_114582.1 |
| Bacillus cereus strain JCM 2152 16S ribosomal RNA gene, partial sequence | 2482 | 2482 | 99% | 0.0 | 99% | NR_113266.1 |
| Bacillus cereus strain IAM 12605 16S ribosomal RNA gene, partial sequence | 2482 | 2482 | 99% | 0.0 | 99% | NR_115526.1 |
| Bacillus cereus strain NBRC 15305 16S ribosomal RNA gene, partial sequence | 2480 | 2480 | 99% | 0.0 | 99% | NR_112630.1 |
| Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence | 2479 | 2479 | 100% | 0.0 | 99% | NR_121761.1 |
| Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA gene, partial sequence | 2479 | 2479 | 100% | 0.0 | 99% | NR_114581.1 |
| Bacillus thuringiensis Bt407 16S ribosomal RNA, complete sequence | 2473 | 2473 | 100% | 0.0 | 99% | NR_102506.1 |
| Bacillus anthracis str. Ames strain Ames 16S ribosomal RNA, complete sequence | 2473 | 2473 | 99% | 0.0 | 99% | NR_074453.1 |

A

http://www.ncbi.nlm.nih.gov/nuccore/444304116,636559654,636558525,631252068,636559466,631251433,659364953,636558524,485099110,444304029




B

**Figure 4.4** (A) Details of potential chitinolytic bacteria for RBLG1 strain using BLASTn; (B) Phylogenetic tree for RBLG1 strain.

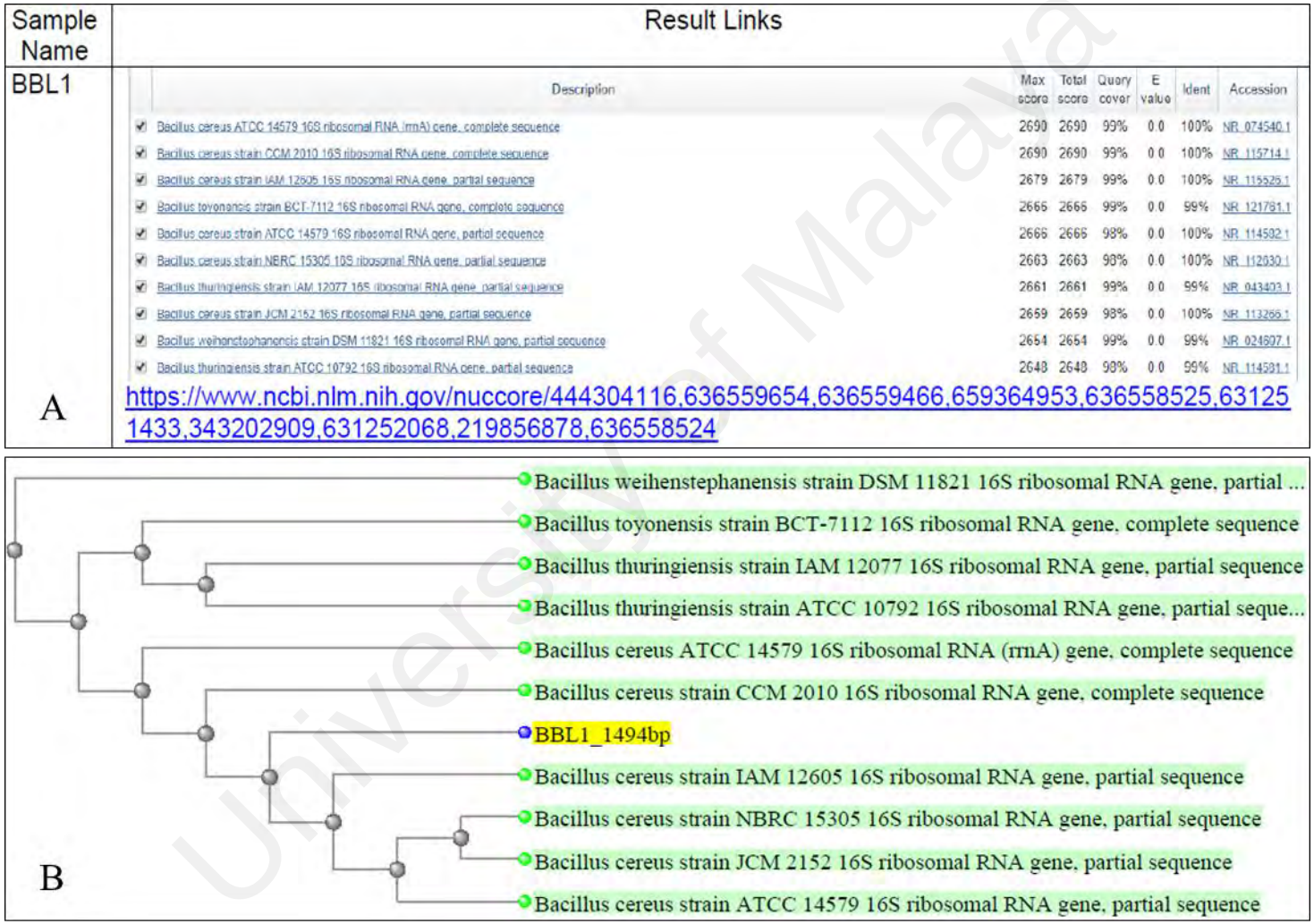



**Figure 4.5** (A) Details of potential chitinolytic bacteria for BBL1 strain using BLASTn; (B) Phylogenetic tree for BBL1 strain.

## 4.2 Bacterial Growth Curve Identification

The RBLG1 and BBL1 were incubated for three days (72 hours) at 37°C and at pH 7. Every 6 hours, enzyme assay for both strains were done and recorded. The growth curves were plotted as shown in Figure 4.6. The highest enzymatic activities for both strains were at 48 hours after incubation period. After 48 hours, enzyme activities for RBLG1 and BBL1 were declined. The values are mean ± SD, n=3. The growth curve consist of lag phase, log phase, stationary phase and death phase. Figure 4.6 shows log phase or exponential phase during 0 to 48 hour. During these hours, bacteria were rapidly divided until they reached the stationary phase at 48 hour to 54 hour. Enzyme activity for bacteria stays the same at stationary phase as the nutrients were depleted and finally the enzyme activity was slowly declined as they enter the death phase at 60 hour. The growth curve data were used as indicator for how long the bacterial culture should be incubated before proceeds with purification process. As highest enzyme activity for both RBLG1 and BBL1 were at 48 hour, RBLG1 and BBL1 stock culture were cultured in CCB and incubated for 48 hour in incubator shaker. After 48 hour, the bacterial cultured for both strains were taken for chitinases purification.

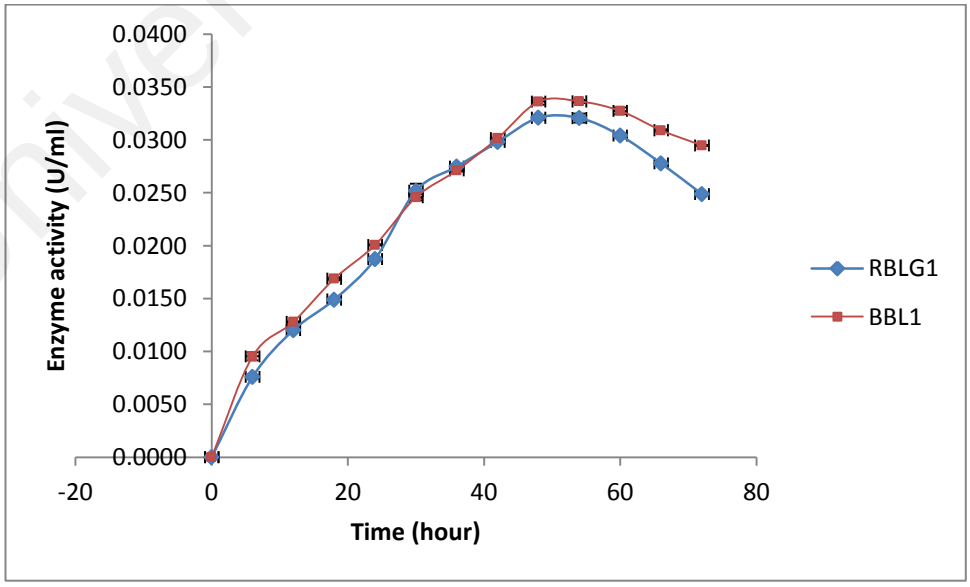



**Figure 4.6** RBLG1 and BBL1 growth curves. Enzyme activities were recorded at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, and 72 hour.

### 4.3 Purification of the Chitinases from RBLG1 and BBL1 strains.

After incubated for 48 hours at 37°C in media containing colloidal chitin with constant shaking, chitinases from two strains were purified by ammonium sulphate precipitation followed by two step chromatographic techniques which are ion exchange chromatography and gel filtration chromatography. Enzyme purification for the two strains; RBLG1 and BBL1 were performed at 37°C ± 2°C and the summary of the steps were presented in Table 4.2 and Table 4.3.

**Table 4.2** RBLG1 enzyme purification table.

| Step | Protein (mg) | Total Activity (U) | Specific Activity (U/mg) | Purification (Fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude | 68.86 | 24.95 | 0.36 | 1.00 | 100.0 |
| Ammonium sulphate fractionation | 31.61 | 11.77 | 0.37 | 1.03 | 47.2 |
| Gel filtration | 14.36 | 7.19 | 0.50 | 1.38 | 28.8 |
| Ion exchange | 4.91 | 3.38 | 0.69 | 1.90 | 13.5 |

**Table 4.3** BBL1 enzyme purification table.

| Step | Protein (mg) | Total Activity (U) | Specific Activity (U/mg) | Purification (Fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude | 72.65 | 28.77 | 0.40 | 1.00 | 100.0 |
| Ammonium sulphate fractionation | 31.40 | 13.25 | 0.42 | 1.07 | 46.1 |
| Gel filtration | 17.40 | 7.82 | 0.45 | 1.13 | 27.2 |
| Ion exchange | 3.53 | 3.88 | 1.10 | 2.78 | 13.5 |

Protein concentration and chitinase activity were measured for all samples from different fractions. The specific activity for chitinase from RBLG1 was 0.36 U/mg in crude extract, and 0.69 U/mg in gel filtration, which gives 1.9 purification fold. The specific activity for chitinase from BBL1 was 0.40 U/mg in crude extract and 1.10 U/mg in gel filtration that gives 2.78 purification fold. The yield for both RBLG1 and BBL1 are 13.5%. The recovery of chitinase activity was low throughout the enzyme purification processes might be due to several reasons which are synergistic action of different isoforms in crude enzyme and a loss of chitinase activity upon purification (Liang, et al., 2014). The loss of protein after each step also contributed to the low chitinase activity. Many investigators have used purification table in their researchs and most of them used ammonium sulphate to get the premilinary precipitation of chitinase enzyme. The saturation levels of precipitation were differing between investigators and ranged from 30% to 85% (Aida, 2014).

### 4.4 Chitinase Molecular Weight Determination

Molecular weight for chitinases from both strains in each fraction was identified. SDS-Page was done for both purified chitinases and the result was shown in Figure 4.7. The purified chitinases from both strains were showing molecular mass approximately 40 kDa. The molecular mass of the purified chitinases from RBLG1 and BBL1 were relatively similar to the masses of chitinases from other strain or species of *Bacillus* for example *B. subtilis* IMR-NK1 (41 kDa) *B. cereus* TKU030 (43 kDa), *B. cereus* S1 (45 kDa), and *Bacillus* sp. P16 (45 kDa) (Liang et al., 2014). There were also chitinases from *Bacillus* sp. that have different molecular mass such as *Bacillus* sp. Hu1 (80.8 kDa) and *B. subtilis* (27 kDa) (Dai et al., 2011; Karunya, 2011).

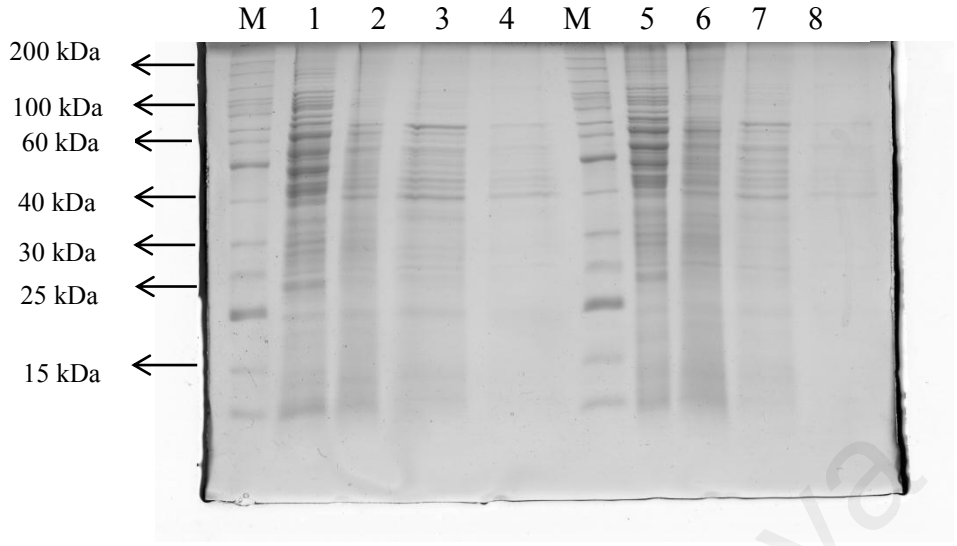



**Figure 4.7** SDS-PAGE analysis of chitinase fractions produced by RBLG1 and BBL1. Lanes : M, molecular markers; 1, culture supernatant (RBLG1); 2, crude enzyme (RBLG1); 3, chitinase fraction after Sephacryl S-100 (RBLG1); 4, chitinase fraction after DEAE-Sepharose CL-6B chromatography (RBLG1); 5, culture supernatant (BBL1); 6, crude enzyme (BBL1); 7, chitinase fraction after Sephacryl S-100 (BBL1); 8, chitinase fraction after DEAE-Sepharose CL-6B chromatography (BBL1).

## 4.5 The Effect of Temperature on Purified Chitinase Activity

Figure 4.8 and 4.9 show the effects of temperature on chitinase activity and stability for RBLG1 and BBL1 respectively. RBLG1 shows the highest chitinase activity which is 0.0354 U/mL at 60°C. The highest chitinase activity for RBLG1 at 60°C might be due to the environmental conditions where the rhinoceros beetle larvae were harvested. During larvae collection, temperature of composted sawdust has been measured and the recorded temperatures were between 50°C to 60°C. Previous study from Kudan (2009), reported that *Bacillus* sp. could grow at 60°C (Kudan & Pichyangkura, 2009). Meanwhile, the highest chitinase activity observed for BBL1 was 0.0367 U/mL at 45°C as the BBL1 strain was isolated from marine environment. Previous study reported that the optimum temperature for marine bacterial chitinase was at 50°C (Han, 2009). Other study also presented that *Bacillus* sp has an optimum temperature at 40°C (Gomaa, 2012).The thermo stability of chitinase from RBLG1 declined after 60°C while

chitinase from BBL1 shows the declined in stability after 50°C. The values show in both figures are mean ± SD, n=3.

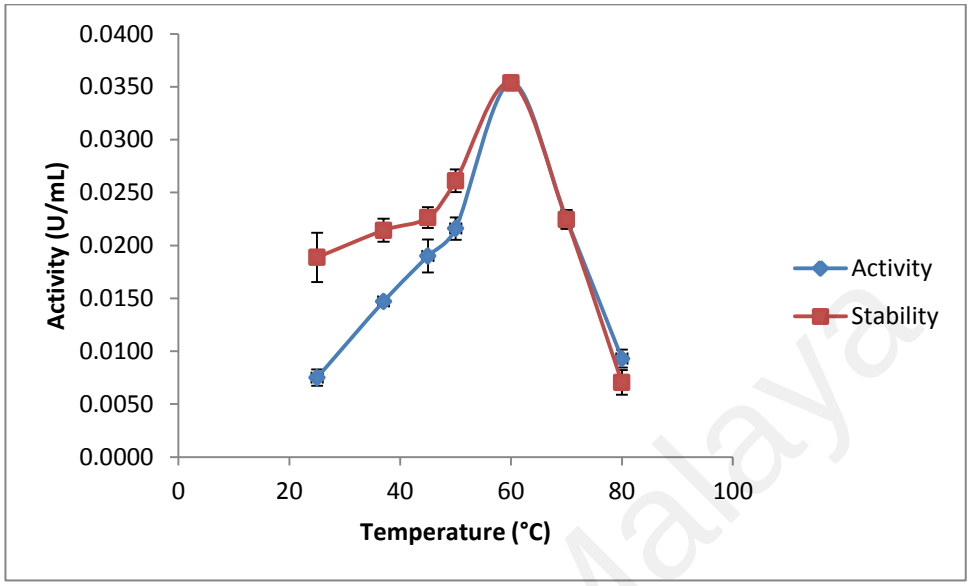



**Figure 4.8** Effect of temperature on chitinase activity and stability for RBLG1.The temperature used were 25°C, 37°C, 45°C, 50°C, 60°C, 70°C and 80°C.

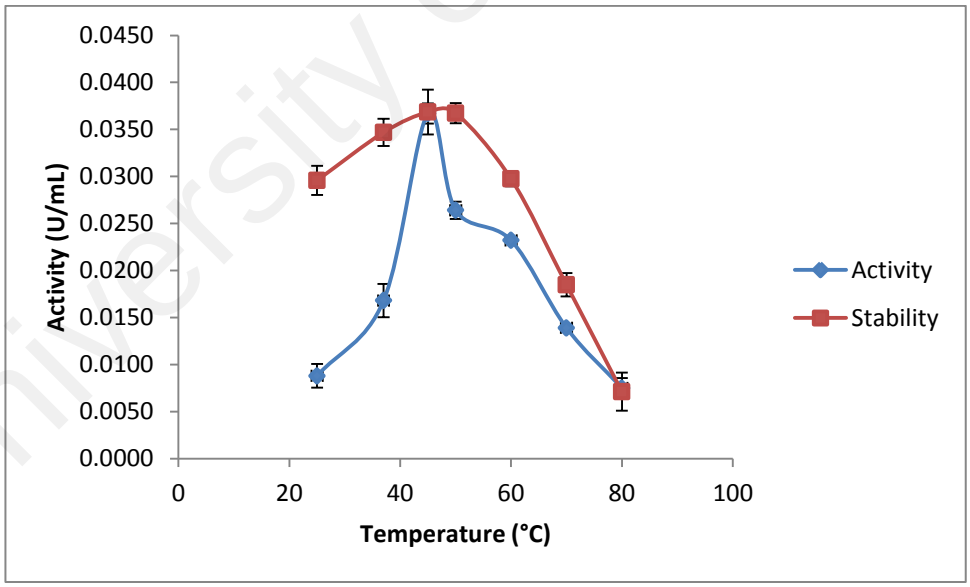



**Figure 4.9** Effect of temperature on chitinase activity and stability for BBL1. The temperature use were 25°C, 37°C, 45°C, 50°C, 60°C, 70°C and 80°C.

## 4.6 The Effect of pH on Purified Chitinase Activity

Figures 4.10 and 4.11 show the effect of pH on chitinase activity and stability for RBLG1 and BBL1 respectively. RBLG1 shows the highest chitinase activity which is 0.0330 U/mL at pH 4. Meanwhile, the highest chitinase activity for BBL1 was 0.0360 U/mL at pH 6. Chitinase from RBLG1 and BBL1 were relatively stable at pH 4-7 and pH 7 respectively. Chitinase stability from both RBLG1 and BBL1 declined starting from pH 7.The values shows in both figures are mean ± SD, n=3. Many studies have reported that pH value for larval gut for most Scarabaeidae was alkaline. Although it might be postulated that the larval gut has alkaline pH, it was observed that the optimum pH for the activity for *N*-acetyl-glucosaminidase was within the range pH 4.1 to pH 5.2 (Wada et al., 2014). As chitinase activity from RBLG1 was showing the highest activity at pH 4, it was relatively similar with study from Wada (2014). Gomaa (2012), reported that optimum pH for *Bacillus* sp. were at pH 7 and pH 8 meanwhile Han (2009), reported that marine bacterial chitinase has an optimum pH at pH 8 (Han, 2009; Gomaa, 2012) .

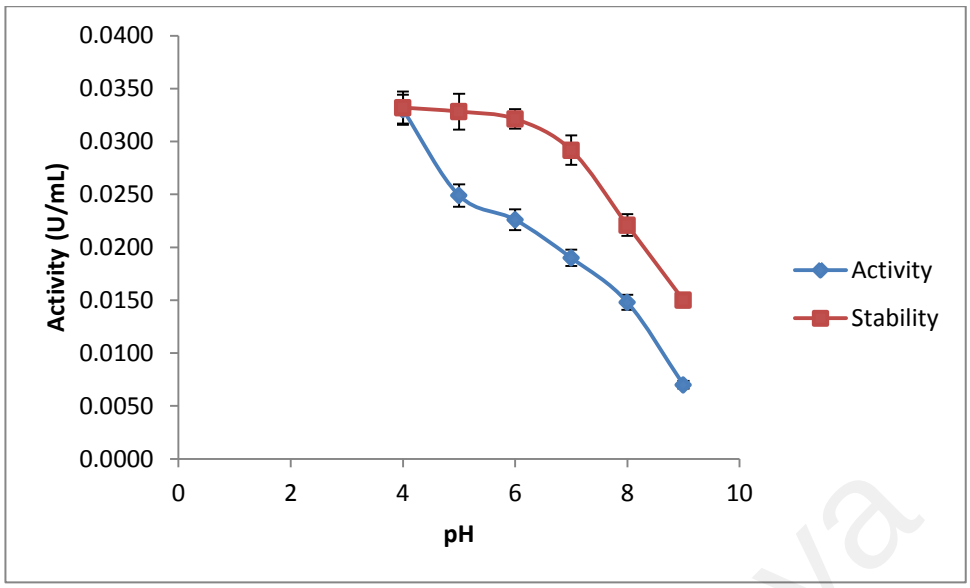



**Figure 4.10** Effect of pH on chitinase activity and stability for RBLG1. The pHs used were pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9.

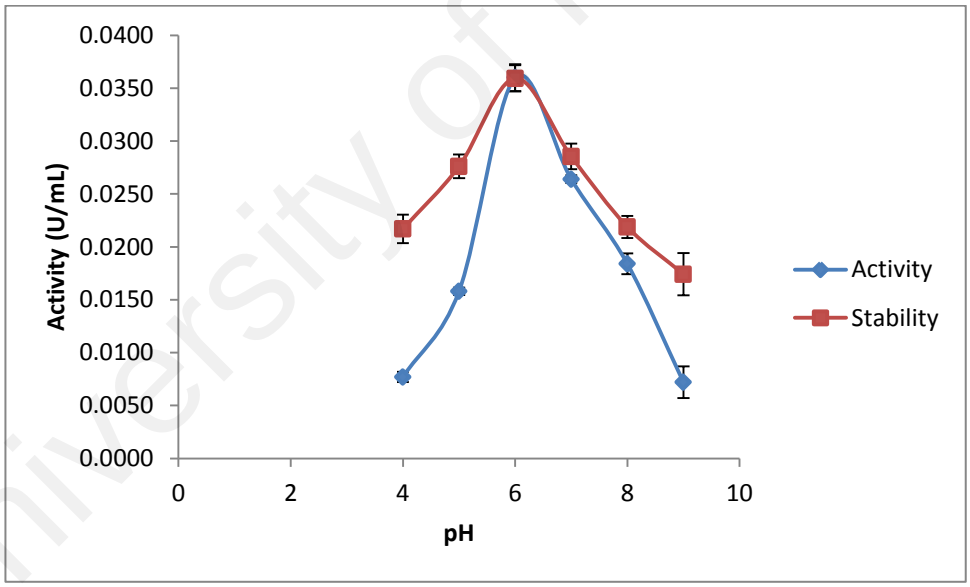



**Figure 4.11** Effect of pH on chitinase activity and stability for BBL1. The pHs used were pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9.

## 4.7 Effect of Colloidal Chitin Concentration on Chitinase Activity

The study of substrate concentration ranging from 0.5% to 2.5% was performed on purified chitinases. The results obtained suggested that when using 1.5% colloidal chitin, it shows high specificity for chitinase from both strains; RBLG1 and BBL1. The values show in Figure 4.12 have mean ± SD, n=3. Literatures on characterization of chitinase using different concentration of colloidal chitin are limited as many investigators opted to use different type of substrates. Previous studies from Dai (2011) and Adrangi (2010) described the used of different types of substrate which include colloidal chitin, chitin from crab shell, flake chitin, chitosan an also carboxymethyl cellulose (CMC) (Adrangi, 2010; Dai, 2011). The used of different concentration of colloidal chitin as substrate for characterization was reported from Anuradha (2013), and the results obtained suggested that 2% colloidal chitin was the ideal substrate concentration for the maximum activity of chitinase (Anuradha, 2013).

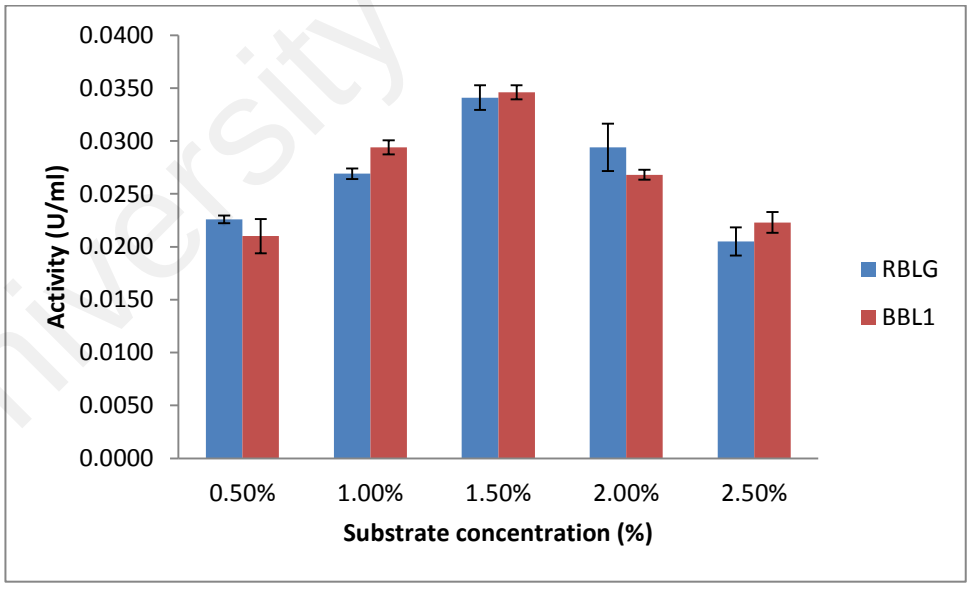



**Figure 4.12** Effect of substrate concentration on chitinases activity. The colloidal chitin concentrations used were 0.5%, 1.0%, 1.5%, 2.0% and 2.5%.

## 4.8 Effect of Metal Ion on Chitinase Activity

Some enzymes can catalyze a reaction by using metal. Metal ions could affect enzymatic activity of bacterial chitinase in either assisting in the catalytic reaction or inhibit enzymes reaction. Metal ion test was conducted to determine the reactions of RBLG1 and BBL1 chitinases towards metal ions and the results were shown in Figure 4.13. For RBLG1, it was observed that chitinase shows highest enzymatic activity when incorporated with $Cu^+$ which is 0.0336 U/mL, while it shows the least activity when incorporated with $Zn^+$ (0.0319 U/mL). The chitinase from BBL1 shows the highest activity that is 0.0343 U/mL when integrated with $Zn^+$ and lowest activity that is 0.0318 U/mL when integrated with $K^+$. Overall, the metal ions used in this experiment were showing no inhibition action toward chitinases and some of the metal ions were increasing the chitinase activity. The values are mean ± SD, n=3. As referred to Wang (2015), some metal ions had an obvious promoting effect on chitinase activity and almost all the metal ions used in the experiment shows no significant effect on chitinase activity (Wang et al., 2015).

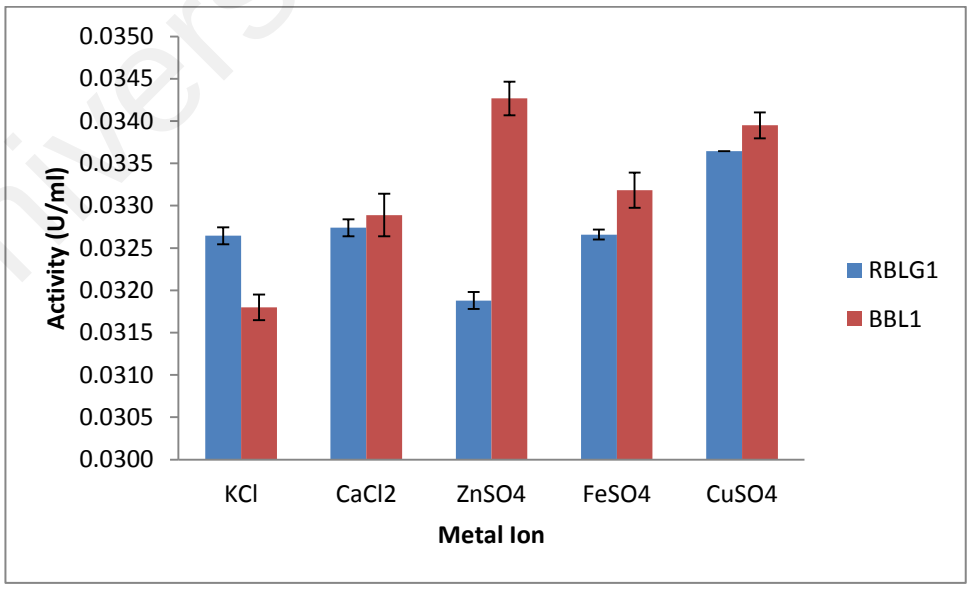



**Figure 4.13** Effect of metal ion on chitinase activity. The metal ions used were $K^+$, $Ca^+$, $Zn^+$, $Fe^+$, and $Cu^+$.

## 4.9 Determination of kinetic for RBLG1 and BBL1 using Michaelis-Menten

### 4.9.1 Polynomial Graph

The kinetic performances of the chitinase enzyme from RBLG1 and BBL1 were determined by using Michaelis-Menten kinetic. Reducing sugar for both strains were calculated for different colloidal chitin concentration at four time intervals. A polynomial 2$^{nd}$ order graphs were plotted based on the reducing sugar production at different concentrations and times (Appendix H). The initial reaction (V) values were determined based on the quadratic differential equation and polynomial graphs were plotted based on initial reaction (V) values against substrate concentration (S) values as shown in Table 4.4, Figure 4.14 and Figure 4.15.

**Table 4.4** Value of S and V for RBLG1 and BBL1.

| **RBLG1** | | **BBL1** | |
|---|---|---|---|
| Substrate concentration (S) (g/L) | Initial reaction (V) ([P](g/L/min) | Substrate concentration (S) (g/L) | Initial reaction (V) ([P](g/L/min) |
| 0.25 | 0.0028 | 0.25 | 0.0034 |
| 0.75 | 0.0040 | 0.75 | 0.0070 |
| 1.25 | 0.0060 | 1.25 | 0.0072 |
| 1.75 | 0.0066 | 1.75 | 0.0080 |

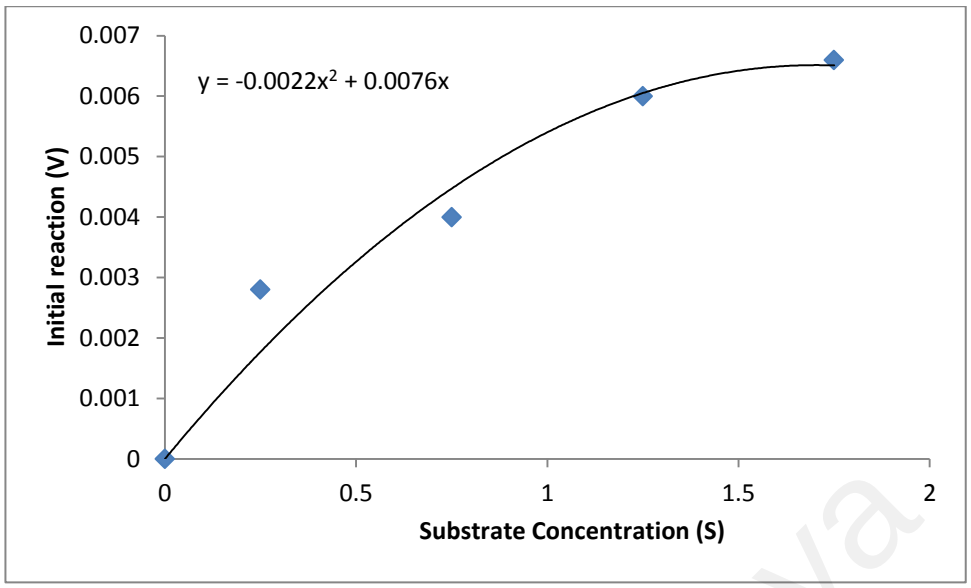



**Figure 4.14** Graph initial reaction (V) against substrate concentration (S) for RBLG1

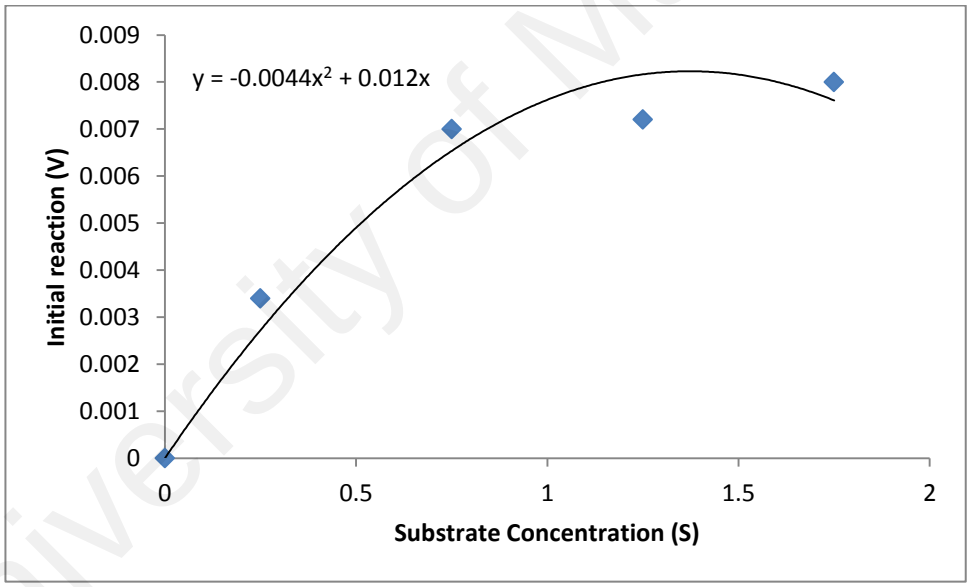



**Figure 4.15** Graph initial reaction (V) against substrate concentration (S) for BBL1

### 4.9.2 Determination of $V_{max}$ and $K_m$

Maximal velocity ($V_{max}$) and substrate concentration at half of maximal velocity ($K_m$) of RBLG1 and BBL1 were calculated using polynomial graphs show in Figure 4.14 and Figure 4.15 respectively. The summary of the $V_{max}$ and $K_m$ values for both strains that had been calculated from the graphs and were shown in Table 4.5;

**Table 4.5** $V_{max}$ and $K_m$ for RBLG1 and BBL1

| Strain | RBLG1 | BBL1 |
|---|---|---|
| $V_{max}$ ([P](g/L/min) | 0.0066 | 0.0080 |
| $K_m$ (g/L) | 0.5093 | 0.3887 |

Affinity is the tendency of certain enzyme to bind with substrate. Different enzyme has different affinity. High $K_m$ indicates the enzyme has low affinity towards its substrate and a lot of substrate must be present to saturate the enzyme. Alternatively, low $K_m$ will use small amount of substrate as the enzyme has high affinity for substrate. RBLG1 shows the high $K_m$ value which is 0.5093 g/L compared to BBL1 which is 0.3887 g/L. $V_{max}$ values for RBLG1 and BBL1 were 0.0066 ([P](g/L/min and 0.0080 ([P](g/L/min respectively. According to Han (2009), the higher $V_{max}$ and lower $K_m$ values indicated that the particular substrate is better for that particular enzyme (Han et al., 2009). $V_{max}$ value basically shows the rate of enzyme break down its substrate. The higher $V_{max}$ value showing the enzymes works effectively. As BBL1 had higher $V_{max}$ and lower $K_m$ values compared to RBLG1, the enzyme from BBL1 strain works more effectively than RBLG1.

Polymath6.0 was used to prove the accuracy of the calculated $V_{max}$ and $K_m$ in Table 4.5. As in Michelis-Menten equation stated that;

$$v = V_{max}[S]/ (K_m + [S])$$

The equation was used as input in the software and the values in Table 4.5 were used as initial guess. The nonlinear regression for RBLG1 and BBL1 were analyzed. Table 4.6 shows the analyzed data for both strains using nonlinear regression. The $R^2$ shows the precision of the initial guess value towards the calculated data using software. The $R^2$ values in RBLG1 and BBL1 show 0.9258 and 0.9598 respectively. The calculated $V_{max}$ and $K_m$ value in the software also showed that BBL1 had higher $V_{max}$ and lower $K_m$ values compared to RBLG1.

**Table 4.6** Nonlinear regression report on RBLG1 and BBL1.

| Strain | Variable | Initial guess | Value | $R^2$ |
|---|---|---|---|---|
| RBLG1 | $V_{max}$ ([P](g/L/min) | 0.0066 | 0.0092914 | 0.9258 |
| | $K_m$ (g/L) | 0.5093 | 0.7257507 | |
| BBL1 | $V_{max}$ ([P](g/L/min) | 0.0080 | 0.0100191 | 0.9568 |
| | $K_m$ (g/L) | 0.3887 | 0.4237398 | |

# CHAPTER 5: CONCLUSION

In this study, several conclusions can be made. The lists are as follow;

- Chitinolytic bacteria can be isolated from several sources using specific substrate that is colloidal chitin.
- Highest chitinases activities from isolates had been identified at different temperature, pH and substrate concentration.
- Kinetic performance for chitinase enzyme from both isolates was determined.

Chitinolytic bacteria could be isolated from *Oryctes rhinoceros* larvae gut and seawater. Colloidal chitin was used as the substrate because it consists of nutrients that chitinolytic bacteria needed to grow. Out of five isolates, two isolates were chosen and identified as *Bacillus* sp, after went through several steps identification process including 16S rDNA.

Before characterization, purification of chitinase enzyme extracted from both isolates had been done, as characterization should use purified enzyme. Several purification steps including ammonium sulphate precipitation, ion exchange chromatography and gel chromatography were performed and the samples from each step were collected and analyzed using SDS-PAGE. The molecular weight for purified chitinase enzymes from both isolates were observed at 40 kDa.

The characterization of the chitinases purified from both isolates show highest chitinase activity at different temperature and pH. For isolate from *Oryctes rhinoceros* larvae gut, the highest chitinase activity was observed at pH 4 and 60°C while isolate from seawater shows highest chitinase activity at pH 6 and 45°C. The difference of chitinase activity exhibit by both isolates is due to the sources where the strains were isolated.

The determinations of kinetic performance for purified chitininase enzyme from both isolates were followed classic Michaelis-Menten equation. The equation used to find $V_{max}$ and $K_m$. Both isolates also show difference performances.

In summary, study on effectiveness of chitinolytic enzymes to react with its substrate that is chitin is crucial. It is important to improve the chitinases role in industry either in biotechnology, medical or other application.

Recommendation for future study regarding the capability of chitinases isolated from several sources should be done in a large scale in order to fit the demand in industries. Further study on conversion of seafood wastes and spent mushroom composed into second generation fermentation feedstock for example biodiesel and bioethanol should be done by using purified enzymes from this experiment, or by using mixed enzymes to make conversion more efficient. This is the time where we should take alternative way to make use all the wastes in order reduce the environmental pollution and as a replacement for non-renewable resources.

## REFERENCES


Adrangi, S., Faramarzi, M. A., Shahverdi, A. R., & Sepehrizadeh, Z. (2010). Purification and characterization of two extracellular endochitinases from *Massilia timonae*. *Carbohydrate Research, 345*(3), 402-407.

Aida, F. M., Al-Nusarie, S., & Taghreed, S. (2014). Production, optimization, characterization and antifungal activity of chitinase produced by *Aspergillus terrus*. *African Journal of Biotechnology*, *13*(14).

Al Sagheer, F., Al-Sughayer, M., Muslim, S., & Elsabee, M. Z. (2009). Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. *Carbohydrate Polymers, 77*(2), 410-419

Annamalai, N., Giji, S., Arumugam, M., & Balasubramanian, T. (2010). Purification and characterization of chitinase from *Micrococcus* sp. AG84 isolated from marine environment. *African Journal of Microbiology Research, 4*(24), 2822-2827.

Antizar-Ladislao, B., & Turrion-Gomez, J. L. (2008). Second-generation biofuels and local bioenergy systems. *Biofuels, Bioproducts and Biorefining, 2*(5), 455-469.

Anuradha, V., & Revathi, K. (2013). Purification and characterization of bacterial chitinase isolated from crustacean shells. *International Journal of Pure Applied & Bioscience, 1*(4), 1-11.

Bedford, G. (2015). *Oryctes rhinoceros* (coconut rhinoceros beetle). *Invasive species compendium*. Wallingford, UK, CABI International.

Bedford, G. O. (2013). Biology and management of palm dynastid beetles: Recent advances. *Annual Review of Entomology, 58*, 353-372.

Beier, S., & Bertilsson, S. (2014). Bacterial chitin degradation mechanisms and ecophysiological strategies. *Frontiers in Microbiology, 4*, 149.

Bhattacharya, D., Nagpure, A., & Gupta, R. K. (2007). Bacterial chitinases: Properties and potential. *Critical Reviews in Biotechnology, 27*(1), 21-28.

Bioenergy, I. E. A. (2008). From 1st to 2nd generation biofuel technologies. *An overview of current industry and RD&D activities. Proceeding IEA-OECD*.

Burgess, R. R., & Deutscher, M. P. (2009). *Guide to protein purification* (Vol. 463). Academic Press.

Cheba, B. A., Zaghloul, T. I., EL-Mahdy, A. R., & EL-Massry, M. H. (2016). Effect of pH and temperature on *Bacillus* sp. R2 chitinase activity and stability. *Procedia Technology, 22*, 471-477.

Dai, D., Hu, W., Huang, G., & Li, W. (2011). Purification and characterization of a novel extracellular chitinase from thermophilic *Bacillus* sp. Hu1. *African Journal of Biotechnology, 10*(13), 2476-2485.

Determann, H. (2012). *Gel Chromatography.* Springer Science & Business Media.

Duskova, J., Tishchenko, G., Ponomareva, E., Simunek, J., Koppova, I., Skalova, T., & Dohnalek, J. (2011). Chitinolytic enzymes from bacterium inhabiting human gastrointestinal tract-critical parameters of protein isolation from anaerobic culture. *Acta Biochimica Polonica, 58*(2), 261-263.

Engel, P. C. (2013). *Enzyme kinetics: The steady-state approach*. Springer.

Gomaa, E. Z. (2012). Chitinase production by *Bacillus thuringiensis* and *Bacillus licheniformis*: Their potential in antifungal biocontrol. *The Journal of Microbiology*, *50*(1), 103-111.

Hamid, R., Khan, M. A., Ahmad, M., Ahmad, M. M., Abdin, M. Z., Musarrat, J., & Javed, S. (2013). Chitinases: an update. *Journal of Pharmacy and Bioallied Sciences, 5*(1), 21.

Han, Y., Yang, B., Zhang, F., Miao, X., & Li, Z. (2009). Characterization of antifungal chitinase from marine *Streptomyces* sp. DA11 associated with South China Sea sponge *Craniella australiensis*. *Marine Biotechnology, 11*(1), 132.

Hinckley, A. D. (1973). Ecology of the coconut rhinoceros beetle, *Oryctes rhinoceros* (L.)(Coleoptera: Dynastidae). *Biotropica*, 111-116.

Ikeda, M., Miyauchi, K., Mochizuki, A., & Matsumiya, M. (2009). Purification and characterization of chitinase from the stomach of silver croaker *Pennahia argentatus*. *Protein Expression and Purification, 65*(2), 214-222.

Jalal, A., Risheed, C., & Ibrahim, B. (2012). Optimization of chitin extraction from chicken feet. *Journal of Analytical & Bioanalytical Techniques, 3*, 1-5.

Janson, J. C. (2012). *Protein purification: Principles, high resolution methods, and applications* (Vol. 151). John Wiley & Sons.

Karunya, S. K. (2011). Optimization and purification of chitinase produced by *Bacillus subtilis* and its antifungal activity against plant pathogens. *International Journal of Pharmaceutical & Biological Archive, 2*(6).

Khan, H. U. (2012). The role of ion exchange chromatography in purification and characterization of molecules. *Ion Exchange Technologies*. Intech.

Kim, Y. H., Park, S. K., Hur, J. Y., & Kim, Y. C. (2017). Purification and characterization of a major extracellular chitinase from a biocontrol bacterium, *Paenibacillus elgii* HOA73. *The Plant Pathology Journal, 33*(3), 318.

Kudan, S., & Pichyangkura, R. (2009). Purification and characterization of thermostable chitinase from *Bacillus licheniformis* SK-1. *Applied Biochemistry and Biotechnology, 157*(1), 23.

Kuddus, M., & Ahmad, I. (2013). Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. *Journal of Genetic Engineering and Biotechnology, 11*(1), 39-46.

Lee, Y. S., Park, I. H., Yoo, J. S., Chung, S. Y., Lee, Y. C., Cho, Y. S., & Choi, Y. L. (2007). Cloning, purification, and characterization of chitinase from *Bacillus* sp. DAU101. *Bioresource Technology, 98*(14), 2734-2741.

Lee, Y. G., Chung, K. C., Wi, S. G., Lee, J. C., & Bae, H. J. (2009). Purification and properties of a chitinase from *Penicillium* sp. LYG 0704. *Protein Expression and Purification, 65*(2), 244-250.

Lennartsson, P. R., Erlandsson, P., & Taherzadeh, M. J. (2014). Integration of the first and second generation bioethanol processes and the importance of by-products. *Bioresource Technology, 165*, 3-8.

Liang, T. W., Chen, Y. Y., Pan, P. S., & Wang, S. L. (2014). Purification of chitinase/chitosanase from *Bacillus cereus* and discovery of an enzyme inhibitor. *International Journal of Biological Macromolecules, 63*, 8-14.

Liang, T. W., Hsieh, T. Y., & Wang, S. L. (2014). Purification of a thermostable chitinase from *Bacillus cereus* by chitin affinity and its application in microbial community changes in soil. *Bioprocess and Biosystems Engineering, 37*(6), 1201-1209.

Liu, M., Zeng, X., Ma, C., Yi, H., Ali, Z., Mou, X., & He, N. (2017). Injectable hydrogels for cartilage and bone tissue engineering. *Bone Research*, *5*, 17014.

Manjeri, G., Muhamad, R., & Tan, S. G. (2014). *Oryctes rhinoceros* beetles, an oil palm pest in Malaysia. *Annual Research & Review in Biology, 4*(22), 3429.

Matroodi, S., Zamani, M., Haghbeen, K., Motallebi, M., & Aminzadeh, S. (2013). Physicochemical study of a novel chimeric chitinase with enhanced binding ability. *Acta Biochimica Biophysica Sinica (Shanghai), 45*, 845-856.

Minagawa, T., Okamura, Y., Shigemasa, Y., Minami, S., & Okamoto, Y. (2007). Effect of molecular weight and deacetylation degree of chitin/chitosan on wound healing. *Carbohydrate Polymers, 67*(4), 640-644.

Moslim, R., Wahid, M. B., Kamarudin, N., Ali, S. R. A., & Hamid, N. H. (2006). Research into the commercialization of *Metarhizium anisopliae* (Hyphomycetes) for biocontrol of the rhinoceros beetle, *Oryctes rhinoceros* (Scarabaeidae), in oil palm. *Oil Palm Journal of Oil Palm Research (Special Issue-April 2006)*, 37-49.

Paulsen, S. S., Andersen, B., Gram, L., & Machado, H. (2016). Biological potential of chitinolytic marine bacteria. *Marine Drugs, 14*(12), 230.

Pillai, C., Paul, W., & Sharma, C. P. (2009). Chitin and chitosan polymers: Chemistry, solubility and fiber formation. *Progress in Polymer Science, 34*(7), 641-678.

Plant Heroes. (2012). Coconut rhinoceros beetle (CRB). Retrieved on 10 October 2017, from http://plantheroes.org/coconut-rhinoceros-beetle.

Priya, C., Jagannathan, N., & Kalaichelvan, P. (2011). Production of chitinase by *Streptomyces hygroscopicus* VMCH2 by optimisation of cultural conditions. *International Journal of Pharma and Bio Sciences, 2*, 210-219.

Radha, S., & Gunasekaran, P. (2009). Purification and characterization of keratinase from recombinant *Pichia* and *Bacillus* strains. *Protein Expression and Purification, 64*(1), 24-31.

Reuveni, S., Urbakh, M., & Klafter, J. (2014). Role of substrate unbinding in Michaelis–Menten enzymatic reactions. *Proceedings of the National Academy of Sciences, 111*(12), 4391-4396.

Roskoski, R. (2014). *Biomedical Sciences*. Elsevier.

Scopes, R. K. (2013). *Protein purification: Principles and practice*. Springer Science & Business Media.

Senol, M., Nadaroglu, H., Dikbas, N., & Kotan, R. (2014). Purification of chitinase enzymes from *Bacillus subtilis* bacteria TV-125, investigation of kinetic properties and antifungal activity against *Fusarium culmorum*. *Annals of Clinical Microbiology and Antimicrobials, 13*(1), 35.

Shivakumar, S., Karmali, A. N., & Ruhimbana, C. (2014). Partial purification, characterization, and kinetic studies of a low-molecular-weight, alkali-tolerant chitinase enzyme from *Bacillus subtilis* JN032305, a potential biocontrol strain.*Preparative Biochemistry and Biotechnology, 44*(6), 617-632.

Sims, R. E., Mabee, W., Saddler, J. N., & Taylor, M. (2010). An overview of second generation biofuel technologies. *Bioresource Technology, 101*(6), 1570-1580.

Soltani, R., Chaieb, I., & Hamouda, M. H. B. (2008). The life cycle of the root borer, *Oryctes agamemnon*, under laboratory conditions. *Journal of Insect Science, 8*(1), 61.

Souza, C. P., Almeida, B. C., Colwell, R. R., & Rivera, I. N. (2011). The importance of chitin in the marine environment. *Marine Biotechnology, 13*(5), 823
.

Souza, C. P., Burbano-Rosero, E., Almeida, B. C., Martins, G. G., Albertini, L. S., & Rivera, I. N. (2009). Culture medium for isolating chitinolytic bacteria from seawater and plankton. *World Journal of Microbiology and Biotechnology, 25*(11), 2079-2082.

Sudhakar, P., & Nagarajan, P. (2010). Production of chitinase by solid state fermentation from rice bran. *International Journal of Environmental Science and Development, 1*(5), 435-440.

Suresh, P. (2012). Biodegradation of shrimp processing bio-waste and concomitant production of chitinase enzyme and N-acetyl-D-glucosamine by marine bacteria: Production and process optimization. *World Journal of Microbiology and Biotechnology, 28*(10), 2945-2962.

Suryanto, D., Irawati, N., & Munir, E. (2012). Isolation and characterization of chitinolytic bacteria and their potential to inhibit plant pathogenic fungi. *Microbiology Indonesia, 5*(3), 8.

Tan, S. C., & Yiap, B. C. (2009). DNA, RNA, and protein extraction: The past and the present. *BioMed Research International, 2009*.

Wada, N., Sunairi, M., Anzai, H., Iwata, R., Yamane, A., & Nakajima, M. (2014). Glycolytic activities in the larval digestive tract of *Trypoxylus dichotomus* (Coleoptera: Scarabaeidae). *Insects, 5*(2), 351-363.

Wang, J., Zhang, J., Song, F., Gui, T., & Xiang, J. (2015). Purification and characterization of chitinases from ridgetail white prawn *Exopalaemon carinicauda*. *Molecules, 20*(2), 1955-1967.

Wu, C. S. (2003). *Handbook of size exclusion chromatography and related techniques: Revised and expanded* (Vol. 91): CRC Press.

Xie, X. S. (2013). Enzyme kinetics, past and present. *Science, 342*(6165), 1457-1459.

Yan, N., & Chen, X. (2015). Don't waste seafood waste: Turning cast-off shells into nitrogen-rich chemicals would benefit economies and the environment. *Nature, 524*(7564), 155-158.

Yan, Q., & Fong, S. S. (2015). Bacterial chitinase: Nature and perspectives for sustainable bioproduction. *Bioresources and Bioprocessing, 2*(1), 31.

Yang, T. L. (2011). Chitin-based materials in tissue engineering: Applications in soft tissue and epithelial organ. *International Journal of Molecular Sciences*, *12*(3), 1936–1963.

Zhou, H., Zhu, J., Luo, X., Leu, S.-Y., Wu, X., Gleisner, R., & Qiu, X. (2013). Bioconversion of beetle-killed lodgepole pine using SPORL: Process scale-up design, lignin coproduct, and high solids fermentation without detoxification. *Industrial & Engineering Chemistry Research, 52*(45), 16057-16065.