ISOLATION OF MYCOLYTIC ENZYME PRODUCING BACTERIA FROM THE ENVIRONMENT IN SARAWAK, AND EVALUATION OF THEIR POTENTIALS AS BIOCONTROL AGENTS AGAINST \textit{GANODERMA BONINENSE}.

by

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Abstract

The greatest threat to oil palm (Elaeis guineensis Jacq.) cultivation in Malaysia is the basal stem rot (BSR) disease caused by white rot fungus, Ganoderma boninense. The BSR causes a great economic loss to the oil palm industries in Malaysia and other South-East Asian countries. Traditionally, chemical fungicides are used to control BSR diseases, however, they do carry other side effects to both the environment and human health. Hence, this brings in the suggestion of the application of biocontrol agents, as they are a more environmentally friendly approach. The aims of this thesis study were to isolate and characterise the mycolytic enzyme producing microbes from the soil ecosystem in Sarawak and to evaluate their effectiveness as biological control agents against G. boninense. Mycolytic enzyme-producing microbes were targeted due to their ability to degrade the fungal cell wall composition. The proteases and glucanases producing microbes were the focus in this study. The proteases and glucanases enzymes producing microbes were successfully isolated out from the local soils samples collected in Sarawak. A total of 21 out of 46 isolates were protease producing microbes whereas another 25 isolates were glucanases producing microbes. The selective media Carboxymethylcellulose agar and Skim milk agar were used in the screening experiment. The BLAST results obtained from the partial 16s rRNA sequence comparison with Genbank, classified the isolates to be the members of the genus of Bacillus, Delftia, Acinetobacter, Stenotrophomonas, Ralstonia, Burkholderia, Pseudomonas, Serratia, Staphylococcus, and Chryseobacterium. A number of 16 different strains were found out of the 46 isolates, Bacillus cereus, Bacillus coagulans, Bacillus flexus, Bacillus anthracis, Delftia tsuruhatensis, Acinetobacter calcoaceticus, Stenotrophomonas maltophilia, Ralstonia pickettii, Burkholderia metallica, Burkholderia cepacia, Pseudomonas plecoglossicida, Pseudomonas mosselii, Pseudomonas putida, Serratia marcescens, Staphylococcus sciuri, and Chryseobacterium indologenes. The efficiency of the 16 different strains as potential biological control candidates were then tested against Ganoderma boninense. Five isolated strains Chryseobacterium indologenes, Acinetobacter calcoaceticus, Serratia marcescens, Pseudomonas putida, and Bacillus cereus were selected for further investigation based on their percentage inhibition of diameter growth (PIDG) in anti-
Ganoderma test. The antibacterial activities of these 5 strains were tested against two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), and yeast (*Saccharomyces cerevisiae*). The antagonistic activities among the selected strains were tested as well. No antagonistic activities were observed among the strains. None of the isolated strains showed antibacterial activity towards the tested standard strains. The antifungal activities of these 5 selected isolated strains were tested against with phytopathogenic fungi *Rhizoctonia solani*, *Aspergillus niger*, and also *G. boninense* at 25°C. All selected strains showed antifungal activity towards the tested standard strains. The culture filtrate assay showed that with the presence of the extracellular metabolites of the isolated bacterial strains, the mycelial germination of the phytopathogenic fungi, *G. boninense* was inhibited. The effectiveness of bacteria consortia consists of *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and *Bacillus cereus* to protect oil palm seedlings against *G. boninense* were evaluated in pot trial experiment. The results obtained in pot trial experiment showed that the consortia bacteria were effective to inhibit the basal stem rot disease caused by *G. boninense* and also could act as a plant growth enhancer for the oil palm seedlings. As a summary, the findings obtained in this study indicated that the isolated mycolytic-producing bacterial have the potential to act as biological control agents against phytopathogenic fungus, *G. boninense*. The aims of this thesis were successfully achieved.
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Declaration

I, Wee Shui Shui, higher degree research student of Master of Science by Research from Faculty of Engineering, Computing and Science in Swinburne University of Technology (Sarawak Campus) hereby declare that the dissertation entitled “Isolation of mycolytic enzyme producing bacteria from the environment in Sarawak, and evaluation of their potentials as biocontrol agents against *Ganoderma boninense*” is original and contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome; to the best of my knowledge, this dissertation contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where the work is based on joint research or publications, the relative contributions of the respective workers or authors has been disclosed.

_____________________________
(WEE SHUI SHUI)

As the principal coordinating supervisor, I hereby acknowledge and verify that the above mentioned statements are legitimate to the best of my knowledge.

___________________________________
(Associate Professor Peter Morin Nissom)
Conference presentations

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CHAPTER 1:
INTRODUCTION AND
LITERATURE REVIEW
1.1 Introduction

Malaysia is currently one of the largest palm oil exporters in the world, about 44% of world exports and the second largest palm oil production in the world, about 39% of palm oil production after Indonesia, which is the world’s largest producer of palm oil. As one of the largest exporter in the world, Malaysia has the responsibilities to produce sustainable palm oil to fulfil the increasing global demand [1]. Sarawak, the largest state in Malaysia is the second largest state in oil palm plantation with 1,442,088 hectares or 24% of the total oil palm planted area in year 2015, with an increment of 14.1% in year 2014 [2]. Sarawak has a humid tropical climate with sunshine throughout the year [3].

There are great potentials for Sarawak in expanding the oil palm industry with an extensive land bank and an ideal climate that is suitable for oil palm to survive. As compared to West Malaysia and Sabah, the lands available for oil palm plantation are limited. Sarawak still has sustainable land to expand for another million hectares of oil palm plantation by the year 2020 [4]. Oil palm is considered as one of the major crop for Sarawak, thus any threat to the industries will cause great economic losses to the state.

The major threat concern to the oil palm plantation in Malaysia is the existing Basal stem rot disease (BSR) which is caused by the white rot fungus known as *Ganoderma boninense*. The estimated economic loss caused by *G. boninense* to the oil palm industry is between RM 225 million to RM 1.5 billion per year [5–7]. *G. boninense* was only found in older oil palm trees in previous year and caused basal stem rot to the oil palm tree [8]. In recent years, the *G. boninense* has been found in younger plants, even the seedlings of the oil palm. The disease symptoms appeared earlier and are more serious [8]. The first sign of the basal stem rot disease shows the yellowing of the young fronds [9]. According to the previous study, the spreading method of basal stem root disease was through root to root method through the contact of the adjacent root [10,11]. Chemical, cultural and mechanical control was conducted in the basal stem disease management in oil palm cultivation but none of the control practices have shown satisfactory results in the disease management [9].

The most common traditionally solutions used to overcome plant diseases issues are by using chemical fungicide. However, the use of chemical fungicides causes adverse
effects towards the environment, health issues to humans and other non-target organisms which also include the beneficial life forms in the environment [12]. Besides that, the chemical fungicides are costly and may induce the pathogen resistance as well. The limitations of the chemical fungicides have increased the research efforts to seek for new, effective and environmental friendly approach to control the phytopathogens in the agriculture industry. Biological control, the used of microorganisms and their secondary metabolites or secretions to suppress the plant diseases is an environmental friendly approach to controlling the phytopathogens in agriculture industry [13].

The most common approach of biological control in plant disease management is by using antagonistic microorganisms. Antagonistic activity involved the secretion of secondary metabolites of one or more microorganisms that causes toxicity towards others microorganisms [14]. The antagonistic microbes possess the ability to secrete the secondary metabolites such as antibiotic, mycolytic enzymes and also volatile compounds with antibacterial or antifungal properties [15]. Numerous of such biological control agents have been reported in previous study such as Pseudomonas spp., Bacillus spp., and Trichoderma spp. [14].

The antagonistic microorganisms have the ability to secrete mycolytic enzymes which are able to degrade the cell walls of pathogenic fungi [14,16]. Chitin and β-1,3-glucan are the major cell wall components of the fungal cell walls [17]. Mycolytic enzymes base formulations consisting of chitinases, proteases, and glucanases, have been used in the control of fungal plant pathogens [12]. The capability of the mycolytic enzymes producing microbes to degrade the fungal cell wall act as an ideal candidate to act as biological control agents in the basal stem rot disease management in oil palm cultivation.

Microorganisms that colonize around rhizosphere are ideal to be used as biological control agents. The rhizosphere serves as the first defence line for the roots system of the plants against attack by the pathogens microorganisms [18]. Therefore, the rhizosphere microorganisms are ideal candidates for biological control agents to suppress the soilborne pathogens. The rhizobacteria Bacillus spp., Serratia spp., and Pseudomonas spp. has shown to be effective biological control agents and has been described in previous study [19]. The rhizobacteria such as Serratia spp. has been
reported to act as biological control agents against nematodes, and also as plant growth enhancer, under greenhouse conditions [20].

The focused of this study were to isolate and characterise the mycolytic enzyme producing microbes from the soil ecosystem in Sarawak and to evaluate their potential to act as biological control agents against plant pathogenic fungus, *G. boninense*. Mycolytic enzyme-producing microbes (proteases and glucanases enzymes) were targeted in this study due to their ability to degrade the fungal cell wall composition. The expected outcomes of this study are successfully isolated out indigenous mycolytic enzyme producing microbes from soil ecosystem in Sarawak and act as potential biological control agents against *G. boninense* in oil palm cultivation.

1.2 Literature review

1.2.1 Oil palm industry in Malaysia

Oil palm tree (*Elaeis guineensis* Jacq.) as shown in Figure 1 which originates from West Africa, was first introduced to Malaysia by British in the early of 1870’s to serve as ornamental plants. The first commercial planting took place in 1917 at Tennamaran Estate in Selangor. This start serves as a foundation for the future expansion for oil palm industry in Malaysia. Agricultural diversification programme was introduced by the Malaysian government in early 1960’s to reduce the country’s economic dependence on rubber and tin, which made the cultivation of oil palm to increase at a fast pace. The Malaysian government introduced land settlement schemes for oil palm plantation in late 1960’s to overcome poverty issues for the landless farmer and smallholders. The oil palm industries in Malaysia are basically based on the estate management system and smallholder scheme [1].
Malaysia is one of the largest producers and exporters of palm oil in the world. According to Malaysian Palm Oil Board 2015, the total oil palm planted area in 2015 reached 5.64 million hectares with an increment of 4.6 % compared to the 2014 records. The increment was due to the new cultivation areas especially in Sarawak with the increment of 14.1% if compared to the 2014 records. The largest oil palm planted state in Malaysia is Sabah with 1.54 million hectares or 27% of the total oil palm planted area. Sarawak is the second largest oil palm planted state in Malaysia with 1.44 million hectares or 26% of the total cultivation area. Peninsular Malaysia accounted for 2.66 million hectares or 47 % of the total oil palm planted area in Malaysia [2].

Figure 1: The oil palm tree (*Elaeis guineensis* Jacq.) plantation estate [21].
The Asia region is the main production of palm oil to fulfil the demand in the market all around the world as shown in Figure 2. Malaysia and Indonesia cover about 83% of the world total production in palm oil industry as shown in Figure 3. The production of palm oil and palm oil kernel in the world had increased rapidly in recent decades to meet the increase in demand worldwide which rose from about 2 million metric ("tonnes") in 1961 to over 56 million tonnes in 2012. The main reason that triggered the high demand for palm oil was due to the development of the application of palm oil besides the use of palm oil in traditional food preparation, and the biodiesel production. It is being estimated by World Bank that in 2020, the world consumption of palm oil will double up [22].

Figure 2: Palm oil world production statistics from year 1970 to 2010 [22].
The increase in global demand towards palm oil made significant contributions to the economics of Malaysia and Indonesia as both countries cover about 83% of the palm oil production in the world. At the moment, palm oil is the major source of sustainable and renewable raw materials available in the global market [23]. Oil palm considers as a highly productive oil-bearing crop in the world in terms of land utilization and productivity. The mesocarp, fruit of oil palm trees can generate about 3 to 4 tonnes/ha/year by contrast the yield of the others oil-bearing crops are normally less than one tonne/ha/year [24]. The comparison of average oil yield (t/ha/year) of different oil-bearing crops is shown in Figure 4. The oil palm crops are able to produce about ten and five times more oil than a soybean and rapeseed with the same amount of land used. The superior productivity of oil palm required less amount of land to meet the global demand on oils and fats for different industries [25]. Therefore, to supply 20% of the world demand for oil and fats (approximately 1.09 billion tonnes), oil palm only required 7 millions hectares of the land area to supply the demand of oil and fats in the world. In contrast, the other oil-bearing crops require 80 million hectares of the land area to supply another 24% of the demand [24,26]. Furthermore, oil palm also generates kernel oil besides mesocarp oil (palm oil) [24].

**Figure 3: Ten largest palm oil producers in the world in year 2011** [22].
1.2.2 Basal stem rot disease (BSR), a major challenge faced by the oil palm industry in Malaysia.

The major challenges face by the sustainability oil palm industry is the basal stem rot disease (BSR). The BSR disease causes economic losses in oil palm industry in various regions around the world which include South-East Asian countries as well [27]. At the present time it is known as the most serious and deadly oil palm disease in Southeast Asian countries. Economics of both Malaysia and Indonesia are affected by this BSR disease. The BSR disease will also result in the decrease in the palm oil production, leading to inability of the industries to fulfil the demand required in the global market [28].
1.2.2.1 Soil borne fungus: Challenges in agricultural industry

The fungal genus *Ganoderma* is a member of the Ganodermataceae, a family notable by a unique double-walled basidiospores and approximately 214 species has been described [29,30]. The fruiting bodies commonly grown in a fan or hood like shape on the trunks of the trees. The basidiocarps form on the trunks have double wall, truncated spores with yellow to brownish ornamented inner layers [5]. Phytopathogenic fungus, *Ganoderma boninense* also known as white rot fungus is soil-borne fungus that causes economic loss in oil palm cultivation. *G. boninense* is the main *Ganoderma* species causing basal stem rot in oil palm plantation in Malaysia. The ‘white rot’ term come from the *Ganoderma* spp. degradation process of the wood components such as lignin (wood components) leaving the white cellulose exposed. Normally *Ganoderma* spp. only attacks old and weakened oil palm trees [27]. *Ganoderma* spp. has many forms of resistance stages which include resistant mycelium, basidiospores, chlamydospores and pseudosclerotia. This increases the difficulty to control the spread of the BSR disease [8,10,31]. The fungus can grow for entrance into the host plants causing spreading of disease through spore or root to root contact whenever the present of the suitable food source such as dead or felled oil palm [9,10,27]. Currently, no satisfactory approach exists to control BSR in the field.

1.2.2.2 Basal stem rot disease

The basal stem rot disease (BSR) was first reported and recognized since 1928. Previously, the BSR disease was only reported to attack old oil palm trees aged 30 years and above. It appears that after 1957, the young oil palm trees (10 to 15 years) were also been infected by BSR disease as well and slowly followed by spreading into the nursery stage in oil palm cultivation. This phenomenon exists mainly in replanted land area. This demonstrates the adaptation ability of the pathogenic fungi to the environmental conditions and the food source available. This resulted in the total infected area of oil palm cultivation to increase gradually but no solutions were able to address the issues effectively. Malaysia as one of the largest producer and exporter of oil palm in the world, it is a serious concern to ensure the oil palm trees are healthy, to deliver maximum production of palm oil to prevent any economic losses to the country [28].
The BSR disease is a lethal plant disease. The spread methods of BSR disease has been described in previous study either through root to root contact [10,11] or another possibility is the cut wound on oil palm as an infection site for *Ganoderma* spore [32,33]. Once infected by the disease, the young oil palms tree normally will die within 6 to 24 months after the first appearance of symptoms, as for mature oil palm trees, these can survive for 2 to 3 years or even longer [11]. The high lethal rate of this disease is due to the difficulties of detection at the early stages which are symptomless. The visible disease symptoms such as basidiomata will only appear at the advanced stages of infection, by that time more than 50% of the internal tissue are already rotten [32,34]. There are several symptoms shown associated with BSR disease. The first sign of the BSR infection show the slight yellowing of the young fronds. Followed by the accompanied of multiple spears on the frond as the infection progresses and cause the frond die back and collapsed [9].

### 1.2.2.3 The traditional practice in basal stem rot disease management

There are several methods that have been described in previous studies: (I) the trench system control, (II) replanting technique and (III) chemical control. The trench system has been recommended as one of the control approach. It works by the technique of digging trenches around the infected palms tree to prevent the spread of mycelia by root contact with the roots of the healthy oil palm tree. There are disadvantages to this system, the digging of trenches depends on the types of soils of the plantation or estate. Furthermore, the digging cost and maintenance for the trenches are high [10,35]. Other than trench system control, the replanting technique of the oil palm trees I also employed. The sanitation procedure before the replanting process is considered as an essential practice to control BSR disease. The assumption has been made that the BSR disease infection is caused by mycelial development through the root contact. The result of this technique shows decrease in disease incidence of oil palm cultivation but does not minimize the BSR disease to a satisfactory rate [10,35].

The traditional practice of using chemical fungicide, with the injection of the combination of carboxin and quintozene fungicides into the trunk, shows significant result in reducing BSR incidence [10,36]. Nevertheless, the use of chemical fungicides
causes harmful effects to the environment and also health and safety concerns. It will also affect the growth of other non-targeted and beneficial microorganisms [13]. Nowadays, the increasing concerns regarding environmental issues and the high cost of the chemicals have stimulated the interest of farmers and researchers to search for an alternative approach to solve the BSR incidence which is environmental friendly [13]. Hence, the suggestion of using biological control agents for BSR disease has drawn notable attention.

1.2.3 The management of plant diseases incidents

The growing world population triggers the demand for food resources in global market to increase rapidly. This phenomenon influences the agriculture system used in current times. Monoculture system has been implemented so that the production of high demand crops is able to meet the requirement of the global market throughout the year [37,38]. In addition, the use of genetically uniform crops in continuous monoculture system, the plant cultivars that susceptible to pathogens and with the use of high nitrogenous concentrations fertilizers have increased the susceptibility to plant disease [39]. The modern agricultural practices used had brought along disadvantages as it is ecological unnatural, thus this enhance the chance of the crops being infected by plants pests, weeds and diseases as well [37,38].

Plant diseases issues in the agricultural industry have attracted global concerns because of the close relationship between plant crops health and the welfare of people, animals, and the environment. The continued improvement in the development of sustainable plant disease management is necessary to ensure the productions of the food crops that are capable of fulfilling the requirements in the global market [40–42]. Plant diseases incidence will cause great economic losses in the agriculture industry due to the reduction of agriculture production in term of both quality and quantity of the products. Furthermore, the infected plants in the fields or in post-harvest storage will affect human and livestock health, the plant pathogenic microorganisms might produce toxic residues in or on the consumable products [40,43–45].

Traditionally practice, chemical fungicides have been selected as active agents to overcome the plant diseases incidences in the agricultural industry. Considering the
limitations of the chemical fungicides has increased the global interest in seeking for new and effective which also environmental friendly control approach to overcome the plant diseases incidences in the agricultural industry. Biological control has been suggested to be used as an alternative approach in the management of the plant diseases incident which also bring zero negative impacts towards the environmental. Biological control strategy has become an important approach for controlling the phytopathogens in sustainable agriculture industry with the used of microorganisms and their secondary metabolites or secretions to suppress the plant diseases which also claim as environmental friendly approach compares to chemical control [13].

1.2.4 The biological control agents
The biological control in the management of plant disease is a strategy that has been proposed half century ago as described in previous study [14]. In recent years, the development of the biological control of plant diseases is still in an early stage although this has been proposed for quite number of years ago. There are only few biological control agents available in the European market [14]. Biological control in plant disease management is by the use of specific microorganisms to interfere with plants pathogen and pests [46]. Biological control is the environment friendly alternative approach to overcome the adverse effects caused by traditional practices, chemical fungicide in plant protection.

1.2.4.1 Mechanism of biological control agents
The mechanisms of biological control activities can be divided into direct and indirect interactions towards plant pathogen. There are 3 main types of direct interactions: antibiosis, parasitism, and competition of the nutrients [14]. Indirect interactions involve the morphological and biochemical changes in the host plant, for example, tolerance to stress by root enhancement and development of plants, solubilisation of the inorganic nutrients and induced the resistance [46].

Antibiosis is the antagonistic activities resulting from the secretion of secondary metabolites by the microorganisms that cause toxic effects towards other
microorganisms. Antibiosis is the most common biological interactions of many biological control agents such as Pseudomonas spp., Bacillus spp., Trichoderma spp., and Serratia spp. [14]. Numerous secondary metabolites can be generated by a strain of biological control agents that are responsible for different functions and are effective against different pathogenic fungi. For example, several secondary metabolites produce by the strain CHAO of Pseudomonas fluorescens: siderophores, phenazines, 2, 4-diacetylphloroglucinol and cyanide. The different combination of the secondary metabolites described are responsible for the antagonism activities towards Gaeumannomyces graminis var. tritici and also Chalara elegans [14,47].

Parasitism is another direct interactions mechanism that is involved in biological control. Parasitism of the plant pathogen by other microorganisms including viruses is a common occurrence [14]. Trichoderma spp. showing parasitic activity towards plant pathogen such as Rhizoctonia solani has been reported in previous study [14,48]. The parasitic activity requires a specific recognition between the antagonist and targeted pathogen and the secretion of several types of cell wall degrading enzymes which enable the parasite to enter the hyphae of the plant pathogen [14]. The mode of actions involved in hyphae interaction for Trichoderma spp. as reported in previous study is by the attachment of the fungus to the host hyphae via coiling, hooks and secretion of lytic enzymes to degrade the target pathogen fungal cell wall. This process inhibits the growth and activity of the plant pathogenic fungi [16].

Competition is one of the direct interactions mechanisms involve in biological control. The competition for nutrient such as carbon in rhizosphere zone is common in soil ecological niche [14]. The capability of biocontrol agents to compete in the rhizosphere is very important and critical in the control of soil-borne pathogenic fungus. This is important because, if the biological control agents are unable to compete for the space and nutrients, the biological control agents are unable to grow in the rhizosphere area [16]. The rhizosphere area is critical as the plant infection normally start from the infection of roots system by soil-borne pathogenic fungus.
1.2.4.2 The rhizosphere microorganisms serve as biological control agents

The rhizosphere compatible fungi and bacteria are potential biological control candidates which exhibit antagonistic activity towards plant pathogens [39]. The rhizosphere is the narrow zone of soil that surrounds the root of the plants as shown in Figure 5. The plants will release a substantial amount of organic compounds named as rhizodeposits into the soil environment through plant root systems [49,50]. Therefore, rhizosphere is the hotspot for activities of the soil microorganisms. Most of the rhizosphere microorganisms are known as plant growth promoting rhizobacteria (PGPR) [49,51]. The benefits contributes by PGPR bacteria towards the plants are increased availability of mineral nutrients for plants, secretion of the plant growth stimulating compounds and also act as first line of defence to provide protection against soil-borne pathogenic fungus [49,52,53].

![Figure 5: The graphical illustrations overview of mycorrhizosphere and the zones of influence (rhizosphere and hyphosphere) [54].](image-url)
The mechanism of PGPR bacteria in plant growth activities are divided into direct and indirect effects towards the plants. The direct effects show the enhanced delivery of the nutrients and the production phytohormones such as auxins, cytokines, and gibberellins [55,56]. The secretion of phytohormones is able to trigger the development of the root system such as the lateral root length, number of root hair, yield and also the height of shoots [55]. The rhizobacteria such as Bacillus spp., Serratia spp., and Pseudomonas spp. have been reported as effective biological control agents and their roles to suppress the soil-borne plant pathogenic fungus has been documented in previous study [19].

*Bacillus* species are gram-positive rhizobacteria possessing several advantages properties that made this species as potential candidates to be biological control agents. The characteristics of *Bacillus* spp. such as omnipresence in soil environments, tolerance with high heat temperature, grow rapidly in liquid culture and the formation of resistant spores made this species as having high potential as biological control agents [57]. The spores of this bacteria species can tolerate high thermal condition, UV light and also organic solvents which makes *Bacillus* spp. to be able to tolerate adverse environmental conditions [58,59]. *Bacillus* spp. has been reported to act as biological control agents in previous study and the mode of action involve in the antagonistic activity has been described as well. The mode of actions involve the production of antibiotic (iturin, surfactin and fengycin), the secretion of the mycolytic enzymes (chitinases, glucanases and proteases) that have the capability to degrade fungi cell wall, and also volatile compounds that possess antifungal or antimicrobial properties [58,60]. In addition, the secondary metabolite secreted are able to enhance the plant growth and the defence responses systems in the host plant [58,61].

*Serratia* species are gram-negative rhizobacteria commonly found at rhizosphere zone and possess antifungal activities [62,63]. Several studies have described the use of *Serratia* spp. as biological control agents against plant pathogenic fungi. One of the strain examples of *Serratia* spp use in plant disease management is *Serratia marcescens* bacteria strain culture. The efficiency of *S. marcescens* as biological control agents has been described in previous study as against nematodes under greenhouse conditions. Besides as biological control agents, *S. marcescens* also act as plant growth enhancer in the study [20]. In addition, *S. marcescens* B2 also showed antagonistic activity towards soil-borne fungus *Rhizoctonia solani* in cyclamen plants in previous study [64]. The
antifungal mechanism of *Serratia* spp. was described in previous study, the direct antifungal properties of this species were based on the antibiosis activity and the production of mycolytic enzymes such as chitinases and β-1, 3-glucanases [65].

*Pseudomonas* species are aerobic, gram-negative rhizobacteria that have been described as potential biological control agents in recent years. *Pseudomonas* spp. have been described to be able produce antifungal antibiotics and enhance the defence system in host plants [66]. Several characteristics of *Pseudomonas* spp. such as rapid growth rate, ability to colonize and grow at rhizosphere environment, the ability to synthesize a variety of bioactive metabolites and the ability to adapt to environment stress made these species suitable candidates to use as a biological control agent in plant disease management [67]. Furthermore, *Pseudomonas* spp. has been described to be responsible for the natural suppression in some plant disease caused by soil-borne pathogens [67,68].

### 1.2.5 Mycolytic enzyme producing microorganisms

Soil also known as earth is one of the major reservoirs of biological diversity. Soil is a unique medium which sustains immense diversity of microbes and yet in a large extent of livings habitat in the soil remains unexplored [69]. The diversity of the soil ecosystems induced the interest in discovers new potential strains from local soil environment. The further investigation of the microorganisms inhabits in diversity soil ecosystems which have the ability to synthesize various biologically active secondary metabolites such as antibiotics and mycolytic enzymes have attracted the attention of the researcher. The potential microorganisms strain will be applied in agriculture and different industries areas. Normally, numerous of bacteria is discovered in the rhizosphere than in the root free soil environment as reported in previous study [50]. The microorganisms at rhizosphere attract the attention for new strains discovery in Sarawak.

Previous studies showed that the ‘reaction zones’ in the infected area were the results of some defence mechanism of the plants to the infection [10,70,71]. As showed in the previous studies, the defence response gene will increase the mycolytic enzymes secretion at the infected area thus this brings out the interest to further investigate the
effects of the enzymes against plant pathogenic fungi. These studies were focused on the isolation of indigenous mycolytic enzymes producing microorganism in Sarawak which possesses the ability to inhibit or killed plant pathogenic fungi.

Biological control by using antagonistic microbes is the most common approach used in plant disease control recently. Antagonistic microbes play an important role in microbial equilibrium which implies direct interactions between two microorganisms. The direct interactions antagonism can be separate to three main types which are parasitism, antibiosis, and competition for nutrients. The antagonism result from the secretion of secondary metabolites by individual microorganisms which is toxic to other microorganism is known as antibiosis. Antagonistic microbes such as fluorescent Pseudomonas spp., Bacillus spp., Trichoderma spp. and others more act as biological control agents. A great amount of various molecules secreted by the antagonistic microbes has been studied and their role in suppression of different plant pathogens has been recorded [14,72–74].

Antagonistic microbes will produce secondary metabolites such as antibiotic, mycolytic enzymes and volatile compounds with antifungal properties. The mycolytic enzymes producing microorganisms were focused in this study due to its ability to produce enzymes that able to degrade cell walls of pathogenic fungi [14–16]. Fungal cell walls are strengthened by chitin whereas major components of plant cell wall are cellulose. Chitin and β-1,3-glucan are the major cell wall components of the fungal cell walls [17]. Mycolytic enzymes base formulations consist of chitinases, proteases, and glucanases which have been used in the control of fungal plant pathogens [12]. The Stenotrophomonas maltophilia is the example of the bacteria strain that has the ability to secrete mycolytic enzymes (chitinases, glucanases, and proteases) as reported in previous study [75,76].
1.2.5.1 Chitinases enzyme

Chitins are flexible but strong nitrogen-containing polysaccharides which also can be found in the external skeletons of the insects and other arthropods. Besides, chitin has wide spectrum distribution in the biosphere, in shells of crustaceans such as crabs so on, the exoskeleton of marine zoo-planktons such as jellyfish and corals, as for insects such as ladybugs and butterfly. Recent years, chitinases, chitin-degrading enzymes have received great attention due to its wide applications in different industrial activities. The ability of this enzymes to degrade chitin which is a major component in fungal cell wall are being studies to be used as the great potential biological control agents in agriculture industry [12]. Chitinases have been divided into three different principles class as describes in previous study. The three different principles class of chitinases enzymes are 1, 4-β-N-acetylyglucosaminidases, endochitinases and exochitinases [46,77]. The oligo-N-actetyl glucosamines which function as elicitors that can lead for the activation of defense-related responses in plant cells [12,78]. Among 3 different groups of mycolytic enzymes, chitinases are the most intensively studied as reported in previous study [15]. The main chitin degraders in soil environment are by fungi and bacteria which involve in the recycling of the carbon and nitrogen resources in the soil ecosystem [15,79]. The strong antagonistic activity of the chitinolytic bacteria of Serratia spp., Choromobacterium sp. and Lysobacter sp against plant pathogenic fungi, Rhizoctonia solani, Fusarium spp. and also Phytophthora capsici has been described in previous study [75].

1.2.5.2 Glucanases enzyme

Glucanases are another key mycolytic enzymes group involve in antagonistic activity in plant disease management. β-Glucans are the homopolymers of D-glucose and are linked in the β-configuration [39]. Both β-1, 3-glucan (laminarin) and β-1, 4-D-glucan (cellulose) are the major components of fungal cell wall beside chitin [46]. Glucanases mycolytic enzymes have the ability to hydrolyze β-1, 3-glucan (laminarin) and β-1, 4-glucan (cellulose). β-1, 3-glucanases enzymes are responsible for the hydrolyse activity of laminarin and the enzymes are further divided into exo-β-glucanases and endo-β-glucanases [46]. Endoglucanases responsible for the hydrolyse activity of β-glucan chain which the cleaving process of the glucose residue is started at the non-reducing
end of the chain. Therefore the products of the cleaving process by endoglucanases are monomer, normally glucose [39,80]. The exoglucanases enzymes involve in the cleaving process of β-linkages at the random site of the chain and result in the release of smaller oligosaccharides [39,80]. *Trichoderma* spp. has been described in previous study produce glucanases enzymes at the certain condition to hydrolyze the minor structure of the cell wall. Besides that, glucanases enzymes also involve in the development and differentiation and the mobilization of the cell wall under the energy source exhaustion condition [46,81]. The antagonistic activity involves by glucanases enzymes has been described in previous study, for example, the *Trichoderma* spp. against *Phytium* plant pathogenic oomycetes [46]. The major cell wall components of *Phytium* consist of β-(1, 3)-(1, 6)-glucans and cellulose instead of chitin [46].

### 1.2.5.3 Proteases enzyme

Proteases are another mycolytic enzymes group play a significant role in the cell wall lysis during antagonistic activity against plant pathogenic fungi [39]. Besides the major cell wall components chitin and glucan, the minor part of the filamentous cell wall are made up of protein and lipids [39,82]. Proteases produced from microorganism play different roles in many industries and also environmental bioremediation. A wide range of fungi (for example genus *Aspergillus*, *Mucor*, and *Rhizopus*) and bacteria (for example genus *Clostridium*, *Bacillus* and *Pseudomonas*) are known to date have the ability to produce proteases enzymes [83]. Although there are quite a number of microorganism has the ability to produce proteases but the commercially available alkaline proteases are mostly derived from the *Bacillus* strains. *Bacillus* strains have the ability to produce a large amount of alkaline proteases enzymes which have great proteolysis activity and able to maintain it stability at quite high pH and temperature condition [83]. In previous study, *Trichoderma* spp. has been reported to play a significant role in the lysis activity by attacking the lipids and proteins, the components of cell-wall skeleton [46].
1.2.6 Sarawak, Borneo, Malaysia

Borneo is the largest island in Asia and third largest island in the world. It is located in South East Asia with a total coverage area of approximately 745,567 square kilometres. Borneo is well known as the oldest and mega biodiversity in the world. Borneo consists of large swathe of diverse forest habitats which include mangrove areas, peat swamp, freshwater swamp, lowland dipterocarp forests, ironwood forests and also hill dipterocarp forests [84]. Besides that, Borneo is also known as the largest heath (kerangas) forests in Southeast Asia. The diverse rainforest located in Borneo were estimated to consist of more than 15,000 flowering plants species, 2000 species of orchids and at least 50 species of the carnivorous pitcher plant. The *Rafflesia* and *Amorphophallus* are two of the largest flowers in the world and can be found in Borneo [84].

The rich biodiversity in Borneo might be due to the diverse habitats areas and ideal climate which supports the growth of different plant species. Borneo has a humid tropical climate with sunshine throughout the year. The temperature range is quite uniform about 22ºC to 33ºC with an evenly distributed annual rainfall of around 3861mm which is ideal for the development and growth of various flora and fauna. Borneo is divided into three nations Malaysia (Sarawak and Sabah), Indonesia and Brunei. The ideal ecosystem makes Borneo a perfect spots for new discovery research. Around 80 percent or 10 million hectares of the total land area in Sarawak is still covered with forest (natural and also secondary forests), this make Sarawak, a part of Borneo as an ideal spot for research and discovery [85].

Sarawak is the largest state in Malaysia located on the Equator and stretches out to around 800 kilometres along the northwest coast of Borneo island with a total area around 124,449.51 square kilometres which is about 37.5 percent of the Malaysia total land area. Sarawak is separated into three regions, coastal lowlands comprising peat swap as well as narrow deltaic and alluvial plains; a huge region of undulating hills (vary to about 300 metres); and also the mountain highlands which stretch out to Kalimantan border. Sarawak also consists of largest peatland area about 1.5 million hectares in Malaysia. Furthermore, Sarawak has an extensive protected area (Figure 6) which comprises 30 national parks, 4 wildlife sanctuaries and 10 natural reserves with the total protect area of 837,553.80 hectares (602,035.8 hectares for land area and
229,789 hectares for water body) [86]. Gunung Mulu National Park located at South of Sarawak is well known as most studied tropical karst area and largest known cave chamber in the world (The Sarawak Chamber, 600 metres by 415 metres and 80 metres high). Besides that, Gunung Mulu National Park also a UNESCO (United Nations Educational, Scientific and Cultural Organization) World Heritage site as its comprises high biodiversity and karst features [85]. The high diversity of the plant species in Sarawak and the ideal ecosystem increase the possibility of the discovery of new or potential species to be utilized in agricultural and industry sectors to boost up the economics of the country. This perception was made according to the discovered of more than 361 new species in Borneo in ten year times from 1994 to 2004 [84].

Figure 6: Summary of existing totally protected areas (TPAs) in Sarawak - Updated at 31 December 2014 [86].
1.2.6.1 Recent new strain discovery in Sarawak, Borneo

Borneo has been known as oldest and mega biodiversity in the world. Sarawak located within Borneo has been selected to conduct the research to discover new species that exist locally due to the broad range of habitats, rich in various plants and animal resources available. Dr Noreha Mahidi has successfully discovered two new *Muscodor*-like isolates from Sarawak, Malaysia which has been named as *Muscodor sarawak* and *Muscodor padawan*. The endophytes isolate has the ability to produce antibiotic VOC’s as well. The further in-vitro screening test of the isolates showed promising results in anti-*Ganoderma* activities, *Ganoderma* spp. is the plant pathogen fungi that caused economic loss in oil palm industry [87]. The previous research showed that different *Muscodor* spp. secreted different mixture of VOC’s. The ability of *Muscodor* spp. to synthesize a broad range of VOC’s mixture has attracted the interest of the researcher’s with the assumption of that yet still other isolate of this species may exist in elsewhere which have the ability to produce other undiscovered compounds in the VOC’s mixture that have been reported so far [88].

Endophytic fungi are the microorganisms live within the living plant tissue but no negative effects caused towards the plants itself have attracted the interest of the researcher as a potential biological control agent. *Muscodor* spp. is the endophyte fungus that so far has only been discovered from certain tropical tress and vine species. *Muscodor* spp. is non-sporulating fungus with the whitish appearance and possess the ability to produce vary volatile organic compounds (VOC’s) with antibiotic properties. The VOC’s secreted by *Muscodor* spp. possess the ability to inhibit and even kill a wide range of both bacterial and fungal pathogens in plant and human which included the soil-borne fungi as well. The characteristics of the *Muscodor* spp. describe gives the potentiality to this fungi genus to be used in agricultural and other industry activities as a biological control agent or biofumigant agent [88].
1.3 Research aims and objectives

The aim of this thesis study was to isolate and characterise the mycolytic enzyme producing bacterial isolates from the soil ecosystem in Sarawak as the biological control agents against plant phytopathogenic, *Ganoderma boninense*. Several objectives were set to achieve the aim of this study, the objectives set were listed as follow:

- To isolate and identification of mycolytic enzymes producing bacterial isolates from the soil samples collected in Sarawak
- To evaluate the efficiency of isolates strains as biological control agents against plant pathogenic fungi, *Ganoderma boninense*
- To evaluate the efficiency of the consortia isolates strains as biological control agents against plant pathogenic fungi, *Ganoderma boninense* in pot trial condition

1.4 Thesis outline

This thesis study is reported in five chapters and is listed as follow:

**Chapter 1 – Introduction and literature review**

Chapter 1 provides the brief introduction on the background of the study. The literature review of the information related to the project which has been reported and described in previous study by the other researchers. The aim and objectives of the research were described in this chapter.

**Chapter 2 – Screening and characterization of the mycolytic enzyme producing microorganisms isolated in Sarawak**

Chapter 2 describes the detailed result of the study on the screening, isolation and characterization of the mycolytic enzymes producing microorganisms with the used of selective media method. This chapter focused on the isolation of proteases and glucanases enzymes producing bacteria from soil ecosystem in Sarawak.
Chapter 3 – Evaluating the efficiency of bacteria strains as biological control agents against *Ganoderma boninense*

Chapter 3 present the details results on the efficiency of the mycolytic enzymes producing isolated strains against *Ganoderma boninense* by dual culture assay. Different assays were carried out to analysis the efficiency of the isolated strains as biological control agents. The focus on this chapter was to evaluate the effectiveness of the isolated strains as potential candidates for biological control agents.

Chapter 4 – Small scale pot trial evaluation of mycolytic enzymes producing bacterial against phytopathogenic fungus, *Ganoderma boninense*

Chapter 4 described the efficiency of the selected mycolytic enzymes producing strains against *Ganoderma boninense* in the small scale pot trial experiment on oil palm seedlings. This chapter focus on the changes of oil palm seedlings over 3 month’s timeframe under different treatments.

Chapter 5 – General conclusions and recommendations

Chapter 5 present the summary of the findings of this research study. The future work and recommendations of this study are described in this chapter as well.
CHAPTER 2: SCREENING AND CHARACTERIZATION OF MYCOLYTIC ENZYME PRODUCING MICROORGANISMS ISOLATED IN SARAWAK
2.1 Introduction

The disadvantages of the traditional practice of using chemical fungicides as active agents to control incidences of plant diseases in the agricultural industry has brought up the suggestion to seek for alternate approach. The application of chemical fungicides will bring various negative effects towards the environment and human health. It also enhanced the possibility of the pathogen acquiring resistance towards the chemical fungicide [13]. Biological control agents have been suggested to be an alternative approach to plant disease management which is also environmental friendly. Biological control with the used of antagonistic microbes is the most common approach used recently [14]. Antagonistic microbes will synthesize secondary metabolites such as antibiotic, mycolytic enzymes and also volatile compounds which consist of antibacterial or antifungal properties. The mycolytic enzymes producing microorganisms was focused in this study due to their ability to synthesize enzymes which have the capability to degrade the cell wall and functional proteins of the pathogenic fungus [14–16].

Previous studies reported that the defence response gene in the plant itself will increase defence mechanism at plant infection areas when plants were attacked by plant pathogenic fungi [10,70,71]. The defence mechanism will trigger the release of mycolytic enzymes in the infected areas. This interesting finding had led to the design of this thesis, to further investigate the effects of mycolytic enzymes towards plant pathogenic fungi. The focused of these studies were on the screening and isolation of mycolytic enzymes producing strains from local soil ecosystem.

Soil is one of the main reservoirs of biological diversity and is also a unique medium that sustains immense diversity of microbes yet to be discovered [69]. Sarawak is the largest state in Malaysia with total land area 124,449.51 square kilometres (37.5% of the Malaysia total land). Additional to that, Sarawak has 512,387.47 hectares of protected area which comprises 18 National Parks, 4 wildlife sanctuaries and 5 nature reserves [86]. Gunung Mulu National Park which is located at south of Sarawak was acknowledged as the most studied tropical karsts area and the largest cave chamber in the world. It is also a UNESCO world heritage site due to the high diversity and karsts features [85]. Sarawak is also located in Borneo, which is one of the twelve mega biodiversity region and third largest island in the world with a total area 743,330 square
kilometres. Borneo is still covered with dense tropical forests. Besides that, diverse habitats such as mangrove swamps, peat swamps, heath (kerangas), dipterocarp forests are also found in Borneo [84].

Borneo is well known in the world due to its oldest and mega diverse rainforests [84]. The diversity of the soil ecosystems was the motivating factor to conduct further investigation in discovering new potential antifungal microorganisms from the local soil environment in Sarawak. The focused of this chapter was on the screening of potential mycolytic producing bacterial from local soil environment and the characterization of the potential isolates obtained.
2.2 Materials and methodology

2.2.1 Soil samples collection from various locations
Soil samples were collected by Karen Yong as part of her final year project in 2015 from various locations within Sarawak. The soil samples were kept in the cold room at 4°C prior to the start of the experiments. The 4 different locations for sampling are as listed below:

1. Fairy cave nature reserve park
2. Betong
3. Padawan area
4. Swinburne University of Technology, Sarawak campus

2.2.2 Media preparation

2.2.2 (a) Skim milk agar (SMA) preparation.
The SMA media was prepared accordingly: 23 grams of Nutrient agar (NA) powder, (Himedia, Mumbai, India) was added to 900mL of deionized water in a 1 litre Schott bottle. 20 grams of skimmed milk powder (Difco, New Jersey, NY) was weighed and added to 100mL of deionized water in a 250mL Schott bottle. Both bottles were autoclaved at 121°C for 15 minutes. After letting the media cooled down to about 50°C, the sterilized skimmed milk solution was added to the sterilized nutrient agar and made up to total volume of 1 litre SMA. The SMA was mixed well before being dispensed on the 90mm petri plates.
2.2.2 (b) Carboxymethylcellulose agar (CMC) preparation.

The recipe of CMC media was shown in Table 1.

**Table 1: Ingredients involve in preparation of CMC media.**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount needed for 1 litre CMC agar</th>
</tr>
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<tbody>
<tr>
<td>Magnesium Sulfate Heptahydrate, MgSO₄·7H₂O (Sigma-Aldrich, St Louis., USA)</td>
<td>0.5 gram</td>
</tr>
<tr>
<td>Potassium chloride, KCl (R&amp;M, Edmonton, Canada)</td>
<td>1.0 gram</td>
</tr>
<tr>
<td>Carboxymethylcellulose powder (Sigma-Aldrich, St Louis., USA)</td>
<td>8.0 gram</td>
</tr>
<tr>
<td>Ammonium dihydrogen phosphate, NH₄H₂PO₄ (Sigma-Aldrich, St Louis., USA)</td>
<td>1.0 gram</td>
</tr>
<tr>
<td>Yeast powder (Himedia, Mumbai, India)</td>
<td>1.0 gram</td>
</tr>
<tr>
<td>Glucose (Bendosen, Selangor, Malaysia)</td>
<td>1.0 gram</td>
</tr>
<tr>
<td>Agar-agar powder (Bendosen, Selangor, Malaysia)</td>
<td>17.0 gram</td>
</tr>
<tr>
<td>Deionized water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

CMC powder and agar powder were added last to the 1 litre Duran, Schott bottle after all other ingredients were added including deionized water. The CMC media was autoclaved at 121°C for 15 minutes.
2.2.3 Screening of potential mycolytic enzymes producing microbes from soil samples

Agar plate assay using Skim milk agar (SMA) and Carboxymethylcellulose agar (CMC) was conducted to screen for the mycolytic enzyme producing microbes.

2.2.3.1 Serial dilution

The serial dilution plating technique was carried up in the initial screening experiment. A ten-fold serial dilution of each soil samples were carried out in sterilized deionized water.

A total volume of 9 mL sterile deionized water was pipetted into 6, 15 mL falcon tubes. Approximately 1 g of soil samples was weighed and transferred into the first falcon tube and labelled as tube 1. The tube was then vortexed using a vortex machine, (Thermolyne). One mL of the mixture solution from tube 1 was transferred to tube 2 by using 1000 µL micropipettes, (Eppendorf) and mixed well. This step was repeated until tube 6, giving a $10^{-6}$ dilution.

2.2.3.2 Screening of potential microbes using selective media

A 0.1 mL of the selected dilution factors solution was spread onto the selective agar for screening process. The dilution factor $10^{-1}$, $10^{-3}$, $10^{-5}$, and $10^{-6}$ was chosen. Agar plate assay using Skim milk agar (SMA) and Carboxymethylcellulose agar (CMC) was conducted.

2.2.3.2 (a) Protease screening

Skim milk agar (SMA) was used to screen for proteases producing microbes. 100µL micropipette, Eppendorf was used to transfer 100µLof the soil suspension from the appropriate dilution to the SMA plates prepared. The suspension was spread evenly by the using of a sterilized spreader. The spread plates were sealed and incubated in the incubator room at $35^\circ C \pm 2^\circ C$ for one day. The plates observed for the presence of the protease producing microbes by the formation of clear zone on SMA plates. The SMA plates with the formation of clear zone were kept in 4°C.
2.2.3.2 (b)  Glucanases screening

CMC agar selective media was used to screen for glucanases producing microbes. 100µL micropipette was used to transfer 100µL of the soil suspension from the appropriate dilution to the CMC plates. The suspension was spread evenly by the using of a sterilized spreader. The spread plates were sealed and incubated in the incubator room at 35ºC ± 2ºC for 3 days. After 3 days of incubation, the incubated plates were stained with 1% congo red dye for about 30 to 60 minutes followed by a de-staining process with the use of sterilized 1 M sodium chloride (NaCl), Bendosen solution for approximately 15 to 20 minutes. After the staining and de-staining process, the plates were observed for the formation of clear zone on CMC plates. The CMC plates with the formation of clear zone were kept in 4ºC.

2.2.4  Isolation of pure culture which produce protease and glucanases enzymes

The potential microbial producers of proteases and glucanases enzymes were picked from the clear zone formation area on the SMA and CMC plates. Sterilized Inoculating loop was used to inoculate the potential bacteria to empty SMA and CMC plates for pure culture isolation. The SMA streaked plates were incubated in incubator room at 35ºC ± 2ºC for one day. CMC streaked plates were incubated for 3 days under same condition and followed by staining and de-staining process. The incubated plates were observed for the purity of the bacteria based on morphology and enzymes activities on the selective media.

The purification step was done by repeated streaking of bacteria onto the selective media until the growth of colony was uniformed on the plates. Contaminated microbes were eliminated at each of the streaked process. Pure cultures of mycolytic enzymes obtained were coded. Glucanases enzyme producing microbes were coded as C1 until C 24 and protease enzymes producing microbes were coded as S1 to S21.
2.2.5 Storage and preservation
Long term storage of bacteria isolates was done by preparation of 25% glycerol stock culture and stored in -80ºC Freezer (Thermo Scientific) for future use. The isolated pure bacteria culture was inoculated into a 10mL sterilized Nutrient broth (NB) (Himedia, Mumbai, India) in a Universal bottle and incubated in an incubator shaker (Sartorious Stedim Biotech), overnight at 35ºC, 120 rpm. The overnight culture was observed for growth and 70 µL of the culture broth was pipetted into sterilized pre-labelled 1.5 mL centrifuge tube with 70 µL of 50% glycerol, (HMBG, Hamburg, Germany) giving a concentration of 25% glycerol. The glycerol stock mixture was sealed and mixed well before being kept in a -80ºC Freezer. For short term storage, isolated pure cultures plates on NA plates were stored at 4ºC in a refrigerator (LG, Korea).

2.2.6 Molecular identification of isolated strains

2.2.6 (a) Crude DNA extraction

(a) DNA extraction by the using Fermentas genomic kit
Fermentas Genomic DNA purification kit was used to extract DNA from the cultures. The steps are as follows:

The isolated cultures were inoculated in sterilized nutrient broth (NB) for overnight in the incubator shaker at 35ºC, 120 rpm. 1000µL of overnight bacteria culture was transferred into sterilized 1.5mL microcentrifuge tube and centrifuged for 10 minutes at 7500 rpm in a microcentrifuge machine (Eppendorf). The supernatant was decanted carefully and making sure the pellet was not discarded. The pellet was then suspended with 200µL of TE buffer by vortexing using a vortexer. 400µL of lysis solution was added into the tube with the 200µL suspended pellet and incubated in a water bath (Memmert) at 65ºC for 5 minutes. After 5 minutes, 600µL of chloroform was added to the tube and inverted for 3 to 5 times to obtain an emulsion. The mixture was then centrifuged at 10,000rpm for 2 minutes. The upper aqueous phase in the tube which contained DNA was transferred to a new labelled
microcentrifuge tube after the centrifuged process was done. An 800µL of 1 X precipitation solution was added into it and was gently mixed by inverted the tube for few times in room temperature about 1 to 2 minutes. The 1 X precipitation solution was prepared by mixing 720 µL sterilized deionized water with 80 µL of 10 X concentrated precipitated solution provided in the kit. The tube was centrifuged for 2 minutes at 10,000 rpm. The supernatant was discarded completely from the tube. The DNA pellet was dissolved in 100µL of the 1.2M sodium chloride (NaCl) solution provided in the kit and was vortexed gently to ensure the pellet was dissolved completely. A 300µL of cold ethanol was then added into the tube. The tube was then placed in -20ºC LG freezer for 10 minutes to precipitate the DNA. The DNA precipitate was spun down at 10,000 rpm for 3 to 4 minutes. After the centrifuging process, the ethanol was removed from the tube and the DNA pellet was air-dried and dissolved in 100µL sterilized deionized water. The procedure described above was repeated for all the isolated bacteria samples.

(b) DNA extraction by freeze and thaw method [89]

The bacteria cultures were prepared as described above. The cell pellet was re-suspended in 1000µL sterilized distilled water by triturating with micropipettes. The mixture was vortex gently to ensure the pellet was completely suspended in the sterilized deionized water. The suspension was subjected to freeze and thaw cycles. A total of 4 cycles were carried out, the condition and time taken for each cycles was as listed.

1st cycle:

8 minutes Freeze at -80ºC in -80ºC freezer
3 minutes Thaw at 85ºC in water bath

2nd to 4th cycles

5 minutes Freeze at -80ºC in -80ºC freezer
3 minutes Thaw at 85ºC in water bath
After the freeze and thaw process, the labelled tube sample was spun down at 10,000 rpm for 5 minutes. The supernatant was transferred carefully to the labelled new sterilized microcentrifuge tube. The labelled tube was then stored at -20ºC until needed. During the transferred process, only the supernatant was transferred. The pellet, as it contains cellular debris, was discarded.

2.2.6 (b) **DNA amplification by Polymerase Chain Reaction (PCR)**

The bacteria DNAs were amplified by polymerase chain reaction (PCR). PCR master mix was prepared accordingly as listed in Table 2 below. Universal primer sets, 8 F and 519 R, targeting 16s rRNA sequences, were selected. The primers sequence for 8 F and 519 R [90] are as listed.

**Primer sequence used:**

8 F 5’-AGAGTTTGATCCTGGCTCAG- 3’

519 R 5’ –GWATTACCGCGGCKGCTG- 3’

<table>
<thead>
<tr>
<th>Composition</th>
<th>Amount per reaction</th>
<th>Amount for 50 reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioline, MyTaq Red Mix</td>
<td>25 µL</td>
<td>1250 µL</td>
</tr>
<tr>
<td>Forward primer, 20 µM (8F)</td>
<td>1 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Reverse primer, 20 µM (519R)</td>
<td>1 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Sterilized MilliQ water</td>
<td>18 µL</td>
<td>900 µL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>45 µL</td>
<td>2250 µL</td>
</tr>
</tbody>
</table>
The prepared master mix was aliquot into sterilized PCR tubes and the tubes were labelled accordingly. Each PCR tubes contained 45 µL of master mix and were added with 5 µL of crude DNA extract. DNA amplification was performed in a PCR machine, (Eppendorf) with following conditions for 30 cycles:

**PCR conditions used:**

- Initial denaturation at 95°C for 1 minute
- Condition for the 29 cycles:
  - Denaturation process: 95°C for 15 seconds
  - Annealing process: 55°C for 15 seconds
  - Extension process: 72°C for 15 seconds
  - Final elongation at 72°C for 5 minutes
- The final PCR products were on hold at 4°C

The PCR products was analysed on a 1 % (w/v) agarose gel with 3 µL of Midori stain (Nippon Genetics Europe GmBH, Duren, Germany) added to it for visualization under UV light. Bio-Rad Gel Documentation EQ System for DNA/RNA Gel Photos was used to visual and captured the gel image of the PCR products.
2.2.6 (e) Sequencing analysis

The PCR products were sent for DNA sequencing. Sequencing was performed by First Base Laboratories Sdn Bhd, Selangor, Malaysia. The sequence results obtained were analysed by matching to the known 16s rRNA gene sequences in the Genbank database [91] using BLAST (Basic Local Alignment Search Tool) of the National Centre for Biotechnology Information [92]. Mega 6 was used to create phylogenetic tree by the used of maximum likelihood method [93]. An overview of the steps involved in the molecular identification of the bacteria isolates is shown in Figure 7.

Figure 7: The flow chart showing the overview of the steps involved in molecular identification of isolated strains. The crude DNA extraction was performed by using Fermentas Genomic kit and freeze and thaw method. The universal sets primer 8F and 519 R, 16s rRNA was used in DNA amplification process. DNA sequencing of PCR products was performed by First Base Laboratories Sdn Bhd, Selangor, Malaysia. Then, the sequence obtained was matched with known 16s rRNA gene sequences in the Genbank database using BLAST.
2.2.7 Gram stain analysis

Gram staining technique was used in this study to differentiate between the gram positive and gram negative of the bacterial strains. The strains were cultured in nutrient broth and incubated overnight at 35°C prior the gram staining analysis. The standard protocol for gram staining was adapted from Beveridge (2001) and which is described as follows:

1. A drop of bacterial suspension was placed on a glass microscope slide and attached by gentle heating.
2. Crystal violet (1.24g in 100mL) was added to the specimen and allowed to stand for 30 seconds.
3. The specimen slide was rinsed with deionized water and Gram’s iodine (the mixture of 0.33g iodine and 0.67g potassium iodide in 100 mL water) was added to the specimen slide and allowed to stand for 30 seconds.
4. The specimen slide was rinsed with deionized water and a few drops of acetone (decolourizing agent) for 5 seconds and rinsed with deionized water immediately after 5 seconds.
5. Safranin was added to the specimen slide and allowed to stand for 30 seconds. The specimen slide was rinsed with deionized water to wash off the excess Safranin and let to dry.
6. The specimen slide was then ready for microscopy analysis.
2.3 Result and discussion

2.3.1 Preliminary screening of mycolytic enzymes producing strain from local soil environment

The soil samples were collected from various locations within Sarawak. Mycolytic enzymes producing bacterial were targeted due to its ability to synthesize enzymes which has the capability to degrade cell wall and functional protein of the plant pathogenic fungus [14–16]. Previous studies have shown that, the mycolytic enzymes increased in amount at infected areas of plants, triggered by the defence response gene of the plants itself [10,70,71]. Mycolytic enzymes base formulations consisting of chitinases, proteases, and glucanases have been used in the control of plant pathogen fungal [12]. Chitinases were not targeted in these studies, as there were ample previous research on this enzymes but less on glucanases and proteases enzymes as biological control agents [12]. Therefore, the focused of this study was to screen and isolated the bacterial that has the ability to secrete protease and glucanase enzymes as potential biological control agents.

Skim milk agar (SMA) and Carboxymethylcellulose agar (CMC) were chosen to be used for primary screening of protease and glucanase producing strains from the soil samples collected. SMA plates were used to screen for protease producing bacteria. Skim milk agar was commonly used to demonstrate coagulation and proteolysis of casein [95]. The clear zones formation on SMA plates indicates the presence of protease enzymes as the protein content within the media, which was casein has been degraded. The isolated bacteria have hydrolyzed casein into soluble nitrogenous compounds thus resulting in the formation of the clear zone observed [95]. As for CMC agar plates, the hydrolysis activities of β-1,3-glucan caused the formation of clear zone on CMC plates after being stained and de-stained, which indicates the presence of glucanase enzymes [96].

The clear zone formation surrounding the microbial colonies acted as a strong evidence of positive results. The microbes with clear zone formation were inoculated out to new SMA and CMC agar plates for pure culture isolation purposes. The microbes of interests were distinguished by the observation of the clear zone formation around the colonies on the selective media as shown in Figure 8. A total of 46 potential isolates
were isolated out from the soil samples collected from the different locations. A total of 21 out of 46 were protease-producing microbes whereas the remaining 25 were glucanase-producing microbes.

Figure 8: Screening for potential proteases and glucanases producing microbes. The formation of clear zone by potential isolates on selective media SMA (A) and CMC (B) agar plates indicate positive mycolytic enzyme production.
2.3.2 Identification of the isolates

A total of 46 isolates were identified using molecular identification via DNA sequencing of the 16s rRNA sequence. The crude DNA for 46 isolates was successfully extracted by the use of DNA extractions kits and also the freeze and thaw approach. Universal primer sets, 8 F (5'AGAGTTTGATCMTGGCTCAG-3') and 519 R (GWATTACCGCGGCKGCTG) [90] was selected to be used which targeted the 16s rRNA sequences. PCR products size obtained for the isolates were approximately 500bp as shown in Figure 9. PCR products were then sent for Sanger sequencing and the sequence results obtained were compared to the sequences in the GenBank database using the BLAST algorithm. BLAST is a sequence similarity search program that used to match user’s query to database sequence which is one of the most broadly used bioinformatics research tools [92].

The partial 16s rRNA sequence comparison results for each isolate were tabulated in Table 3 and 4. The selection of closest match species towards isolates were based on the reference of query cover and identity percentage which not less than 70 % for both percentages. The closest species towards the isolates were selected and the accession number for the closest match species were indicated in Table 3 and 4. On the basis of the comparison results, the total isolates of 46 were identified to be members of the following genus: Bacillus, Delftia, Acinetobacter, Stenotrophomonas, Ralstonia, Burkholderia, Pseudomonas, Serratia, Staphylococcus, and Chryseobacterium. Out of 46 isolates, 16 different strains were found as listed in Table 5.

The phylogenetic tree for protease and glucanase enzymes producing isolates in Figure 10 and 11 showing the position of the isolates based on the results obtained from the partial 16s rRNA sequence comparison with Genbank. The phylogenetic tree was generated by the used of maximum likelihood method based on Tamura-Nei model [97]. Mega 6 software was used to analyse the sequence results and to generate the phylogenetic tree. The phylogenetic tree was constructed to organize the data of the isolates. It is a diagram that portrayed the lines of the evolutionary origin of different species, organisms or genes which shared a common ancestor [98].
Figure 9: The visualization of PCR products for protease enzyme producing isolates (A) S1 to S20 and glucanase isolates (B) C1 to C25 were observed on 1 % agarose gel by gel electrophoresis method. The gel image was viewed and captured by Bio–Rad Gel Documentation EQ system.
Table 3: The closest match obtained from Genbank database based on partial 16s rRNA sequence for glucanases enzymes producing isolates. The closest match was selected based on query cover percentage (%) in base pairs and identity percentage (%).

<table>
<thead>
<tr>
<th>Glucanase enzyme producing isolates</th>
<th>Glucanase Isolates</th>
<th>Closest match</th>
<th>Accession no. of closest match</th>
<th>Query cover (%)</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>KJ396861.1</td>
<td>96%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td><em>Bacillus coagulans</em></td>
<td>JN646037.1</td>
<td>97%</td>
<td>70%</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>KF530799.1</td>
<td>95%</td>
<td>71%</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td><em>Pseudomonas putida</em></td>
<td>HQ455003.1</td>
<td>89%</td>
<td>73%</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td><em>Bacillus coagulans</em></td>
<td>JN646037.1</td>
<td>94%</td>
<td>72%</td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td><em>Delftia tsuruhatensis</em></td>
<td>HM003216.1</td>
<td>96%</td>
<td>78%</td>
<td></td>
</tr>
<tr>
<td>C7</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>KT229742.1</td>
<td>92%</td>
<td>95%</td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td><em>Pseudomonas putida</em></td>
<td>KF367465.1</td>
<td>97%</td>
<td>90%</td>
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</tr>
<tr>
<td>C9</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>FJ976573.1</td>
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<tr>
<td>C10</td>
<td><em>Bacillus cereus</em></td>
<td>KF376341.1</td>
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<tr>
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<td><em>Bacillus anthracis</em></td>
<td>KU510075.1</td>
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<td>84%</td>
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<tr>
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<td>KF376341.1</td>
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<tr>
<td>C13</td>
<td><em>Ralstonia pickettii</em></td>
<td>FR873796.1</td>
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<td>92%</td>
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<tr>
<td>C14</td>
<td><em>Bacillus cereus</em></td>
<td>JN411396.1</td>
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<td>85%</td>
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<tr>
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<td><em>Bacillus anthracis</em></td>
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<tr>
<td>C16</td>
<td><em>Ralstonia pickettii</em></td>
<td>KP744132.1</td>
<td>100%</td>
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<tr>
<td>C17</td>
<td><em>Burkholderia metallica</em></td>
<td>LN890069.1</td>
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<td>99%</td>
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</tr>
<tr>
<td></td>
<td>Organism</td>
<td>Accession</td>
<td>Similarity</td>
<td>Percent</td>
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</tr>
<tr>
<td>---</td>
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<td>-------------</td>
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</tr>
<tr>
<td>C18</td>
<td><em>Burkholderia cepacia</em></td>
<td>AY268157.1</td>
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<td>73%</td>
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</tr>
<tr>
<td>C19</td>
<td><em>Burkholderia cepacia</em></td>
<td>HQ236034.1</td>
<td>92%</td>
<td>88%</td>
<td></td>
</tr>
<tr>
<td>C20</td>
<td><em>Bacillus cereus</em></td>
<td>JX901104.1</td>
<td>94%</td>
<td>90%</td>
<td></td>
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<tr>
<td>C21</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>KT228276.1</td>
<td>96%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>C22</td>
<td><em>Pseudomonas plecoglossicida</em></td>
<td>KC210863.1</td>
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<td></td>
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<tr>
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<td><em>Bacillus cereus</em></td>
<td>KP772338.1</td>
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<td>85%</td>
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<tr>
<td>C24</td>
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<tr>
<td>C25</td>
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<td>JX030412.1</td>
<td>86%</td>
<td>90%</td>
<td></td>
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</table>
Table 4: The closest match obtained from Genbank database based on partial 16s rRNA sequence for protease enzymes producing isolates. The closest match was selected based on query cover percentage (%) in base pairs and identity percentage (%).

<table>
<thead>
<tr>
<th>Protease enzyme producing isolates</th>
<th>Closest match</th>
<th>Accession no. of closest match</th>
<th>Query cover (%)</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td><em>Bacillus cereus</em></td>
<td>KJ586282.1</td>
<td>98%</td>
<td>98%</td>
</tr>
<tr>
<td>S2</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>KU563543.1</td>
<td>96%</td>
<td>99%</td>
</tr>
<tr>
<td>S3</td>
<td><em>Chryseobacterium indologenes</em></td>
<td>LN866620.1</td>
<td>98%</td>
<td>81%</td>
</tr>
<tr>
<td>S4</td>
<td><em>Chryseobacterium indologenes</em></td>
<td>LN681561.1</td>
<td>97%</td>
<td>99%</td>
</tr>
<tr>
<td>S5</td>
<td><em>Bacillus cereus</em></td>
<td>FJ217203.1</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>S6</td>
<td><em>Bacillus cereus</em></td>
<td>KT588298.1</td>
<td>91%</td>
<td>84%</td>
</tr>
<tr>
<td>S7</td>
<td><em>Burkholderia cepacia</em></td>
<td>GQ169807.1</td>
<td>87%</td>
<td>81%</td>
</tr>
<tr>
<td>S8</td>
<td><em>Staphylococcus sciuri</em></td>
<td>KC153123.1</td>
<td>76%</td>
<td>87%</td>
</tr>
<tr>
<td>S9</td>
<td><em>Burkholderia cepacia</em></td>
<td>HQ236034.1</td>
<td>96%</td>
<td>99%</td>
</tr>
<tr>
<td>S10</td>
<td><em>Staphylococcus sciuri</em></td>
<td>NR_041328.1</td>
<td>94%</td>
<td>99%</td>
</tr>
<tr>
<td>S11</td>
<td><em>Staphylococcus sciuri</em></td>
<td>LC090517.1</td>
<td>81%</td>
<td>96%</td>
</tr>
<tr>
<td>S12</td>
<td><em>Serratia marcescens</em></td>
<td>KJ721215.1</td>
<td>98%</td>
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<tr>
<td>S13</td>
<td><em>Burkholderia cepacia</em></td>
<td>GQ169807.1</td>
<td>95%</td>
<td>96%</td>
</tr>
<tr>
<td>S14</td>
<td><em>Serratia marcescens</em></td>
<td>KT992358.1</td>
<td>94%</td>
<td>98%</td>
</tr>
<tr>
<td>S15</td>
<td><em>Bacillus coagulans</em></td>
<td>JN646037.1</td>
<td>85%</td>
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<tr>
<td>S16</td>
<td><em>Bacillus flexus</em></td>
<td>JQ977706.1</td>
<td>86%</td>
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<td>S17</td>
<td><em>Pseudomonas mosselii</em></td>
<td>HQ455008.1</td>
<td>92%</td>
<td>94%</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>Accession Number</td>
<td>Similarity 1</td>
<td>Similarity 2</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------</td>
<td>------------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>S18</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>KM585587.1</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>S19</td>
<td><em>Bacillus cereus</em></td>
<td>EF633277.1</td>
<td>94%</td>
<td>98%</td>
</tr>
<tr>
<td>S20</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>JN700142.1</td>
<td>97%</td>
<td>99%</td>
</tr>
<tr>
<td>S21</td>
<td><em>Bacillus cereus</em></td>
<td>KJ524513.1</td>
<td>98%</td>
<td>99%</td>
</tr>
</tbody>
</table>
Table 5: The closest match of strains identity obtained after comparison with Genbank database.

<table>
<thead>
<tr>
<th>No</th>
<th>Strains identity obtained</th>
<th>Number of isolates</th>
<th>Strain species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Bacillus cereus</em></td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Bacillus coagulans</em></td>
<td>3</td>
<td><em>Bacillus</em> spp.</td>
</tr>
<tr>
<td>3</td>
<td><em>Bacillus flexus</em></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>Bacillus anthracis</em></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>Delftia tsuruhatensis</em></td>
<td>1</td>
<td><em>Delftia</em> spp.</td>
</tr>
<tr>
<td>6</td>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>6</td>
<td><em>Acinetobacter</em> spp.</td>
</tr>
<tr>
<td>7</td>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>2</td>
<td><em>Stenotrophomonas</em> spp.</td>
</tr>
<tr>
<td>8</td>
<td><em>Ralstonia pickettii</em></td>
<td>2</td>
<td><em>Ralstonia</em> spp.</td>
</tr>
<tr>
<td>9</td>
<td><em>Burkholderia metallica</em></td>
<td>1</td>
<td><em>Burkholderia</em> spp.</td>
</tr>
<tr>
<td>10</td>
<td><em>Burkholderia cepacia</em></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td><em>Pseudomonas plecglossicida</em></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td><em>Pseudomonas mosselii</em></td>
<td>1</td>
<td><em>Pseudomonas</em> spp.</td>
</tr>
<tr>
<td>13</td>
<td><em>Pseudomonas putida</em></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td><em>Serratia marcescens</em></td>
<td>2</td>
<td><em>Serratia</em> spp.</td>
</tr>
<tr>
<td>15</td>
<td><em>Staphylococcus sciuri</em></td>
<td>3</td>
<td><em>Staphylococcus</em> spp.</td>
</tr>
<tr>
<td>16</td>
<td><em>Chryseobacterium indologenes</em></td>
<td>2</td>
<td><em>Chryseobacterium</em> spp.</td>
</tr>
</tbody>
</table>
Figure 10: Phylogenetic tree shows the position of the glucanases enzyme producing isolates, based on the partial 16s rRNA sequence comparison. The phylogenetic tree was constructed based on Maximum Likelihood method.
Figure 11: Phylogenetic tree shows the position of the proteases enzyme producing isolates, based on the partial 16S rRNA sequence comparison. The phylogenetic tree was constructed based on Maximum Likelihood method.
2.3.3 Gram stain analysis on the isolated strains

The gram stain analysis was conducted in this study to differentiate the bacteria based on the two fundamental varieties of cells. Gram stain technique is an important light microscopy stain that is useful in the initial classification of bacteria isolates. This technique help to determine the overall structure of the bacterial cells (for example: cocci, rods, spirals and others) [94]. The gram stain results for 16 different isolated strains were tabulated and presented in Table 6.

Table 6: The gram stain analysis for the bacterial isolates strains found.

<table>
<thead>
<tr>
<th>No</th>
<th>Strains identity obtained</th>
<th>Gram stain</th>
<th>Cell shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacillus cereus</td>
<td>+ve, purple</td>
<td>Rod</td>
</tr>
<tr>
<td>2</td>
<td>Bacillus coagulans</td>
<td>+ve, purple</td>
<td>Rod</td>
</tr>
<tr>
<td>3</td>
<td>Bacillus flexus</td>
<td>+ve, purple</td>
<td>Rod</td>
</tr>
<tr>
<td>4</td>
<td>Bacillus anthracis</td>
<td>+ve, purple</td>
<td>Rod</td>
</tr>
<tr>
<td>5</td>
<td>Delfia tsuruhatensis</td>
<td>-ve, pink</td>
<td>Rod</td>
</tr>
<tr>
<td>6</td>
<td>Acinetobacter calcoaceticus</td>
<td>-ve, pink</td>
<td>Coccobacilli</td>
</tr>
<tr>
<td>7</td>
<td>Stenotrophomonas maltophilia</td>
<td>-ve, pink</td>
<td>Rod</td>
</tr>
<tr>
<td>8</td>
<td>Ralstonia pickettii</td>
<td>-ve, pink</td>
<td>Rod</td>
</tr>
<tr>
<td>9</td>
<td>Burkholderia metallica</td>
<td>-ve, pink</td>
<td>Rod</td>
</tr>
<tr>
<td>10</td>
<td>Burkholderia cepacia</td>
<td>-ve, pink</td>
<td>Rod</td>
</tr>
<tr>
<td>11</td>
<td>Pseudomonas plecoglossicida</td>
<td>-ve, pink</td>
<td>Rod</td>
</tr>
<tr>
<td>12</td>
<td>Pseudomonas mosselii</td>
<td>-ve, pink</td>
<td>Rod</td>
</tr>
<tr>
<td>13</td>
<td>Pseudomonas putida</td>
<td>-ve, pink</td>
<td>Rod</td>
</tr>
<tr>
<td>14</td>
<td>Serratia marcescens</td>
<td>-ve, pink</td>
<td>Rod</td>
</tr>
<tr>
<td>15</td>
<td>Staphylococcus sciuri</td>
<td>+ve, purple</td>
<td>Cocci</td>
</tr>
<tr>
<td>16</td>
<td>Chryseobacterium indologenes</td>
<td>-ve, pink</td>
<td>Rod</td>
</tr>
</tbody>
</table>

**Note:** Gram-positive (+ve) Gram-negative(-ve)

Out of total of 16 different isolates strains, only five were Gram-positive bacterial strains, majority was Gram-negative bacteria. The ‘Gram-positive’ bacterial retain the crystal violet stain (purple) whereas ‘Gram-negative’ bacterial retain the safranin stain (pink). The staining analysis was based on the chemical and structural design of the cell.
walls of both ‘Gram-positive’ and ‘Gram-negative’ bacterial [94]. Gram-positive bacterial consist of thick and impermeable cell wall composed of peptidoglycan and the secondary polymers, the thick cell wall resists the decolourization by acetone in the gram-stain analysis. However, Gram-negative bacterial only have a thin and permeable cell membrane composed thin peptidoglycan layer with an overlying lipid-protein bilayer. Gram-negative bacterial have no cell wall to resist the entrance of acetone into the cell therefore it only retain the safranin stain at the end of the analysis [94].

2.4 Summary

The study of chapter 2 focused on the screening and characterization of the mycolytic enzymes producing microbes. The proteases and glucanases enzymes producing microbes were successfully isolated out from local soils samples collected in Sarawak. Out of 46 isolates, 21 isolates were proteases enzyme producing microbes while another 25 isolates were glucanases enzyme producing microbes. On the basis of the partial 16s rRNA sequence comparison results, total of 46 isolates were classified to be members of the genus of Bacillus, Delftia, Acinetobacter, Stenotrophomonas, Ralstonia, Burkholderia, Pseudomonas, Serratia, Staphylococcus, and Chryseobacterium. Out of 46 isolates, 16 different strains of were found. The 16 different strains found were Bacillus cereus, Bacillus coagulans, Bacillus flexus, Bacillus anthracis, Delftia tsuruhatensis, Acinetobacter calcoaceticus, Stenotrophomonas maltophilia, Ralstonia pickettii, Burkholderia metallica, Burkholderia cepacia, Pseudomonas plecoglossicida, Pseudomonas mosselii, Pseudomonas putida, Serratia marcescens, Staphylococcus sciuri, and Chryseobacterium indologenes. Majority of the isolated strains were Gram-negative bacterial, 5 out of 16 strains were Gram-positive bacterial. Further evaluations for the 16 different strains mentioned were carried out and described in the following chapter. The efficiency of the 16 different strains as a potential biological control agent was evaluated in chapter 3.
CHAPTER 3: EVALUATING THE EFFICIENCY OF ISOLATED MYCOLOYTIC BACTERIAL STRAINS AS BIOCONTROL AGENTS AGAINST GANODERMA BONINENSE
3.1 Introduction

The white rot fungus, *Ganoderma boninense* is the greatest threat in oil palm (*Elaeis guineensis*) cultivation in South East Asia region. The phytopathogenic fungus, *G. boninense* will cause stem rot disease in oil palm trees. Basal stem rot (BSR) is the most common symptoms of this disease, where infect the root system of oil palm trees and decay gradually. The disease symptoms only can be visualised when the decay process finally reach the lower stem of the oil palm trees [99]. Besides the decay of the stem of oil palm trees, the disease symptoms also included the water stress, multiple unopened spears, flattening of the crown, one side mottling of the canopy and the formation of basidiocarps on the lower stem of the plants [70,99].

Previously, the stem rot disease only attacked weak and old oil palm trees. Nevertheless, recently the stem rot disease has been found to infect the young oil palms trees as young as 12 to 24 months after planting [8,35,100]. The 4 to 5 years old oil palm trees showed serious infection by the stem rot disease especially at the replanted areas [35,100]. The traditional techniques in stem rot disease management of oil palm include the mechanical technique (the trench system), cultural technique (sanitation in replanting) and also the chemical method (chemical fungicide) [8,10].

However, none of the control techniques reach satisfactory rate in disease management of stem rot disease in oil palm cultivation [8,101]. The adverse effects raised by the use of chemical fungicides include pollution towards the environment, health hazards towards humans and others non-targeted organisms, and it also increases the resistance of pathogenic fungi to fungicides [100,101]. The alternate approach suggested to solve the *Ganoderma* problem in oil palm cultivation recently is with the use of biological control agents based on the observation of the low disease incidences of the stem rot disease in some natural stand [8,100,101].

Biological control is environmentally friendly, safe and may give long-term protection to the plants crops. Antagonistic microbes are the most common biological control agents used in plantation disease management recently. Antagonistic microbes will produce secondary metabolites such as antibiotic, mycolytic enzymes and volatile compounds with antifungal properties. Antagonistic microbes such as fluorescent *Pseudomonas* spp., *Bacillus* spp., *Trichoderma* spp. and others more act as biological
control agents which categorize as antibiosis types. A number of various molecules secreted by the antagonistic microbes has been studied and their role in suppression of different plant pathogens has been recorded [14,72–74].

Chapter 3 focused on the effectiveness of the 16 different bacteria isolated strains act as biological control agent against the plant pathogenic fungus, *G. boninense* that causes basal stem rot disease (BSR) in oil palm cultivation industry in Malaysia. Different assays were conducted to analyse the efficiency of the isolates strain as biological control agents. The results obtained from the studies were presented and discussed in this chapter.
3.2 Materials and methods

3.2.1 Preparation of strains

(I) Phytopathogenic fungi, *Ganoderma boninense*

*Ganoderma boninense* was grown on Potato Dextrose Agar (PDA, Himedia, Mumbai, India) from slant agar stock culture. The culture PDA plates were then incubated in an incubator (Binder GmbH, Tuttlingen, Germany) at 25°C for 5 days prior to the start of the experiments. The plates were observed to confirm the growth of *G. boninense*.

(I) Mycolytic enzymes producing bacterial

The 16 different strains were cultured on Nutrient agar (NA, Himedia, Mumbai, India) from glycerol stock prior the start of the experiments. The culture NA plates were incubated in incubator room at 35°C ± 2°C for one day. The plates were observed and kept at 4°C in a LG refrigerator.

3.2.2 Evaluating the effectiveness of mycolytic enzymes producing bacterial against phytopathogenic fungi, *Ganoderma boninense*

The Dual culture test was adapted to be used for the evaluation of the effectiveness of mycolytic enzymes producing bacterial against plant pytopathogenic fungi, *Ganoderma boninense* [19]. Nutrient agar (NA) media was selected to be used in this experiment. Prior the test, *G. boninense* was grown on the centre of PDA media for 5 days and incubated at 25°C in the incubator. NA plate was divided into half and labelled. The 16 different isolated strains were inoculated onto NA plate by a single streak in the middle on the half side of the NA plates. An agar plug from a 5-day old *G. boninense* culture plate was placed in the middle of another half side of the NA plate. As for control plates, an agar plug was transferred from 5 days old *G. boninense* culture plate and placed in the middle of NA plate without the presence of isolated strains. The inoculated plates were then incubated in the incubation room at 35°C ± 2°C for 7 days. The plates were observed and measured on the 3rd and 7th day. Three replicates were made for each test strain plates and also the control plates.
The microbial inhibitions activities were observed by measuring the diameter of the inhibition zones. After 7 days, the diameter growth of *G. boninense* was measured and the percentage inhibition of diameter growth (PIDG) was calculated according to the equation [102]:

\[
\text{Percentage Inhibition of Diameter Growth} = \frac{C-T}{C} \times 100
\]

Where;

C – Diameter growth of *Ganoderma boninense* in the control plate

T – Diameter growth of *Ganoderma boninense* in the test plate

3.2.3 Growth rate and total plate count of bacterial strains

(I) Indirect growth measurements – Optical density by the use of the Spectrophotometer

Overnight broth culture for the 5 selected isolated strains was prepared. The overnight culture broth was prepared by inoculating the isolates into sterilized 10mL nutrient broth and incubated in the incubator shaker at 35ºC with 160rpm speed. Spectrophotometer (Thermo Scientific Genesys™ 20, Waltham, MA USA) was used to measure the optical density of the bacterial culture. The wavelength used was 600nm and empty nutrient broth was used as a blank solution prior to the measurement. The overnight culture was added into a shake flask with 90mL sterilized nutrient broth and mixed well. The optical density (OD) of the initial reading, time zero, was taken immediately after the overnight culture was added. The OD reading was taken every 1-hour interval thereafter, until the OD readings reach approximately 1.000 OD. The final OD reading taken was at time 24 hours. One millilitre of the broth culture was pipetted out and discarded after the reading was taken for each measurement. Triplicates were made for this test.
(II) Direct growth measurements – Serial dilutions and total plate counts

Colony-forming unit of the 5 selected strains were obtained by direct growth measurements method and total plate counts. Ten-fold serial dilution was carried out in sterilized deionized water for the broth culture pipetted out from the shake flask at the exponential growth phase of culture strains. A total volume of 9 mL sterile deionized water was pipetted into 6, 15mL falcon tubes. One millilitre of the broth culture from shake flask was pipetted into the first falcon tubes and labelled as tube 1. The tube was inverted for few times for complete mixed of the solution. One millilitre of the mixture solution from tube 1 was transferred to tube 2 by using 1000µL micropipettes and mixed well. This step was repeated until tube 6, giving a $10^{-6}$ dilution for low OD reading and tube 7, giving a $10^{-7}$ dilution for high OD reading. The dilution factor $10^{-5}$, $10^{-6}$, and $10^{-7}$ was spread on nutrient agar and incubate in incubator room at 35ºC ± 2ºC for one day. The distinct colonies on each plate were counted on the second day. The colony forming unit per millilitre (CFU/mL) was calculated according to the equation as follow:

\[
\text{CFU/mL} = \frac{\text{number of colonies per mL plated}}{\text{Total dilution factor}}
\]

3.2.4 Antimicrobial and antifungal activities of selected strains

The 5 selected isolated strains that gave high PIDG results in dual culture test are listed as follow:

1. *Pseudomonas putida*, C8
2. *Bacillus cereus*, C12
3. *Acinetobacter calcoaceticus*, S2
4. *Chryseobacterium indologenes*, S4
5. *Serratia marcescens*, S12
(I) **Antimicrobial activities**

Preliminary screening of antimicrobial activities was modified from a cross streak method described in a previous study [103]. The standard bacteria test strains used in the screening were *Escherichia coli* NBRC 3301, *Pseudomonas aeruginosa* NBRC 12689, *Staphylococcus aureus* NBRC 12732 and *Bacillus subtilis* NBRC 3134. A yeast strain was *Saccharomyces cerevisiae* ATCC 9763.

Nutrient agar (NA) media was used in cross streak method. The selected strains of isolates were streaked at the side of the plate and the standard bacteria test strains were streaked perpendicularly towards the selected isolated strains. NA plates without the presence of the selected strains served as the control in this test. Triplicates were made for the test and the inoculated plates were incubated in the incubation room at 35°C ± 2°C for one day. The inhibition of selected strains against test strains were observed and recorded after 24 hours of incubation.

**Note:**

EC - *Escherichia coli* NBRC 3301  
PA - *Pseudomonas aeruginosa* NBRC 12689  
SA - *Staphylococcus aureus* NBRC 12732  
BS - *Bacillus subtilis* NBRC 3134  
SC - *Saccharomyces cerevisiae* ATCC 9763

(II) **Antifungal activities**

The fungi test strains selected to be used in the screening were *Aspergillus niger*, *Rhizoctonia solani*, and *Ganoderma boninense*. The growth rates of plant pathogenic fungi on the potato dextrose agar (PDA) were measured prior to the preparation of inoculum for the test. *R. solani* and *A. niger* were grown on PDA for one day and 5 days for *G. boninense* prior the start of the experiment. The culture plates were then incubated at 25°C condition.
Nutrient agar (NA) media was selected to be used in this test. NA plate was separated into half and labelled. The selected isolated strains were inoculated onto NA plate by single streaked in the middle on half side of the NA plates. The agar plug of fungi test strains were placed in the middle of another half side of the NA plate. The control plate was prepared by placing an agar plug of the fungi test strains in the middle of NA plate, without the presence of the selected isolated strains. The inoculated plates were then incubated in the incubation room at 35°C ± 2°C for 7 days. The plates were observed and measured on the 3rd and 7th day. Three replicates were made for each testing strain plates and also the control plates.

The microbial inhibitions activities were observed by the diameter of the inhibition zones. After 7 days, the diameter growth of test fungi was measured and the percentage inhibition of diameter growth (PIDG) was calculated.

** Note:

AN - *Aspergillus niger*

RS - *Rhizoctonia solani*

GB - *Ganoderma boninense*

### 3.2.5 Evaluation on the ability of the selected isolated strains to produce volatile antimicrobial compounds

Double plate assay was carried out to evaluate the ability of the selected isolated strains to produce volatile compounds which possessed anti-*Ganoderma* activities besides through the secretion of secondary metabolites [87]. Nutrient agar (NA) media was selected to be used in this assay. Prior the test, *Ganoderma boninense* was grown on potato dextrose agar (PDA) for 5 days and incubated at 25°C.

The selected isolated strain was streaked on the labelled NA plate and in a new separated NA plate, 5 days old *G. boninense* culture agar plug was placed in the middle of the plate. The lids of both plates were removed and the plate with the selected isolates strains was inverted over the plate with *G. boninense*. The plates were sealed and incubated for 7 days in the incubation room at 35°C ± 2°C. The inoculated *G.
*boninense* plate was sealed together with an empty NA plate which served as control plate in this assay. Triplicate was made for this assay. This assay was conducted for all 5 selected isolated strains. After 7 days, the diameter growth of *G. boninense* was measured and the percentage inhibition of diameter growth was calculated.

### 3.2.6 Culture filtrate test

The culture filtrate test used in this study was adapted from Elmahdi et al. 2015. The 5 selected isolated strains that gave high PIDG results in dual culture test were inoculated in 250mL nutrient broth and incubated in the incubator shaker at 35°C ± 2°C for 72 hours or 3 days. The culture broth was centrifuged at 10000 rpm for 10 min, after 3 days. After the centrifuging process, the supernatant was collected and the pellets were discarded. The collected supernatant was then filtered through a sterilized 0.22µm membrane filter (Sartorius Stedim Biotech, Goettingen, Germany). The filtrates were then added into sterilized PDA in 20% (v/v) concentration. The amended agar was mixed well before being dispensed on 90mm petri dish plates (Favorit, Malaysia). Approximately 20mL of the filtrates PDA agars were dispensed onto petri dish plates. The amended agars were allowed to cool down and solidify before being used. Prior to the test, *Ganoderma boninense* was grown on potato dextrose agar (PDA) for 5 days and incubated at 25°C in the incubator. The mycelial plug of *G. boninense* was transferred from a 5-day old *G. boninense* culture plates and inoculated onto the center of each plate. Control plates were prepared with the non-amended PDA. The inoculated plates were then incubated at 25°C for 5 days. The plates were observed and measured on the 3rd and 5th days. Triplicates were made for each testing strain plates and also control plates. The antagonistic activity was quantified by measuring the diameter growth of *G. boninense* after 5 days and the percentage inhibition of diameter growth was calculated.
3.2.7 Chitinases, proteases and glucanases activity for selected bacterial strains

The capability for the selected bacterial strains to produce chitinases, proteases and glucanases enzymes were tested by using selective media methods. The method involved in chitinases assay was described in section 3.2.7.1. As for proteases and glucanases assay, the methods used were same as described in section 2.2.3.2 (a) and (b).

3.2.7.1 Chitinases assay for selected bacterial strains

Five selected bacterial strains were checked for the ability to produce chitinases enzyme as well by the used of chitin agar. The bacterial strains were inoculated onto middle of chitin agar. The chitin media plates were sealed incubated in the incubator room at 35°C ± 2°C for 3 days. After 3 days incubation period, the plates were observed for the formation of clear zone on chitin agar plates. The recipe of chitin agar [104] used was listed in Appendix.

3.2.8 Data analysis

Experiments results were analyzed using analysis of t-Test: Two-sample assuming unequal variances, with statistical significance taken as p ≤ 0.05. Data analysis programme in Statistical Package for the Social Sciences (SPSS) software was used for the data analysis in this experiment.
3.3 Result and discussion

Phytopathogenic fungi, *Ganoderma boninense* was selected to be tested in this study, as it is the major species that caused basal stem rot disease (BSR) of oil palm in plantation. The BSR disease causes economic losses in oil palm industry in Malaysia as the country is the major producer and exporter for palm oil industry worldwide [28]. As described in the previous study, none of the traditional control technique showed significant results in stem rot disease management in oil palm cultivation [8,101]. Biological control of the used of antagonistic microbes were commonly used in plant disease management recently and has attracted the interest of researcher to discover more new potential strains as biological control candidates.

Antagonistic activity of the 16 different isolates were evaluated and discussed in this study. The negative impacts brought by chemical fungicides towards the environment and human health issues contributed to the development of biological sources such as antagonistic microorganisms as biological control agents [105]. Antagonistic microbes play an important role in microbial equilibrium and also act as efficient biological control agents in plant diseases management [14–16]. The secondary metabolites synthesized by antagonistic microbes included antibiotic, mycolytic enzymes and also volatile compounds. The secondary metabolite synthesized possessed antibacterial or antifungal properties [14–16].

The growth rate for the fungi strain used was studied prior to the test, as this affects the determination of the preparation time needed to grow the phytopathogenic fungi for further used in the experiment before the start of the assays. *G. boninense* required 7 days to cover the whole PDA agar plate at 25°C as refer to the previous study [87]. In this study, the shorter incubation time was chosen to harvest fungi strain to prevent the strain mycelia touched and reached the edge of the petri plates. The contamination of culture plates by other filamentous fungi was typically detected started from the edge of the petri plate [87]. Therefore, as a prevention of culture contamination, *G. boninense* was harvested at Day 5 for further used in the study.
3.3.1 Evaluate the effectiveness of mycolytic enzymes producing bacterial against phytopathogenic fungi, *Ganoderma boninense*

Dual culture plate assay was adapted to be used in this study. Dual culture plate assay is an established technique used to differentiate isolated strains with antagonistic potential from large sample size [19]. Nutrient agar was selected to be used in this study for the optimum growth of 16 different strains obtained. The growth diameter (cm) of phytopathogenic fungi, *Ganoderma boninense* in both control and test plate was measured and recorded after 7 days. The comparison of the growth of phytopathogenic fungi, *G. boninense* in control and test plate is presented in Figure 12, which shows the growth of the phytopathogenic fungi, *G. boninense* at day 7 of the anti- *Ganoderma* experiment.

The percentage inhibition of diameter growth (PIDG) was calculated based on the equation mentioned in section 3.2.2. The PIDG of 16 different isolated strains was calculated according to the growth diameter (cm) results at day 7 of the anti- *Ganoderma* experiment. The results of PIDG for 16 different isolated strains are presented in Figure 13.

![Figure 12: Dual culture plate assay to evaluate the efficiency of the isolated strains inhibiting the growth of the phytopathogenic fungi, *Ganoderma boninense*. The comparison of the mycelial growth of phytopathogenic fungi, *Ganoderma boninense* in control plate (A) and test plate with the presence of *Bacillus cereus*, C12 (B) at day 7.](image)
Figure 13: Percentage inhibition of diameter growth (PIDG) results on the 7th day of the anti-\textit{Ganoderma} experiment. The 16 different isolated strains tested against phytopathogenic fungi, \textit{Ganoderma boninense}.

The results of the anti-\textit{Ganoderma} test after 7 days by the used of dual culture assay is shown in Figure 12. The comparison of the growth of phytopathogenic fungi, \textit{Ganoderma boninense} in control and test plates showed that with the presence of the isolated strains the growth of the phytopathogenic fungi, \textit{G. boninense} was inhibited. The percentage inhibition of diameter growth (PIDG) results was calculated and presented in Figure 13. The results showed that \textit{Pseudomonas putida}, \textit{Bacillus cereus}, \textit{Serratia marcescens}, \textit{Chryseobacterium indologenes}, \textit{Acinetobacter calcoaceticus}, and \textit{Bacillus flexus} had higher PIDG rate compared to the other isolated strains. The PIDG for \textit{P. putida} showed highest PIDG among all isolated strains which was 86.3 %. Followed by \textit{B. cereus} and \textit{S. marcescens} the PIDG for both strains were 85.8% and
84.7%. *C. indologenes, A. calcoaceticus*, and *B. flexus* isolates showed similar PIDG results in the anti-*Ganoderma* test which were 83.7%, 82.8%, and 82.0%.

The isolated strains *Bacillus coagulans, Bacillus anthracis, Stenotrophomonas maltophilia*, and *Ralstonia pickettii* showed lower PIDG in the anti-*Ganoderma* study. The PIDG for this 4 isolated strains were 26.9%, 36.3%, 35.7% and 60.5% respectively. According to the percentage inhibition of diameter growth results, only 5 best antagonistic mycolytic enzymes producing isolates out of 16 different isolated strains were selected for further evaluation in this study. Among 6 isolates strains that showed high PIDG results, *B. flexus* isolates strain was eliminated due to the unstable growth in the previous experiment. *C. indologenes, A. calcoaceticus, S. marcescens, P. putida*, and also *B. cereus* were selected to be further evaluated in this study.

### 3.3.2 Growth rate and total plate count

Evaluation of the growth kinetics for the 5 selected isolated strains *Chryseobacterium indologenes, Acinetobacter calcoaceticus, Serratia marcescens, Pseudomonas putida*, and also *Bacillus cereus* were conducted based on indirect growth measurement technique by the used of spectrophotometer and direct growth measurements technique, the viable counts or known as total plate count method. The total populations of the isolated strains were determined by measured the turbidity of the culture at 600 nm wavelength in Nutrient broth at 35 °C, 160 rpm. The 5 selected isolated strains growth kinetics characteristic were summarized in Table 7.
The growth kinetics characteristics for the 5 selected isolated strains *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and also *Bacillus cereus* were evaluated based on spectrophotometer assay and total plate count techniques. The specific growth rate (k) for the isolated strains were calculated based on absorbance results obtained in spectrophotometer assay and were presented in Table 7. *P. putida* showed the highest value in specific growth rate which was 0.337 hr⁻¹. *S. marcescens* showed the lowest value in specific growth rate among 5 selected isolated strains which was 0.129 hr⁻¹.

The doubling time for each isolated strains was studied as well in this experiment. The doubling time for each isolated strains were calculated based on the specific growth rate obtained. The double time is proportional to the specific growth rate of the bacteria. The highest the specific growth rate of the isolated strains, the shortest doubling time needed for the isolates strains. *P. putida* has the highest specific growth rate among 5 selected isolated strains, time taken for this isolate to double up the population was 2.06 hour.
The doubling time required for \textit{S. marcescens} was 5.36 hour, as this isolate has lowest specific growth rate among 5 selected isolates strains.

The absorbance value of 5 selected isolated strains at 24 hours were taken and recorded in Table 7. The total plate counts were conducted in the log or exponential phase of the isolated strains. The colony forming units per millilitres (cfu/mL) at 24 hours were shown in Table 7. The colony forming units per millilitres for the 5 selected isolated reached $10^7$ cfu per mL at 24 hours. The growth kinetics results served as the reference for the experiment designed in the pot trial section which was further discussed in chapter 4.

3.3.3 Antimicrobial and antifungal activities of selected strains

Antimicrobial and antifungal experiment has been carried out for the selected strains listed as follow:

1. \textit{Chryseobacterium indologenes}, S4
2. \textit{Acinetobacter calcoaceticus} S2
3. \textit{Serratia marcescens}, S12
4. \textit{Pseudomonas putida}, C8
5. \textit{Bacillus cereus}, C12

The isolated strains were selected based on the PIDG results in section 3.3.1 and the growth of the strains.

(I) Antimicrobial activities of the selected strains against standard bacterial test strains

Varieties of laboratory techniques can be used for evaluating or detecting \textit{in vitro} antimicrobial activities. Disk diffusion and broth or agar dilution techniques were the most common and basic methods used for the screening of antimicrobial activity [106]. Cross-streaking technique was easy and comparatively rapid methods to be used to screen for antimicrobial activity of the pure isolated strains against standard test strains [107]. Cross-streaking method was selected to be used in this study.
The selected isolated strains from section 3.3.1 *Chryseobacterium indologenes, Acinetobacter calcoaceticus, Serratia marcescens, Pseudomonas putida*, and also *Bacillus cereus* were tested for antimicrobial activity by their ability to inhibit the growth of the standard bacterial test strains. Two gram-negative and two gram-positive standard bacterial strains and a yeast strain were selected to be used in this study. The standard bacterial strains for gram-negative bacteria were *Escherichia coli* NBRC 3301 and *Pseudomonas aeruginosa* NBRC 12689. The standard bacterial strains for gram-positive bacteria were *Staphylococcus aureus* NBRC 12732 and *Bacillus subtilis* NBRC 3134. The yeast strain selected to be used in this study was *Saccharomyces cerevisiae* ATCC 9763.

The antimicrobial activity of 5 selected isolated strains *C. indologenes, A. calcoaceticus, S. marcescens, P. putida*, and also *B. cereus* against the standard bacterial strains *E. coli* NBRC 3301, *P. aeruginosa* NBRC 12689, *S. aureus* NBRC 12732 and *B. subtilis* NBRC 3134 and the yeast strains *S. cerevisiae* ATCC 9763 were investigated in this study. The inhibition activities among each isolates were tested as well. No inhibition activities were observed among each isolates. The results of the antimicrobial test among each isolates were shown in Figure 14 (A). The results obtained for this test served as a guideline for the experimental designed in pot trial section which was further discussed in chapter 4.
Figure 14: Antimicrobial activities evaluation for the 5 selected isolated strains. The selected 5 isolated strains (A), Chryseobacterium indologenes, S4, Acinetobacter calcoaceticus, S2, Serratia marcescens, S12, Pseudomonas putida, C8, and also Bacillus cereus, C12 were streak on the same nutrient agar plate. The standard bacterial strains Escherichia coli (EC), Pseudomonas aeruginosa (PA), Staphylococcus aureus (SA) and Bacillus subtilis (BS) and also the yeast strains Saccharomyces cerevisiae (SC) were streak perpendicular to the 5 selected isolated strains in nutrient agar plates separately (B, C, D, E and F).
As described in the previous study, to improve the efficiency of biological control agents, the use of multiple biological control agents has attracted the interest of the researcher recently instead of using single biological agents [108]. The use of consortia in most cases has been reported in previous studies to be unsuccessful due to the selected strains often being antagonistic towards each other [108,109]. As shown in Figure 14, no antagonistic activities were observed among 5 selected strains in this study. Therefore, the 5 selected strains were potential to be used as biological control consortia in following experiment in this study.

Five selected mycolytic enzyme producing isolated strains *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and also *Bacillus cereus* isolates all showed no antibacterial activity against the standard testing strains. The results obtained served as an indication that the selected isolated strains had no antibacterial properties. Therefore, the further application of the selected isolated strain as biological control agents on the field would not affect the balance of the ecosystem in the applied area. The traditional practice used to overcome plant diseases incidents were by the use of chemical fungicide which brings adverse environmental effects that caused health hazards to humans and other non-targeted organisms, included beneficial life-form as well [12]. The results of the antibacterial test of 5 isolated strains against the standard bacterial strains were shown in Figure 14 (B, C, D, E, and F).
Antifungal activities of the selected strains against standard fungi test strains

Effective bacteria isolates tested and discussed in section 3.3.1 that showed higher PIDG were further re-evaluated by dual culture assay towards *Rhizoctonia solani, Aspergillus niger*, and *Ganoderma boninense*. The selected isolated strains from section 3.3.1 *Chryseobacterium indologenes, Acinetobacter calcoaceticus, Serratia marcescens, Pseudomonas putida*, and *Bacillus cereus*. The antagonistic activity of the selected isolated strains towards 3 different fungi strains on nutrient agar plates at 25°C were evaluated and discussed in this study.

Determination of the growth rate for the fungi strain used was important as this affects the time to harvest the fungi strains for further used in the experiment. A shorter incubation time was chosen to harvest the fungi strain in order to prevent the hyphae of fungi strains for reaching the edge of the plates. Previous study showed that the detection of contamination by other filamentous fungi strains has typically occurred at the edge of the culture plates [87]. Plant pathogenic fungi *R. solani* and *A. niger* would cover the whole PDA plates within 3 days at 25 ºC. Meanwhile, *G. boninense* required 7 days to cover whole PDA plates at 25 ºC. Therefore, as a prevention of culture contamination, *R. solani* and *A. niger* was harvested at Day 2, whereas *G. boninense* was harvested at Day 5 for further use in the study.

The percentage inhibition of diameter growth (PIDG) was calculated based on the equation mentioned in section 3.2.2. The antagonistic activity of the 5 selected isolated strains towards 3 different fungi strains in PIDG were calculated according to the growth diameter (cm) results at day 7 of the antifungal experiment. The results of PIDG for the 5 selected isolated strains were presented in Figure 18.
Figure 15: Evaluation of the efficiency for 5 selected isolated strains obtained against phytopathogenic fungi, *Rhizoctonia solani* by dual culture plate assay on Nutrient agar (NA) at 25°C. The comparison of the mycelial growth of phytopathogenic fungi, *Rhizoctonia solani* in control plate (A) and test plate (B) at day 7.

Figure 16: Evaluation of the efficiency for 5 selected isolated strains obtained against phytopathogenic fungi, *Aspergillus niger* by dual culture plate assay on Nutrient agar (NA) at 25°C. The comparison of the mycelial growth of phytopathogenic fungi, *Aspergillus niger* in control plate (A) and test plate (B) at day 7.
Figure 17: Evaluation of the efficiency for 5 selected isolated strains obtained against phytopathogenic fungi, *Ganoderma boninense* by dual culture plate assay on Nutrient agar (NA) at 25°C. The comparison results of the mycelial growth of phytopathogenic fungi, *Ganoderma boninense* in control plate (A) and test plate (B) at day 7.
Figure 18: Percentage inhibition of diameter growth (PIDG) results on the 7th day of the antifungal experiment. The 5 selected isolated strains, Chryseobacterium indologenes, Acinetobacter calcoaceticus, Serratia marcescens, Pseudomonas putida, and also Bacillus cereus against phytopathogenic fungi, Rhizoctonia solani, Aspergillus niger, and also Ganoderma boninense.

The phytopathogenic fungi tested in this study were Rhizoctonia solani, Aspergillus niger, and also Ganoderma boninense. R. solani is one of the common soil-borne plant pathogens that caused great loss in the agricultural industry. R. solani infected a wide range of agricultural crops such as cauliflower, corn, tomato and a lot more [62]. A. niger plant pathogenic fungus causes black mould in onion, garlic and shallot; stem rot to Dracaena, boll rot of cotton and others agricultural crops [110]. The basal stem rot disease causes great economic losses in oil palm industry in various regions around the world which included South-East Asia countries as well [27]. Basal stem rot disease in Malaysian oil palm cultivation is mainly caused by Ganoderma species. G. boninense has been identified as the major plant pathogen fungi in oil palm cultivation in Malaysia [111–113]. The plants diseases caused by phytopathogenic fungi caused great economic losses in the agricultural industry worldwide. Therefore, the management of plant
diseases caused by phytopathogenic fungi was the main focus of agricultural research worldwide.

Five bacterial isolates *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and *Bacillus cereus* with high antagonistic activity to *G. boninense* were selected to evaluate the antagonistic activity towards phytopathogenic fungi *R. solani*, *A. niger*, and *G. boninense* in this study. The results of antifungal test after 7 days by the used of dual culture assay was shown in Figure 15, 16, and 17. The comparison example of the growth of phytopathogenic fungi in control and test plates showed that with the presence of the isolated strains, inhibition of growth of the tested fungi strains were observed.

The percentage inhibition of diameter growth (PIDG) results was calculated and presented in Figure 18. The results showed that *C. indologenes*, *A. calcoaceticus*, *S. marcescens*, *P. putida*, and *B. cereus* showed antagonistic activity towards tested fungi strains. The 5 selected isolated strains showed highest PIDG towards *A. niger*. *S. marcescens* showed highest in PIDG in against *A. niger* with 87.7%. *C. indologenes* and *B. cereus* showed with similar PIDG results with 87.0% and 85.5%. The PIDG of *P. putida* and *A. calcoaceticus* towards *A. niger* were slightly lower than others isolate with 85.4% and 84.6%.

*A. calcoaceticus* showed highest in PIDG in against *R. solani* with 89.2% among 5 selected isolated strains. The PIDG of *S. marcescens* and *P. putida* towards *R. solani* were slightly lower than *A. calcoaceticus* with 83.5% and 82.3%. *C. indologenes* and *B. cereus* showed lower PIDG against *R. solani* among the 5 selected isolates with 79.4% and 74.9%. The antagonistic activity towards *G. boninense* in this study showed a dramatically dropped as compared to the PIDG of 5 selected isolated strains at 35°C. The highest PIDG achieved by *P. putida* was 60.0 % only. *B. cereus*, *S. marcescens* and *C. indologenes* showed similar PIDG results against *G. boninense* with 53.7%, 53.5% and 51.3%. *A. calcoaceticus* showed lowest PIDG results towards *G. boninense* with 49.5%.

The results showed that among the 5 selected isolated strains, *S. marcescens* showed highest antagonistic activity towards both *A. niger* and *R. solani*. *P. putida* showed highest antagonistic activity towards *G. boninense*. The difference antagonistic
activities rate of the selected strains towards standard fungi test strains might due to different secondary metabolites secreted of the individual strains. Besides that, different fungus species had different fungal cell wall composition which enables fungus to perform a wide range of essential roles in the interaction of fungus with surrounding environment [114]. The different cell wall composition of different fungus species might be one of the factors that influence the antagonistic activity of the selected bacterial strains. As described in previous study, a biological control strain has the ability to produce several types of secondary metabolites that served different functions and effective against different species types of plant pathogenic fungi [14]. The dramatic drop in antagonistic activity against G. boninense was observed in this study when compared to the PIDG results obtained in section 3.3.1 at 35°C. This drop of value in the PIDG at 25°C incubation may be attributed to the lytic activities of mycolytic enzymes produced by the bacteria isolates not working at their optimal temperature. As reported in a previous study, the chitinases enzymes produced by Salmonella spp. showed optimum chitinolytic activity at 37°C [115]. As described in Bakare et al. (2005), the optimal cellulase enzyme activity secreted by Pseudomonas species was at 35°C [116]. The results reported in previous study served as a strong support that the optimal temperature for enzyme activity was around 35°C and this explained why the inhibition rate of isolated bacterial strains against G. boninense decreased at 25°C.
3.3.4 Evaluation on the ability of selected isolated strains to produce volatile compounds possessing antibiotic activities

The bacterial volatile compounds production for the 5 selected isolated strains against *Ganoderma boninense* were evaluated based on the double plate assay or known as sealed plate methods on Nutrient agar (NA), at 35°C. The 5 selected isolated strains *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and also *Bacillus cereus* showed inhibition in mycelia germination of *G. boninense* in double plate assay. The mycelial growth observations on the control and test plates at day 7 of the experiment were shown in Figure 19.

The percentage inhibition of diameter growth (PIDG) was calculated based on the equation mentioned in section 3.2.2. The PIDG of the volatile compounds production for 5 selected isolated strains against *G. boninense* was calculated according to the mycelial growth diameter (cm) results at day 7 of the double plate assay experiment. The results of PIDG for this study were presented in Figure 20.
Figure 19: Evaluation of the ability of 5 selected isolated strains to produce volatile compounds possesses anti-*Ganoderma* properties by double plate assays on Nutrient agar at 35°C. The mycelial growth of phytopathogenic fungi, *Ganoderma boninense* in control plate (A), test plates with the presence of *Serratia marcescens*, S12 (B), test plate with the presence of *Acinetobacter calcoaceticus*, S2 (C), test plate with the presence of *Bacillus cereus*, C12 (D), test plate with the presence of *Pseudomonas putida*, C8 (E) and test plate with the presence of *Chryseobacterium indologenes*, S4 (F) at day 7 of the experiment.
Figure 20: The percentage inhibition of diameter growth (PIDG) results on the 7th day of the experiment on the evaluation of the ability and efficiency of the volatile compounds produce by the selected isolated strains. *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and *Bacillus cereus* were tested against *Ganoderma boninense*.

The 5 selected isolated strains *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and *Bacillus cereus* produced volatile compounds that showed promising results in inhibition of the mycelial growth of the phytopathogenic fungi, *G. boninense*. The comparison of the mycelial growth for *G. boninense* at day 7 of the experiment in control plates (without the presence of the isolated strains) and the test plates (with the presence of the isolates strains) were presented in Figure 19. Of the 5 isolated strains tested, all of them possessed the ability to produce volatile compounds that inhibited the mycelial growth of *G. boninense*.

Percentage of inhibition of *G. boninense* diameter mycelial growth with the presence of 5 selected strains at day 7 were calculated and presented in Figure 20. *A. calcoaceticus* showed highest percentage inhibition of *G. boninense* mycelial growth among 5 isolated
strains by 86.5%. The diameter mycelial growth of *G. boninense* was inhibited in the presence of *S. marcescens* with 83.8% PIDG. The percentage inhibition of *G. boninense* mycelial growth with the presence of *P. putida*, and also *B. cereus* were quite similar with 80.6%, and 80.3% PIDG. *Chryseobacterium indologenes* showed lowest percentage inhibition of *G. boninense* mycelia growth, 76.0% among 5 isolated strains in VOC assay.

*Pseudomonas* spp. was the common rhizosphere bacteria that produce volatile compounds that possessed antifungal properties has been reported in previous study [117]. Besides that, *Bacillus* spp. had been reported to produce volatile compounds with antifungal effects as well against *Penicillium* strains in a previous study [58]. Bacteria of *Bacillus* spp. have been reported to act as the biological control agent in inhibiting the growth of pathogen through antagonistic activity such as antibiotic production, secretion of lytic enzymes (chitinases, β-1,3-glucanase) and as well as antifungal volatile compounds [58,60]. The antagonistic activity of the volatile compounds production by the bacteria species reported served as a strong support to the results obtained in the present study.

### 3.3.5 Culture filtrate test

Evaluation of the anti-*Ganoderma* activities of the extracellular metabolites of 5 selected isolated strains *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and *Bacillus cereus* were tested by the culture filtrate technique on Potato dextrose agar (PDA) and also amended PDA plates at 25°C. The extracellular metabolites of the isolated strains were tested against *Ganoderma boninense*. The mycelial growth observations on the control and test plates at day 5 were shown in Figure 21. The mycelial growth diameter (cm) of *G. boninense* on the control and test plates were measured at day 5 and presented in Table 8.

The percentage inhibition of diameter growth (PIDG) was calculated based on the equation mentioned in section 3.2.2. The PIDG of the extracellular metabolites for 5 selected isolated strains against *G. boninense* was calculated according to the mycelial growth diameter (cm) results at day 5 of the culture filtrate assay experiment. The results of PIDG for this study were presented in Figure 22.
Figure 21: Evaluation of the efficiency for the culture filtrate from 5 selected isolated strains against *Ganoderma boninense* by culture filtrate assay on potato dextrose agar (PDA) at 25°C. The growth of phytopathogenic fungi, *Ganoderma boninense* in control plate (A), test plates with the presence of *Serratia marcescens*, S12 (B), test plate with the presence of *Pseudomonas putida*, C8 (C), test plate with the presence of *Acinetobacter calcoaceticus*, S2 (D), test plate with the presence of *Chryseobacterium indologenes*, S4 (E) and test plate with the presence of *Bacillus cereus*, C12 (F) at day 5 of the experiment.
Table 8: The mycelial growth diameter of *Ganoderma boninense* in control and test plates at day 5.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>S2</th>
<th>S4</th>
<th>S12</th>
<th>C8</th>
<th>C12</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>8.0</td>
<td>5.2</td>
<td>3.9</td>
<td>3.9</td>
<td>4.0</td>
<td>3.7</td>
</tr>
<tr>
<td>P2</td>
<td>7.8</td>
<td>5.2</td>
<td>4.0</td>
<td>3.9</td>
<td>4.3</td>
<td>3.5</td>
</tr>
<tr>
<td>P3</td>
<td>8.0</td>
<td>5.2</td>
<td>3.8</td>
<td>4.6</td>
<td>4.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Average</td>
<td>7.93 ± 0.12</td>
<td>5.20 ± 0.00</td>
<td>3.90 ± 0.10</td>
<td>4.13 ± 0.40</td>
<td>4.17 ± 0.15</td>
<td>3.57 ± 0.12</td>
</tr>
</tbody>
</table>

**Note: The average value calculated is significantly different at p value p ≤ 0.05**

Figure 22: The percentage inhibition of diameter growth (PIDG) results of 5 selected isolated strains on the 5th day of the culture filtrate experiment. *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and also *Bacillus cereus* were tested against *Ganoderma boninense*. 
The culture filtrate assay results showed that with the presence of the extracellular metabolites of the isolated strains Chryseobacterium indologenes, Acinetobacter calcoaceticus, Serratia marcescens, Pseudomonas putida, and also Bacillus cereus, the mycelial growth of the phytopathogenic fungi, Ganoderma boninense cannot grow well as compare to control plate. The significant difference of the mycelial growth of G. boninense in control plate and test plates were compared and presented in Figure 21 and Table 8 at day 5 of the experiment. All of the 5 selected isolated strains, showed inhibition of mycelial growth of G. boninense in the presence of the extracellular metabolites of each isolates.

Percentage of inhibition of G. boninense diameter mycelial growth with the presence of 5 selected strains at day 5 were calculated and presented in Figure 22. B. cereus and C. indologenes showed highest percentage inhibition of G. boninense mycelial growth among 5 isolated strains by 55.9% and 50.8%. The percentage inhibition of G. boninense mycelial growth with the presence of extracellular metabolites of S. marcescens and P. putida, were quite similar with 47.9% and 47.5%. A. calcoaceticus showed lowest percentage inhibition of the diameter mycelial growth of G. boninense which PIDG obtained was only 34.4%.

The extracellular metabolites secretions by the isolated strains were one of the important mechanisms to inhibit the fungal growth. Similar results were demonstrated and reported in other studies such as the inhibition of Fusarium oxysprum growth which caused Fusarium wilt disease in rock melon by Pseudomonas sp., Bacillus sp. And also Serratia sp. [19]. The PIDG results obtained in the culture filtrate assay were observed to be lower than the PIDG results obtained in dual culture assay. The lower PIDG results in this assay might due to the discontinuous secretion of secondary metabolites by the isolated strains as compared to the dual culture assay.
3.3.6 Chitinases, proteases and glucanases activity for selected bacterial strains

Evaluation of the capability of each selected bacterial strains to secrete chitinases, proteases and glucanases were tested by the used of selective media: chitin agar, skim milk agar and carboxymethylcellulose agar at 35°C ± 2°C. The capability of each individual bacterial strains to produce different enzymes were tabulated in Table 9.

Table 9: Chitinases, proteases and glucanases activity of five selected bacterial strains.

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>A. calcoaceticus</th>
<th>C. indologenes</th>
<th>S. marcescens</th>
<th>P. putida</th>
<th>B. cereus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitinases</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Proteases</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucanases</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

**Note:**
- No secretion of particular enzyme
+ Weak secretion of particular enzyme
++ Strong secretion of particular enzyme

The capability of the individual bacterial strains, *Acinetobacter calcoaceticus*, *Chryseobacterium indologenes*, *Serratia marcescens*, *Pseudomonas putida*, and also *Bacillus cereus* to secrete chitinases, proteases and glucanases enzymes were tested with the used of chitin agar, skim milk agar and carboxymethylcellulose agar. The clear zone formation around the bacteria colonies on the respective plates indicates the presence of the particular enzymes activities.

As showed in table 9, all five bacterial strains had the capability to produce both proteases and glucanases enzymes. *A. calcoaceticus*, *C. indologenes*, and *S. marcescens* showed strong proteases activity among five bacterial strains, whereas *P. putida*, and *B. cereus* showed strong glucanases activity. Of five bacterial strains only *S. marcescens* and *P. putida* had the ability to secrete chitinases enzyme. The absence or weak
chitinases activity by the five bacterial strains as compared to proteases and glucanases enzymes gave a good indication that chitinases enzymes did not play an important role in the inhibition of the pathogenic fungus, *Ganoderma boninense*.

### 3.4 Summary

This chapter discussed on the efficiency of the 16 different strains obtained in the previous chapter as potential biological control agent against phytopathogenic fungi, *Ganoderma boninense*. Phytopathogenic fungi, *G. boninense* was selected to be used in the dual culture plate assay as it is the major strains that caused basal stem rot disease in Malaysia oil palm plantation. The percentage inhibition of diameter growth (PIDG) of the *G. boninense* by 16 different strains was calculated based on the equation described in section 3.2.2. The isolated strains with highest PIDG value were selected for further evaluated the efficiency as the biological control agents. Of 16 different isolated strains, 5 isolated strains were selected based on the PIDG and the stable growth in lab condition. *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and *Bacillus cereus* were the isolated strains selected to be further evaluating in this study. The antibacterial activity of the 5 isolated strains selected was evaluated. The standard bacteria strains used in this assay were *Escherichia coli* NBRC 3301, *Pseudomonas aeruginosa* NBRC 12689, *Staphylococcus aureus* NBRC 12732 and *Bacillus subtilis* NBRC 3134 and the yeast strains *Saccharomyces cerevisiae* ATCC 9763. The inhibition activity among the selected isolated strains was tested as well. No inhibition activity was observed among the selected isolated strains. Out of 5 isolated strains, none of them showed antimicrobial activity towards the tested standard strains. The antifungal activities of the 5 selected isolated strains were evaluated against phytopathogenic fungi *Rhizoctonia solani*, *Aspergillus niger*, and also *Ganoderma boninense* at 25°C. The 5 selected isolated strains showed antagonistic activity towards *R. solani*, *A. niger*, and also *G. boninense* at 25°C. The results obtained in antibacterial and antifungal assay indicated that the isolated strains only inhibit the growth of the targeted phytopathogenic fungi and the antagonistic activity of the isolated strains worked in both temperature 25°C and 35°C. The bacterial volatile compounds productions for 5 selected isolated strains against *G. boninense* were evaluated based on the double plate assay. Of the 5 isolated strains
tested, all of them possessed the ability to produce volatile compounds that inhibited the mycelial germination of *G. boninense*. The efficiency of the extracellular metabolites of 5 selected isolated strains against *G. boninense* were evaluated based on culture filtrate assay. The culture filtrate assay results showed that, inhibition of the mycelial germination of the phytopathogenic fungi, *G. boninense* were observed with the presence of the extracellular metabolites of the isolated strains. The chitinases, proteases and glucanases activities results showed that, five selected bacterial strains showed that ability to secrete both proteases and glucanases enzymes. Of 5 selected bacterial strains, only *S. marcescens* and *P. putida* had the ability to secrete chitinases enzymes. The absence or weak secretion of chitinases enzymes by selected bacterial strains as compared to proteases and glucanases enzymes gave a good indication that chitinases enzymes did not play an important role in the inhibition of the pathogenic fungus, *G. boninense*. The growth kinetics of the 5 selected isolated strains was conducted and the results obtained served as an experimental designed guideline in the pot trial section which was further discussed in chapter 4. The results of the assay conducted in this chapter showed that the 5 selected isolated strains *C. indologenes*, *A. calcoaceticus*, *S. marcescens*, *P. putida*, and also *B. cereus* were effectively as a potential biological control agent to be used in agricultural industry. The efficiency of the 5 selected isolated strains in pot trial condition as biological control agents towards *G. boninense* was further evaluated and discussed in Chapter 4.
CHAPTER 4: SMALL SCALE POT TRIAL EVALUATION OF MYCOLYTIC ENZYMES PRODUCING BACTERIA AGAINST PHYTOPATHOGENIC FUNGUS, _GANODERMA BONINENSE_
4.1 Introduction
The main challenges face in oil palm agricultural industry is the infection of plant pathogenic disease, basal stem rot (BSR) disease. The BSR disease causes great economic losses for oil palm industry in Malaysia as Malaysia is one of the largest palm oil exporter in worldwide [1]. Basal stem rot disease in Malaysia oil palm cultivation is mainly caused by *Ganoderma* species. It is the major disease of oil palm and with no known curing method at the moment. *Ganoderma boninense* has been identified as the major plant pathogen fungi in oil palm cultivation in Malaysia [111–113]. According to the previous study, the spreading method of BSR disease was through root to root method [10,11]. Therefore, the root system of oil palm seedlings was selected as targeted are to introduced *G. boninense* to infect the healthy oil palm seedlings for further investigation in this study.

Biological control by using antagonistic microbes is the most common approach used in plant disease control recently. Antagonistic microbes play an important role in microbial equilibrium which implies direct interactions between two microorganisms. A number of various molecules secreted by the antagonistic microbes have been studied and their role in suppression of different plant pathogens has been recorded [14,72–74]. Based on the percentage inhibition diameter growth results as described in the previous chapter, 5 isolated bacterial were selected to be used in this pot trial study. The selected strains were *Pseudomonas* spp., *Bacillus* spp., *Acinetobacter* spp., *Chryseobacterium* spp. and *Serratia* spp. The previous study has shown that *Bacillus* spp., *Serratia* spp., and *Pseudomonas* spp. were effective biocontrol agents [19].

*Serratia* species is gram negative rhizobacteria that possess the antifungal activities and are commonly found along with plant roots area [62,63]. Numerous studies have reported the use of *Serratia* spp. as biological control agents in plant disease management. *Serratia marcescens* has been described in previous study as biological control agents and plant growth enhancer against nematodes under greenhouse conditions [20]. In addition, *Serratia marcescens* B2 also showed antagonistic activity towards soil-borne fungus *Rhizoctonia solani* in cyclamen plants in previous study [64].

*Bacillus* species is gram positive rhizobacteria that possess few advantages properties that highlighted to be good biological control agent candidates. The first advantage is
the high spore production of the *Bacillus* spp. The spores are resistant to high heat, UV light and organic solvents which allow *Bacillus* spp. to withstand the adverse environmental conditions [58,59]. In addition, the antagonistic activity of *Bacillus* spp. have been described in previous study with numerous mode of action such as the secretion of secondary metabolites possess antifungal or antimicrobial activities which include the antibiotics, enzymes that are able to degrade fungal cell wall and antifungal volatiles [58,60]. The secondary metabolite secretes are able to enhance the plant growth and the defence responses systems in the host plant have been reported in previous study [58,61].

*Pseudomonas* species is gram negative rhizobacteria that have been described as potential biological control agents in recent years. *Pseudomonas* spp. have been described to be able produce antifungal antibiotics and enhance the defence system in host plants [66]. The aim of this chapter was to conduct a small scale pot trial on oil palm seedlings to evaluate the efficiency of the selected mycolytic enzymes producing strains against the plant pathogenic fungus, *G. boninense*. The focused of these studies was on the observation of the changes of oil palm seedlings under different treatments.
4.2 Materials and methodology

4.2.1 Preparation of barley grains infected with *Ganoderma boninense*

Barley grains infected with *Ganoderma boninense* were prepared as follows:

Five grams of barley grains were washed twice with deionized water and placed into Universal bottles followed by 3mL of Potato dextrose broth (PDB, Himedia, Mumbai, India). These were autoclaved twice at 121 °C for 15 minutes. An agar plug from a 5-day old *G. boninense* culture plate was cut and transferred into the sterilized Universal bottles containing the barley grains by the use of sterilized straws. The inoculated grains were incubated for 2 weeks at 25°C, to allow the fungi mycelia to fully cover the barley grains within the Universal bottles. The inoculated bottles were shaken at the intervals of 3 days to detach the barley grains that were attached together by the mycelia during the incubation period [87].

4.2.2 Preparation of bacteria consortia culture

The following 5 isolated bacterial were selected to be used in the pot trials:

1. *Pseudomonas putida*, C8
2. *Bacillus cereus*, C12
3. *Acinetobacter calcoaceticus*, S2
4. *Chryseobacterium indologenes*, S4
5. *Serratia marcescens*, S12

The isolated bacterial strains mentioned above were inoculated in sterilized Nutrient Broth (NB, Himedia, Mumbai, India) in Erlenmeyer shake flask, separately. The NB was prepared and 250ml was aliquot into each flask. The top of the flask was covered with cotton buds and sterilized at 121 °C for 15 minutes. The inoculated shake flask was placed in the incubator shaker (Sartorious Stedim Biotech) for 3 days at 35°C with 120 rpm speed. After 3 days of incubation, the growth culture was poured into a sterilized 2000mL beaker and mixed together to form consortia bacteria cultures to be used in the pot trials.
4.2.3 Preparation of oil palm seedlings and experimental design

One month old oil palm seedlings were selected to be used in this study. The one month old oil palm seedlings were purchased from Igan Plantation Company, Sarawak, and transferred to sterilized soil mixture for 30 days observation. The soil mixture consisted of a mix of topsoil, coconut husk, and sand with the ratio of 3:2:1. Soil mixture (250g) was weighed and distributed into bags and sterilized twice at 121 °C for 15 minutes.

After 30 days, 68 oil palm seedlings with the best conditions were selected. The selected oil palm seedlings were uprooted and replanted into new pots. The pots used in this study were washed with 15% diluted commercial chlorox and rinsed with tap water. Besides that, each pot was also wiped with 70% ethanol. The 68 selected oil palm seedlings were divided into 4 different groups, each group with the replicates of 17 oil palm seedlings. The setup of this study was as follows.

Table 10: The oil palm seedlings were divided into 4 different groups and the brief description of setup for each test group.

<table>
<thead>
<tr>
<th>Experiment group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>The oil palm seedlings were planted without the presence of <em>Ganoderma boninense</em> and the bacteria consortia.</td>
</tr>
<tr>
<td>Positive Control A</td>
<td>The oil palm seedlings were treated with <em>Ganoderma boninense</em> only.</td>
</tr>
<tr>
<td>Positive Control B</td>
<td>The oil palm seedlings treated with the bacteria consortia only.</td>
</tr>
<tr>
<td>Test</td>
<td>The oil palm seedlings treated with both <em>Ganoderma boninense</em> and bacteria consortia.</td>
</tr>
</tbody>
</table>

The sterilized soil mixture was mixed with 30 mL sterilized nutrient broth for the negative control group. The 2-month old oil palm seedlings were replanted into the new pots contained the soil mixture prepared. As for the positive control group A, the sterilized soil mixture was prepared by the mixed of 30 mL of sterilized nutrient broth simultaneously with the barley grain that had been infected with *G. boninense*. After the complete mixture of soil, empty nutrient broth and *G. boninense* infected barley grains, the 2-month old oil palm seedlings were planted into the new pots with the mixture
prepared. The positive control group B was prepared by the mixed of 30 mL bacteria consortia cultures that had incubated in nutrient broth for overnight (24 hours) together with the soil mixture. The 2-month old oil palm seedlings were then replanted into the new pots contained the soil mixture contained both soil and overnight consortium bacterial. The test group was setup by first the mixed of the soil mixture with 30 mL of overnight bacteria consortia cultures. After the mixed of the overnight bacteria consortia cultures, *G. boninense* infected barley grains were added into the soil mixture and mixed thoroughly. The 2-month old oil palm seedlings were then replanted into the new pots together with the mixture of soil, consortium bacterial and *G. boninense* infected barley grains.

The initial physical examination of the seedlings was conducted and recorded prior to the start of the pot trials. The measurements and observations were:

1. The height of the stem was measured from shoots to the base of stem starting from the soil surface.
2. The number of leaves were counted and recorded; only leaves that opened completely were counted.
3. The occurrence of disease symptoms was observed and recorded based on the appearance of yellow brownish or black spots on the leaves.
4. The viability of the seedlings were observe based on the physical appearance, 50% of the physical appearance of the seedlings in green indicated viable seedlings. The physical examination was adapted and modified from the Ph.D. dissertation of Dr. Noreha Mahidi [87].

The pot trials experiment was conducted at 7th floor balcony, Block B, of Swinburne University of Technology Sarawak Campus. After the first week of the experiment conducted, second application of the overnight bacteria consortia culture was carried up. The volume of overnight bacteria consortia culture incubated in nutrient broth applied to positive control group B and also to the test groups was 30mL for each pot in the group. The overnight bacteria consortia culture was applied to positive control B and test groups to serve as a booster, whereas for the negative control and positive control A groups, sterilized nutrient broth (without any cultures) were applied. The amount of sterilized nutrient broth applied to each pot in the group was 30 mL as well. The
duration of the pot trials experiment conducted was 3 months. The physical examination was conducted once a month throughout the 3 months experiment period. The temperature range was about 25°C to 38°C, measured using the temperature monitor sensor (Digital Thermo, Malaysia). The oil palm seedlings pots were watered regularly every day. Tap water was used to water the oil palm seedlings. The amount of water applied to oil palm seedlings were 50 mL for each pot.

**Note:**

Control/Ctrl – Oil palm seedlings without any treatment

GB – Oil palm seedlings treated with *Ganoderma boninense*

C.BAC – Oil palm seedling treated with bacteria consortia only

GB + C.BAC – Oil palm seedlings treated with both *Ganoderma boninense* and bacteria consortia

### 4.2.4 Data analysis

The data were reported as the mean ±SE (standard deviation) of 17 pots replicates from each test group. Experiments results were analyzed using analysis of t-Test: Two-sample assuming unequal variances, with statistical significance taken as p ≤ 0.05. Data analysis programme in Statistical Package for the Social Sciences (SPSS) software was used for the data analysis in this experiment.
4.3 Results and discussion

*Ganoderma boninense* is the major species that caused basal stem rot disease in oil palm cultivation in Malaysia. According to previous studies, there are no well-known treatments to solve the disease incidents in oil palm cultivation [111–113]. The small scale pot trial conducted in this study was to investigate the efficiency of the selected mycolytic enzymes producing strains as biocontrol agents against *G. boninense*. One month old oil palm seedlings were selected to be used in this study. The selection of one month oil seedlings was due to at this stage, less existence of endophytic fungi was discovered in the oil palm seedlings [87].

According to previous study, the basal stem rot disease was spreads through root to root contact [10,11], Therefore, root system of oil palm seedlings were selected as targeted area for introduced phytopathogenic fungi, *G. boninense* to infect the oil palms seedlings for further investigation in this study. Barley grains were selected to be used as a carried for pytopathogenic fungi, *G. boninense* to mix with soil mixture and applied at the root area around oil palm seedlings.

The 5 isolated strains *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and also *Bacillus cereus* were selected to be used in the pot trial experiment. The efficiency of the 5 selected isolated strains in pot trial condition as biological control agents towards *G. boninense* was evaluated and discussed in this study. The selections of 5 isolated bacterial strains were based on the percentage inhibition of mycelial diameter growth of *G. boninense* in the antifungal assay. The stable growth rate of isolated strains in lab condition takes in consideration for the selection process.

The oil palm seedlings were divided into 4 different groups with different treatments as described in the methodology section. Each experiment group was set up with 15 pots of oil palm seedlings to obtain the replicate results. The results obtained from the studies was be presented and discussed in the following sections.
4.3.1 The presence of bacteria consortia showed enhancement in the oil palm seedlings growth.

Plant disease management by the used of biological control agents due to its antagonistic activity towards phytopathogenic fungi has been reported in many studies. Number of bacteria has been reported to able suppress the plant pathogens, the bacteria species reported included *Pseudomonas* spp., *Bacillus* spp., and *Serratia* spp [19]. In the present study, the effectiveness of bacteria consortia consists of *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and also *Bacillus cereus* to protect oil palm seedlings against *Ganoderma boninense* were evaluated.

Besides suppress the plant pathogens growth, biological control agents were reported to be able enhanced the growth of the plants in previous studies. *Serratia* spp. has been described in previous study to be able inhibit the growth of the pathogen fungi cause strawberry disease and also increase the yield of strawberry [63]. It has been reported in previous studies that *Bacillus* spp. also act as growth promoter besides act as biological control agents [118].

(I) The height increment observations of oil palm seedlings for 4 different test groups

The height increment of oil palm seedlings was observed and measured by using a normal ruler. The height increment of seedlings was measured the seedlings stem start from the soil surface until the first leaves internode encountered along the stem of oil palm seedlings. The height increment of oil palm seedling was recorded in unit centimetre (cm). The mean of height increment of oil palm seedling for 4 different groups over 3 months were calculated and tabulated in Table 11. The height increment measurement was conducted once in a month over the pot trial experiment.
Table 11: Mean of height increment of oil palm seedlings for the 4 different groups over a 3 month period.

<table>
<thead>
<tr>
<th>Height Increment of Oil Palm Seedlings (cm)</th>
<th>Control</th>
<th>GB</th>
<th>C.BAC</th>
<th>GB + C.BAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st month</td>
<td>1.23 ± 0.76a</td>
<td>0.30 ± 0.23a</td>
<td>1.31 ± 0.89b</td>
<td>0.59 ± 0.46a</td>
</tr>
<tr>
<td>2nd month</td>
<td>1.63 ± 0.80a</td>
<td>0.65 ± 0.39a</td>
<td>1.72 ± 0.92b</td>
<td>1.01 ± 0.54a</td>
</tr>
<tr>
<td>3rd month</td>
<td>2.18 ± 0.90a</td>
<td>0.88 ± 0.65a</td>
<td>2.37 ± 1.26b</td>
<td>1.41 ± 0.85a</td>
</tr>
</tbody>
</table>

**Note:**

Each value is the mean ± standard deviation of 17 pots from each group

Each value is significantly different at p value p ≤ 0.05 is labelled with ‘a’ letter, whereas labelled with letter ‘b’ indicates no significantly different at p value p ≤ 0.05.

The p value of each comparison group was calculated. All compare group show significantly different with p value p ≤ 0.05 except for control group compare with bacteria consortia group which show no significant different in height increment. The height increment in percentage of oil palm seedlings along 3 months of 4 different groups were showed in Figure 23.
Figure 23: The height increment of oil palm seedling in percentage with 4 different treated groups over 3 months. The oil palm seedlings treated with bacteria consortia showed significant increment in height than the oil palm seedlings of the control group. The oil palm seedlings group treated with both *Ganoderma boninense* and bacteria consortia showed improvement in height growth as compare to the oil palm seedlings group treated with *Ganoderma boninense* only. Each tested group had 17 replicates and the bars represent the standard error of the mean of 17 replicates.

The oil palm seedlings group treated with bacteria consortia only for the first month of pot trial experiment show the highest increment in height which was 78 % of compared to the initial readings. The control group, which was oil palm seedlings without any treatment showed 47.5% increment in height and 28.1% for oil palm seedlings group treated with both bacteria consortia and *Ganoderma boninense*. The oil palm seedlings group treated with *G. boninense* showed the lowest height increment, which was only 9.4% for the first month.

The height increment of oil palm seedlings at the end of 3 months showed the same trend as the first month. The height increment of oil palm seedling treated with bacteria consortia only showed highest increment in height which was 133% compare to the
initial readings recorded. Followed by control group which was 83\% increment in height and oil palm seedlings treated with both *G. boninense* and bacteria consortia was 62.6\% increment in height. The oil palm seedlings treated with *G. boninense* only showed the lowest height increment which was only 28.3\% over three months.

The results obtained showed that the oil palm seedlings treated with bacteria consortia had the highest increment in height as compared to the oil palm seedling without any treatment. This result indicates that the bacteria consortia help to promote the growth of the oil palm seedlings. The height increment for oil palm seedlings treated with *G. boninense* only was lower than oil palm seedlings treated with both *G. boninense* and bacteria consortia. This phenomenon indicates that bacteria consortia can become potential biocontrol agents as with the presence of bacteria consortia the growth of the seedlings was better even though with the presence of plant pathogenic fungus. As shown in previous study, the potential biological control agents, *Bacillus* spp. showed the ability to enhance the growth of plants and also suppress the growth of the plant pathogenic fungi [119].

(II) The health condition of oil palm seedlings leaflets for 4 different test groups.

The number of healthy leaflets for oil palm seedlings of 4 different groups were observed and recorded. The healthy leaflets were determined based on the observations of the colour of leaves and the appearance of disease symptoms such as black spots formation on leaflets. The mean for number of healthy leaflets of oil palm seedlings for 4 different groups over 3 months were calculated and tabulated in Table 12.
Table 12: Mean of number of healthy leaflets of oil palm seedlings for 4 different groups over 3 months.

<table>
<thead>
<tr>
<th>No. of healthy leaflet over 3 months</th>
<th>Control</th>
<th>GB</th>
<th>C.BAC</th>
<th>GB + C.BAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st month</td>
<td>3.88 ± 1.41a</td>
<td>2.12 ± 1.73a</td>
<td>4.18 ± 0.73b</td>
<td>3.35 ± 0.93a</td>
</tr>
<tr>
<td>2nd month</td>
<td>4.82 ± 1.74a</td>
<td>2.29 ± 2.11a</td>
<td>4.53 ± 1.70b</td>
<td>2.71 ± 1.57b</td>
</tr>
<tr>
<td>3rd month</td>
<td>4.88 ± 2.15a</td>
<td>2.24 ± 2.28a</td>
<td>4.94 ± 2.25b</td>
<td>2.76 ± 1.86b</td>
</tr>
</tbody>
</table>

**Note:**

Each value is the mean ± standard deviation of 17 pots from each group.

Each value is significantly different at p value p ≤ 0.05 is labelled with ‘a’ letter, whereas labelled with letter ‘b’ indicates no significantly different at p value p ≤ 0.05.

The p value of each comparison group was calculated. All comparison groups showed significant difference with a p value p ≤ 0.05. Control group compared with oil palm seedlings treated with bacteria consortia showed no significant difference in the number. The oil palm seedlings treated with both *Ganoderma boninense* and bacteria consortia compared with oil palm seedlings treated with *G. boninense* only, also showed no significant difference in the second and third month. This might be due to the populations of bacteria consortia were insufficient to suppress the plant pathogenic fungus for the second and third month as no further booster consortia-culture supernatant was applied after the first month of the experiment.

The percentage of healthy leaflets for oil palm seedlings among 4 different groups for the timeframe of 3 months were calculated based on the results obtained in the experiment (Table 12) and presented in Figure 24. Among 4 different test groups, the percentage of the healthy leaflet for oil palm seedling treated with bacteria consortia showed highest in percentage. Followed by the oil palm seedlings control group showed second high in percentage. The percentage of healthy leaflets for oil palm seedlings treated with *G. boninense* only showed lowest in percentage among 4 test groups.
Figure 24: The numbers of healthy leaflets of oil palm seedlings in percentage as compared to initial results for 4 different groups over 3 months. The percentage of healthy leaflets for oil palm seedlings group treated with bacteria consortia showed slightly higher than the oil palm seedlings control group at 3rd month of the experiment. The percentage of healthy leaflets for oil palm seedlings group treated with both *Ganoderma boninense* and bacteria consortia showed higher in percentage than seedlings group treated with *Ganoderma boninense* at 3rd month of the experiment. Each tested group had 17 replicates and the bars represent the standard error of the mean of 17 replicates.

The oil palm seedlings treated with bacteria consortia only, showed highest percentage of healthy leaflets in the first and third month which was 30.4% and also 55.9%. The percentage of healthy leaflets in the second month was second highest with 42.2 %. As for oil palm seedlings without any treatment, the percentage of healthy leaflets showed second highest in the first and third month at 22.1% and 51.5%. The percentage of healthy leaflets for oil palm seedlings control group was higher than oil palm seedlings group treated with bacteria consortia in the second month at 52.0%.

The percentage of healthy leaflets for the oil palm seedlings group treated with both *Ganoderma boninense* and bacteria consortia was highest in the first month which was 14.2% and lowest by the second month which was -1.0%. The negative value obtained in second months showed that the number of healthy leaflets for the oil palm seedlings
group treated with both *G. boninense* and bacteria consortia have dropped as compared to the first month. On the third month, the percentage of healthy leaflets increases back to 1.0%. The dramatic drop of the percentage might be due to the insufficient populations of bacteria consortia to suppress the plant pathogenic fungus during the second and third month as no further booster consortia-culture supernatant was applied after the first month of the experiment. As for the oil palm seedlings treated with *G. boninense* only, the percentage of healthy leaflets showed lowest among the 4 groups which was -13.7% for the first month, -7.8% for the third month and a slight increment in the second month which was 2.5%. The lowest percentage of value in the first month might be due to the infected grains applied still in active growth condition. Furthermore, the grains applied served as nutrients for the growth for the *G. boninense*. The slight increment in the second month might be due to the adaption for the new nutrient sources as the grains nutrient has been finished and slow growth rate of the pathogenic fungus.

The results obtained suggest that the application of bacteria consortia enhance the health of the oil palm seedlings. This indicates that bacteria consortia have potential to be biological control agents against plant pathogenic fungus, *G. boninense*, but the booster of cultures needs to be applied after a period of time.

(III)  Disease incidence level and viability of oil palm seedlings over a 3 months observation timeframe

The disease incidence level and viability of oil palm seedlings over 3 months for 4 different testing groups were observed and recorded. The measurements for disease incidence level of oil palm seedlings were based on the observations of the formation of black spots and colour appearance of the leaves, which are categorized as one of the disease symptoms for basal stem rot disease (BSR) [87]. The survival of oil palm seedlings after infected by *Ganoderma boninense* was observed over 3 months for 4 different test groups.

Based on the observation of the health condition for the oil palm seedlings, the disease incidence level described as followed was given to each pot of the oil palm seedlings. The mean of the disease incidence level of the oil palm seedlings for 4 different groups
over 3 months was calculated based on the following formula and is shown in Table 13.

Disease incidence level classified as follow based on observation on oil palm seedlings:

1- Good  
2- Fairly Good  
3- Not Good  
4- Very Bad  
5- Dead

Mean disease incidence level

= Average of disease incidence level of each group / Total number of plants

Table 13: Mean of disease incidence level of oil palm seedlings for 4 different groups over 3 months.

<table>
<thead>
<tr>
<th>Disease incidence level over 3 months</th>
<th>Control</th>
<th>GB</th>
<th>C.BAC</th>
<th>GB + C.BAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st month</td>
<td>1.65 ± 0.86\text{a}</td>
<td>2.24 ± 1.15\text{a}</td>
<td>1.24 ± 0.44\text{b}</td>
<td>1.53 ± 0.62\text{a}</td>
</tr>
<tr>
<td>2nd month</td>
<td>1.35 ± 0.70\text{a}</td>
<td>2.82 ± 0.95\text{a}</td>
<td>1.41 ± 0.62\text{b}</td>
<td>2.06 ± 0.97\text{a}</td>
</tr>
<tr>
<td>3rd month</td>
<td>2.00 ± 1.00\text{a}</td>
<td>3.06 ± 1.03\text{a}</td>
<td>1.71 ± 1.05\text{b}</td>
<td>2.41 ± 1.06\text{a}</td>
</tr>
</tbody>
</table>

**Note:**

Each value is the mean ± standard deviation of 17 pots from each group

Each value is significantly different at p value \( p \leq 0.05 \) is labelled with ‘a’ letter, whereas labelled with letter ‘b’ indicates no significantly different at p value \( p \leq 0.05 \).
Oil palm seedlings group treated with *Ganoderma boninense* showed highest in the mean of disease incidence level after 3 months of pot trial experiment which was 3.06. Followed by oil palm seedlings group treated with both *G. boninense* and consortium bacteria which were 2.41. The mean of disease incidence level of oil palm seedlings treated only with consortium bacteria was lowest among 4 tested groups with the mean value of 1.71. By the used of T-test, oil palm seedlings treated with consortium bacteria showed no significant difference as the control group which was without any treatment. The amount of consortium bacteria added initially might decrease gradually after the certain period of time. Booster of consortium bacteria was required to be added constantly after one month of the experiment to maintain the antagonistic activity of the bacteria consortia towards *G. boninense*.

The percentage of the disease incidence level of oil palm seedlings was analysed as shown in Figure 25.

![Figure 25: Disease incidence level of oil palm seedlings in percentage for 4 different groups at 3<sup>rd</sup> month of the experiment. The oil palm seedlings group treated with *Ganoderma boninense* showed the highest disease incidence percentage among 4 test groups. The oil palm seedlings group treated with bacteria consortia showed lowest in the percentage of disease incidence among all test group. Each tested group had 17 replicates and the bars represent the standard error of the mean of 17 replicates.](image)
Oil palm seedlings group treated with only consortium bacteria only showed lowest in the percentage of disease incidence level which was only 18.63 %, followed by the control group with 37.25 %. The oil palm seedlings without any treatment served as control group. Figure 25 showed that oil palm seedlings group treated with *Ganoderma boninense* only, were highest in percentage which was 51.27 % at the third month of pot trial experiment. Oil palm seedlings group treated with both *G. boninense* and consortia bacteria showed 45.59% in disease incidence level. The difference with only 5.68% between *G. boninense* treated oil palm seedlings and oil palm seedlings treated with consortium bacteria might be due to the population of consortium bacteria decrease in the soil environment. The number of loss of the bacteria might happen during the watering step of the plants as part of the bacteria was washed out from the soil environment. This incidence can be overcome by constantly adding booster of consortium bacteria culture to the oil palm seedlings.

Figure 26 showed the viability rate of oil palm seedlings group for the 4 different test groups at 3rd month of the pot trial experiment. Among 4 different groups, only oil palm seedling treated with *G. boninense* showed decreased in viability percentage over 3 months which was 94.12%. Control oil palm seedlings group, oil palm group treated with consortium bacteria only and also the oil palm seedlings group treated with both *G. boninense* and consortium bacteria all three groups showed 100% viability rate. The viability was defined by the number of oil palm seedlings survived over 3 months of experiment timeframe. The viability rate percentage (%) was calculated according to the following formula:

\[
\text{Viability rate} \, (\%) = \frac{\text{Number of oil palm seedlings survive}}{\text{Total number of plants}} \times 100 \%
\]
Figure 26: Viability of oil palm seedlings in percentage for 4 different groups at 3rd month of the pot trial experiment. The oil palm seedlings group treated with *Ganoderma boninense* only showed decrease in the viability percentage. The others 3 test groups remained the same viability percentage. Each tested group had 17 replicates and the bars represent the standard error of the mean of 17 replicates.

As discussed in section 4.3.1 and 4.3.2, the presence of the consortia bacteria showed enhancement towards plant growth in terms of height and health condition of the oil palm seedlings. Based on the results obtained in viability test, the presence of consortia bacteria also enhanced the survival rate of oil palm seedlings after being infected by pytopathogenic fungus *Ganoderma boninense*. These results served as another positive indication that consortia bacterium were suitable to be used as biological control agents against plant pathogenic fungus, *G. boninense*. As discussed earlier further booster of culture supernatant was required after a period of time to boost up the anti-fungus activity.
4.3.2 Physical observations after 3 months of pot trial experiment

The observation on the physical appearance of oil palm seedlings was carried out and recorded along the 3 months timeframe of the pot trial experiment. The changes of physical appearance of each test group were shown as follows:

(a) Oil palm seedlings without any treatment, negative control group
(b) Oil palm seedlings treated with plant pathogenic fungus, *Ganoderma boninense*
(c) Oil palm seedlings treated with consortia bacteria (Top 5 strains)
(d) Oil palm seedlings treated with both *Ganoderma boninense* and consortia bacteria
(e) Comparison between different groups after 3 months pot trial experiment

As the pot trial experiment was conducted in an opened environment thus many unexpected factors might influence the end results. The conditions of the open environment was much more similar to the plantation estate, thus the pot trial experiment was conducted in an open environment instead of in a controlled laboratory environment. The results can act as the reference for further experiment in the field.

(a) Oil palm seedlings without any treatment, negative control group

The physical appearance changes of the oil palm seedlings from initial till the end of the pot trial experiment for oil palm seedlings group without any treatment, the negative control group was compared in Figure 27 and 28. The physical observations conducted included the changes in height, the number of leaves and the health condition of the oil palm seedlings.
Figure 27: The physical observation of oil palm seedlings for control groups at initial of the pot trial experiment and after 3 months of the experiment.
Figure 28: The physical observation of oil palm seedlings for control groups before and after 3 months of pot trial experiment.
The pot images for oil palm seedlings were captured at the beginning of the pot trial experiment. The initial oil palm seedlings pot images captured were used for comparison in the changes of the physical appearance of the oil palm seedlings at the end of the pot trial experiment. As shown in Figure 27 and 28, the height increment of oil palm seedlings in the control group were observed after 3 months of the pot trial experiment with the comparison with the initial oil palm seedlings pot images. The increased in number of leaves for the oil palm seedlings control group was observed after 3 months of the pot trial experiment besides the height increment. The health condition of the oil palm seedlings for the control group were observed to be slightly decreased after 3 months of the pot trial experiment. There was black spot formation on the leaves and also the white rot fungus was observed growing on the soil surface for few pots of oil palm seedlings.

The observations obtained on the oil palm seedlings were suspected to be the disease symptoms for basal stem rot disease (BSR) [87]. The formation of black spots might be also due to the insufficient of nutrient supplied to the oil palm seedlings. No further nutrient was added after the start of the experiment to control the consistency of the nutrient conditions of 4 different test groups. The observations of white fungus on soil surface might be due to some uncontrolled factor such as disturbance by others personnel or the wind factor as the pot trial study was conducted at opened environment as mentioned above. No treatment was received by the oil palm seedlings in control group thus the chances of the seedlings to be infected by phytopathogenic fungus, *Ganoderma boninense* increase.

(b) Oil palm seedlings treated with plant pathogenic fungus, *Ganoderma boninense*

The physical appearance changes of the oil palm seedlings from initial till the end of the pot trial experiment for the oil palm seedlings group treated with plant pathogenic fungus, *Ganoderma boninense* only was compared in Figure 29, 30, and 31. The physical observations conducted included the changes in height, the number of leaves and the health condition of the oil palm seedlings.
Figure 29: The physical observation of the oil palm seedlings treated with *Ganoderma boninense* only before and after 3 months of the pot trial experiment.
Figure 30: The physical observation of the oil palm seedlings treated with *Ganoderma boninense* only before and after 3 months of the pot trial experiment.
Figure 31: The oil palm seedling treated with *Ganoderma boninense* initial (A) and after 3 months of pot trial experiment (B), (C). The growth of white rot fungus was observed on the soils surface. Green fungus was observed colonized around trunk area (D).
The pot images for the oil palm seedlings were captured at the beginning of the pot trial experiment. The pot images of the oil palm seedlings captured at the initial of the experiment were used for comparison in the changes of the physical appearance of the oil palm seedlings at the end of the pot trial experiment. The height of the oil palm seedlings group treated with phytopathogenic fungi, *Ganoderma boninense* only was observed to be increased after 3 months of the pot trial experiment compared with the initial pot images (Figure 29 and 30). The height increment for the oil palm seedlings group treated with *G. boninense* only was less than the control oil palm seedlings group.

The number of leaves of the oil palm seedlings was observed to increase after 3 months of the pot trial experiment. As for the health condition of the oil palm seedlings in this test group showed dramatic dropped. Number of the oil palm seedlings in this test group showed the basal stem rot disease symptoms. The formation of the black spots on the leaves was observed in the middle or at the end of the leaves. The colour of the leaves of the oil palm seedlings showed unhealthy colour which was yellowish or even brownish in colour. The trunk of the oil palm seedlings in this test group was observed to be small and skinny when compared to other test groups in the pot trail experiment. As shown in Figure 30, one of the oil palm seedlings in this test group was observed dead after 3 months of the pot trial experiment. The death of the oil palm seedlings was suspected to be the *G. boninense* infection towards the plant. The bad health conditions of the oil palm seedlings in this group might be due to the plant infection by *G. boninense*.

An exceptional result was observed in this test group as shown in Figure 31. Although the growth of white fungus has been observed on the soil surface but the oil palm seedling was extremely healthy and strong compared to the others oil palm seedlings in the test group. The height increment of this particular oil palm seedling was observed to be higher than others oil palm seedlings in the test group. Besides that, the growth of the trunk was observed to be bigger and stronger as compared to the others oil palm seedlings in the test group. The increase in number of leaves develops was observed for this oil palm seedlings. The colour of the leaves of this oil palm seedling was greenish in colour. No black spot formation was observed on the leaves of this seedling.
Besides the formation of the white rot fungus on the soil surface, greenish fungus was spotted colonized around the trunk of oil palm seedling as circled in Figure 31 (D). The presence of the green fungus showed inhibition towards the growth of white rot fungus. The greenish fungus was suspected to be *Trichoderma* spp.. *Trichoderma* spp. has been widely used as biological control agents in plant disease management and also well known as the growth enhancer for the plants crops [120]. The ability of the *Trichoderma* spp. to synthesize secondary metabolites which possessed antifungal or antibacterial activities has been described in previous study [120]. This explained why with the presence of the white rot fungus, the oil palm seedling still able to grow well. The green fungus enhanced the growth of the oil palm seedling. *Trichoderma* spp. are actively found in root, soil and foliar environments, therefore it was not surprised the growth of this fungi were observed in the experiment [121]. Furthermore, the pot trial study was conducted in open environment, uncontrollable factors such as the wind factor had high chance to carry along the soil particles that contained *Trichoderma* to the pot trial study area.

(c) Oil palm seedlings treated with consortia bacteria (Top 5 strains)

The physical appearance changes of the oil palm seedlings from initial till the end of the pot trial experiment for the oil palm seedlings group treated with consortia bacteria only was compared and presented in Figure 32, 33, and 34. The physical observations conducted included the changes in height, the number of leaves and the health condition of the oil palm seedlings.
Figure 32: The physical observation of the oil palm seedlings treated with consortia bacteria only before and after 3 months of pot trial experiment.
Figure 33: The physical observation of the oil palm seedlings treated with consortia bacteria only. Initial oil palms seedling pot image (A) and after 3 months pot trial experiment (B). The roots of the oil palm seedlings were observed grew out from the pots (C), (D).
Figure 34: The physical observations of the oil palm seedlings treated with consortia bacteria only. The initial pot image (A) and after the 3 months pot trial experiment (B). One new shoot was observed compared to the initial pot image (C).
The oil palm seedlings group treated with consortia bacteria only showed increment in height after 3 months of pot trial experiment. The increased in number of leaves were observed as well when compared to the initial pot images as shown in Figure 32. As compared to negative control group and oil palm seedlings group treated with *Ganoderma boninense* only, the increment of the height and number of leaves of oil palm seedlings for this test group showed highest in increment based on the physical comparison of initial and after 3 months pot images (Figure 32). The health condition of the oil palm seedlings for this test group was majority in good health condition, only a minority of oil palm seedling showed the basal stem rot disease symptoms. The formation of the black spot on leaves was observed in some of the oil palm seedlings in this test group. The leaves colours of some oil palm seedlings showed yellowish in colour after 3 months of the pot trial experiment.

Besides the increment in height and number of leaves, the roots of oil palm seedlings have been observed grew and extend out from the planting pots as circled and shown in Figure 33 (D). This indicates the presence of consortia bacteria had enhanced the root develop of the oil palm seedlings. As this occurrence cannot be observed in the oil plant seedlings pots either in the control group or oil palm seedlings group treated with *G. boninense* only. Furthermore, no white fungus was observed on the soil surface in this test group as compared to the negative control group and less black spot formation was observed on the leaves. This served as another indication that other than enhanced the growth of the oil palm seedlings, with the presence of the consortia bacteria the health of the oil palm seedlings has been improved as well.

Figure 34 showed the interesting finding obtained in this test group. One of the oil palm seedlings pot was observed to have new shoots coming out which initial only have one shoots observed in the planted pot. This result served as strong evidence that the growth and health of oil palm seedlings was enhanced and improved by the presence of consortia bacteria in the soil environment. This occurrence was not found in any other test group in this pot trial experiment. The physical observations obtained from this test group indicated that consortia bacteria were good potential biological control agents to be used in plant disease management in oil palm cultivation.
(d) Oil palm seedlings treated with both *Ganoderma boninense* and consortia bacteria

As for the physical appearance changes observation for the oil palm seedlings group treated with both *Ganoderma boninense* and consortia bacteria before and after 3 months of the pot trial experiment was compared and distributed in Figure 35, 36, and 37. The physical observations conducted included the changes in height, the number of leaves and the health condition of the oil palm seedlings.
Figure 35: The physical observations of the oil palm seedlings treated with both *Ganoderma boninense* and consortia bacteria initial and after 3 months pot trial experiment.
Figure 36: The physical observations of the oil palm seedlings treated with both *Ganoderma boninense* and consortia bacteria initial and after the 3 months pot trial experiment.
Figure 37: The physical observation of the oil palm seedlings treated with both *Ganoderma boninense* and consortia bacteria. The initial pot image (A) and after 3 months pot trial experiment (B). The root of the oil palm seedlings was observed grew out from the pots (C).
After 3 months of the experiment, the noticeable height increment of oil palm seedlings was observed when compared to initial pot images. The significant increment in the number of leaves also has been observed in this test group. The black spot formation on the leaves has been observed but the number of black spots observed in this test group was lesser than oil palm seedlings group treated with *Ganoderma boninense* only. The majority of the leaves of the oil palm seedlings in this test group showed greenish in colour. A minority of them showed yellowish in colour. The health condition of the oil palm seedlings in this test group was better than the oil palm seedlings group treated with *G. boninense* only. This served as evidence that with the treatment of consortia bacteria, the health of the plants showed improvement even with the presence of the pathogenic fungus.

The roots of oil palm seedlings in this test group were found to grow out from the planted pot as circled and shown in Figure 37 (C). This phenomenon was not found in both negative control group and the oil palm seedlings group treated with *G. boninense* only. The improvement in root growth of the oil palm seedlings was only able to be observed in this test group and the oil palm seedlings group treated with consortia bacteria only. This served as strong evidence that consortia bacteria act as a growth enhancer in this study.

The observation obtained for the oil palm seedlings group treated with both *G. boninense* and consortia bacteria showed better growth and health condition than the oil palm seedlings group treated with *G. boninense* only. The observation showed that the consortia bacteria were potential biological control agents against *G. boninense* and also growth enhancer for the oil palm seedlings.
(e) Comparison between 4 different test groups after 3 months pot trial experiment

The overall physical appearance observation for four different test groups after 3 months of the pot trial experiment was compared and shown in Figure 38. The purpose of comparison between each test groups were to give a clear image of the effects bring towards oil palm seedlings with and without the presence of plant pathogenic fungus, *Ganoderma boninense* and consortia bacteria. The physical observations conducted included the changes in height, number of leaves and the health condition of the oil palm seedlings
Figure 38: The physical comparison among 4 different test groups after 3 months pot trial experiment. Oil palm seedlings without any treatment, negative control group (A), oil palm seedlings treated with *Ganoderma boninense* only (B), oil palm seedlings treated only with consortia bacteria (C) and oil palm seedlings treated with both *Ganoderma boninense* and consortia bacteria (D).
The comparison between the oil palm seedlings control group and the oil palm seedlings group treated with *Ganoderma boninense* only was conducted to observe the differences in physical appearance for this two test group. As shown in Figure 38 (B), the health condition for the oil palm seedlings group treated with *G. boninense* only was observed to be in bad condition. As compared to the control group, the leaves of oil palm seedlings for the *Ganoderma* treated group showed black spots formation on the leaves and the leaves was observed to be yellowish in colour. This observations outcome indicates that the health of oil palm seedlings was effected and infected by the presence of plant pathogenic fungus, *G. boninense*.

The second comparison was conducted between oil palm seedlings without any treatment, negative control group and oil palm seedlings treated with consortia bacteria only. The differences of physical appearance for this two test groups were observed and described. The oil palm seedlings group treated with consortia bacteria only shown in Figure 38 (C) showed better growth and health condition when compared to oil seedlings of the control group as shown in Figure 38 (A). The number of black spot formations and yellowish leaves of the oil palm seedlings in consortia bacteria treated group was less than the oil palm seedlings in control groups. The outcome of the comparison for this two test group showed that the growth and health condition of the oil palm seedlings were improved with the presence of consortia bacteria. Consortia bacteria act as potential biological control agents and plant growth enhancer in this study.

The comparison between the oil palm seedlings treated with *G. boninense* only and the oil palm seedlings treated with both *G. boninense* and consortia bacteria showed differences in physical appearance after 3 months of the pot trail experiemnt. The number of black spot formations and yellowish leaves observed for the oil palm seedlings group treated with both *G. boninense* and consortia bacteria was less than *Ganoderma* treated group. The growth and health condition for the oil palm seedlings group treated with both *G. boninense* and consortia bacteria was better than *Ganoderma* treated group. The observation showed that the consortia bacteria served as potential biological control agents and also promote the growth of the oil palm seedlings in this study even though with the presence of the pathogenic fungus *G. boninense*. 
The comparison among four different test groups showed that with the presence of consortia bacteria, the oil palm seedlings were better in term of growth and health conditions. The consortia bacteria had the ability to enhance and improve the health of the oil palm seedlings.

4.4 Summary
This chapter describes the efficacy of using mycolytic producing bacteria as biological control agents against *Ganoderma boninense*. A selection of 5 mycolytic enzyme-producing microbes was used in this study. The selection of the microbes was based on the percentage of inhibition rate in the antifungal assay described in Chapter 3. The bacteria, *Pseudomonas putida*, C8, *Bacillus cereus*, C12, *Acinetobacter calcoaceticus*, S2, *Chryseobacterium indologenes*, S4, and *Serratia marcescens*, S12 were selected to be used in the pot trial experiment. The height, number of leaves and disease symptoms was observed and recorded over a three-month timeframe. The aim of pot trial experiment was achieved at the end of the study. At the end of the experiment, the oil palm seedlings treated with consortia bacteria has the highest increment in height and number of leaves among four different treatment groups in the study. Besides, the disease incidence level was lowest among 4 different test groups as well. The oil palm seedlings treated with plant pathogenic, *G. boninense* only showed the lowest in height increment and number of leaves. The disease incidence level was high and the viability rate drops after 3 months for *Ganoderma* treated group. As for the oil palm seedlings group treated with both *G. boninense* and also consortia bacteria, the increment of height and number of leaves was higher than the oil palm seedlings group treated with *G. boninense* only. The viability percentage for this test group still remains 100% at the end of the pot trial experiment. The results obtained from this study indicates that with the treatment of selected consortia bacteria, the health of oil palm seedlings showed improvement and it also enhanced the growth of oil palm seedlings. The consortia bacteria were effective to inhibit the basal stem rot disease caused by *G. boninense* and also as a plants growth enhancer for the oil palm seedlings.
CHAPTER 5: GENERAL CONCLUSIONS, RECOMMENDATIONS, AND FURTHER WORKS
5.1 General conclusion

5.1.1 Aims of the thesis
The main aim of this thesis is to isolate mycolytic enzyme producing microorganisms from soils of Sarawak, and to evaluate these as potential biological control agents against the plant pathogenic fungi in oil palm cultivation, *Ganoderma boninense*. The fungus, *G. boninense*, is the main species that causes basal stem rot disease (BSR) in oil palms. It is the major disease of oil palm and has no known treatment method at the moment. This thesis proposes using biological control derived from mycolytic enzyme producing microorganisms.

This thesis reports on the approach to isolate and characterise the mycolytic enzyme producing microbes obtained from soil samples collected in Sarawak. Several assays were conducted to study the efficiency of the isolated strains. A selection of 5 isolated strains with high percentage inhibition of diameter growth (PIDG) was selected to be used in pot trial study. Small scale pot trial study was conducted to evaluate the selected isolated strains against the plant pathogenic, *G. boninense* and the effects of the isolated strains towards the oil palm seedlings. This chapter summarised the results obtained in this research study that has been described in Chapter 2, 3 and 4 of the thesis. Further works and recommendations of this study were discussed in this chapter as well.

5.1.2 Biodiversity of Sarawak as sources of industrial microorganisms
The high diversity of the plant species in Sarawak and the ideal ecosystem increase the possibility of the discovery of new or potential species to be utilized in agricultural and industry sectors to boost up the economics of the country. This assumption was made according to the discovery of more than 361 new species in Borneo from 1994 to 2004 [84]. Sarawak was selected in this study due to the ideal ecosystem and the possibility to obtain the new species that is indigenous to the Sarawak environment. Soil samples were collected from various locations within Sarawak to screen for new potential mycolytic enzyme producing strains. A number of 46 potential isolated strains were successfully isolated out from the soil samples collected.
5.1.3 Screening and isolation of mycolytic enzyme producing microorganisms

It has been reported in previous studies that the defence response gene in the plant itself, the defence mechanism were detected to increase at the infection area [10,70,71]. The defence mechanism will trigger the release of the mycolytic enzymes at the infection areas. This finding served as an idea of this study, to investigate the mycolytic producing microorganisms as potential biological control agents towards plant pathogenic fungi, specifically, Ganoderma boninense.

The rhizosphere compatible fungi and bacteria are potential biological control candidates which exhibit antagonistic activity towards plant pathogens [39]. Most of the rhizosphere microorganisms are known as plant growth promoting rhizobacteria (PGPR) [49,51]. The increased availability of mineral nutrients for plants, secretion of the plant growth stimulating compounds and also acting as first defence line to provide protection against soil-borne pathogenic fungus are the benefits contributed by PGPR bacteria towards the plants [49,52,53]. The rhizobacteria such as Bacillus spp., Serratia spp., and Pseudomonas spp. have been reported as effective biological control agents and their roles to suppress the soil-borne plant pathogenic fungus has been documented in a previous study [19].

Skim milk agar (SMA) and Carboxymethylcellulose agar (CMC) were chosen to be used in this study for primary screening of proteases and glucanases producing strains from the soil samples collected. A total of 46 isolated strains were successfully isolated out from the soil samples collected via the used of the selective agar. Out of 46 isolates, 21 were proteases enzyme-producing microbes while another 25 isolates were glucanases enzyme-producing microbes.

Molecular identification on the basis of the partial 16srRNA sequence results showed that the 46 isolates were classified as a member of the genus of Bacillus, Delftia, Acinetobacter, Stenotrophomonas, Ralstonia, Burkholderia, Pseudomonas, Serratia, Staphylococcus, and Chryseobacterium. Out of 46 isolates, 16 different strains were found. The 16 different strains found were Bacillus cereus, Bacillus coagulans, Bacillus flexus, Bacillus anthracis, Delftia tsuruhatensis, Acinetobacter calcoaceticus, Stenotrophomonas maltophilia, Ralstonia pickettii, Burkholderia metallica, Burkholderia cepacia, Pseudomonas plecoglossicida, Pseudomonas mossellii,
*Pseudomonas putida, Serratia marcescens, Staphylococcus sciuri,* and *Chryseobacterium indologenes.* The 16 isolated strains obtained were mainly gram-negative bacteria while only 5 were gram-positive bacteria.

5.1.4 **Evaluate efficiency of the mycolytic enzyme isolated strains against plant pathogenic fungi, *Ganoderma boninense***

The double plate assay was used in this study to evaluate the antagonistic activity of the 16 isolated strains against phytopathogenic fungi, *Ganoderma boninense* [19]. *G. boninense* was selected to be used in the dual culture plate assay as it is the major strains that caused basal stem rot disease in Malaysia oil palm plantation. Nutrient agar media was selected to be used in this assay. The isolated strains with highest PIDG value were selected for further evaluated the efficiency as the biological control agents. Out of 16 different isolates strains, 5 isolated strains were selected based on the PIDG and the stable growth in lab condition. *Chryseobacterium indologenes, Acinetobacter calcoaceticus, Serratia marcescens, Pseudomonas putida,* and also *Bacillus cereus* were the isolated strains selected to be further evaluating in this study.

The antibacterial assay was carried out for the 5 isolated strains selected against with the standard bacteria strains were evaluated in this study. The standard bacteria strains used in the antibacterial assay were *Escherichia coli* NBRC 3301, *Pseudomonas aeruginosa* NBRC 12689, *Staphylococcus aureus* NBRC 12732 and *Bacillus subtilis* NBRC 3134 and the yeast strains *Saccharomyces cerevisiae* ATCC 9763. The inhibition activity among the selected isolated strains was tested as well. No inhibition activity was observed among the selected isolated strains. Of 5 isolated strains, none of them showed antimicrobial activity towards the tested standard strains.

The antifungal activity of the 5 selected isolated strains against with plant pathogenic fungi was evaluated in this study. The pathogenic fungi strain used was *Rhizoctonia solani, Aspergillus niger,* and also *G. boninense.* The 5 selected isolated strains showed antagonistic activity towards *R. solani, A. niger,* and also *G. boninense* at 25°C. Based on the results obtained in the antibacterial and antifungal assay, the isolated strains only inhibited the growth of the targeted phytopathogenic fungi and the antagonistic activity of the isolates strains worked in both temperature 25°C and 35°C.
Double plate assay was conducted to evaluate the bacterial volatile compounds secretion from 5 selected isolated strains against *G. boninense* [87]. All isolated strains possessed the ability to produce volatile compounds that inhibited the mycelial germination of *G. boninense*. Culture filtrate assay was carried out to study the efficiency of the extracellular metabolites of 5 selected isolated strains against *G. boninense* [19]. The inhibition of the mycelial germination of the phytopathogenic fungi, *G. boninense* were observed with the presence of the extracellular metabolites of the isolated strains in the culture filtrate assay. The results in this thesis showed that the 5 selected isolated strains *C. indologenes*, *A. calcoaceticus*, *S. marcescens*, *P. putida*, and also *B. cereus* were potential candidates to be used in agricultural industry act as biological control agents.

5.1.5 Small scale pot trial evaluation of the mycolytic enzyme isolates strains against plant pathogenic fungi, *Ganoderma boninense*

A small scale pot trial was conducted to evaluate the efficiency of the 5 selected strains as biological control candidates against plant pathogenic fungi, *Ganoderma boninense* in this study. The 5 isolated strains *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and *Bacillus cereus* were selected to be used in the pot trial experiment. The one month old oil palm seedlings were purchased from Igan Plantation Company, Sarawak, and selected to be used in this study. The selection of one month old oil palm seedlings were selected as less existence of endophytic fungi was discovered in the oil palm seedlings at this stage [87].

According to previous study, the basal stem rot disease was spreads through root to root contact [10,11]. Therefore, root system of oil palm seedlings were selected as targeted area for introduced phytopathogenic fungi, *Ganoderma boninense* to infect the oil palms seedlings for further investigation in this study. Barley grains were selected to be used as a carried for phytopathogenic fungi, *G. boninense*. The basal stem rot (BSR) disease symptoms were observed on the oil palm seedlings infected by *G. boninense* at the end of the pot trial experiment. This result suggested that the spread method to infect oil palm seedlings through root system was successful conducted in this study.
The height, number of leaves and disease symptoms of oil palm seedlings for 4 different treatment groups were observed and recorded over a three-month timeframe. The results obtained showed that the oil palm seedlings treated with bacteria consortia only had the highest increment in height and number of leaves as compare to the oil palm seedling without any treatment. This result suggested that with the presence of the consortia culture, enhanced the growth of the oil palm seedlings. Furthermore, the disease incidence level for bacteria consortia treated group was lower than the control group. This served as evidence that the presence of the consortia culture improves the health conditions of the oil palm seedlings as well.

The increment of height and number of leaves for the oil palm seedlings group treated with *G. boninense* only was lower than the oil palm seedlings group treated with both *G. boninense* and bacteria consortia in this experiment. The decreased in viability rate for *G. boninense* treated group was obtained at the end of the experiment while the oil palm seedlings group treated with both *G. boninense* and bacteria consortia remains the same as initial of the experiment. The results obtained in this thesis showed that locally isolated consortia culture were potential biological control candidates against plant pathogenic fungi, *G. boninense*. The presence of the consortia culture was able to suppress the growth of *G. boninense* and enhances the growth of oil palm seedlings.
5.2 Further works

The mycolytic enzyme producing isolated strains obtained in this study showed potentials to be used as biological control agents in oil palm cultivation against plant pathogenic fungi *Ganoderma boninense*. The selected 5 isolated strains *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Serratia marcescens*, *Pseudomonas putida*, and also *Bacillus cereus* showed their effectiveness as biological control agent against *G. boninense* in pot trial experiment describes in this thesis. The application of the isolated strains as biological control agent in oil palm plantation estates needs to be further investigated. Field application required large-scale production, therefore, the cost of the production takes into consideration. The development of an optimal low-cost media and the delivery method for biological control agents is suggested to overcome the cost concern issue.

Alternate carbon sources, such as compost product, fine oil palm fibres, and fine shredded oil palm trunk are suggested to be used. These can also serve as a carrier for biological control agents in field application. The carbon sources mentioned will be further investigated for the development of low-cost media. The further work of this project study is described as follow:

I. The design of formulation for the development of low-cost media with the use of different carbon sources
   - Compost products
   - Fine oil palm fibres
   - Fine shredded oil palm trunk

II. The delivery system design and testing in field
   - The culture carriers undergo freeze dry process and dissolve in water prior to spraying on the soil and oil palm plants surface.
   - Soil mixture with culture carriers to be used in oil palm plantation.

The endophytic fungus, *Muscodor sp* which produces volatile organic compounds and possesses antibiotic properties could be incorporated into the biofungicide formulation, together with the isolated mycolytic enzyme producing bacterial. This endophytic fungus has the ability to inhibit and kill various plant and human pathogenic bacteria.
and fungi, including soil-borne fungi. This present a possibility for this genus to be used in oil palm industry as biological control agents or biofumigant agents [88]. Dr. Noreha Mahidi has successfully discovered two new Muscodor-like isolates from Sarawak, Malaysia which has been named as Muscodor sarawak and Muscodor padawan. The endophytes isolate has the ability to produce antibiotic VOC’s as well. The further in-vitro screening test of the isolates showed promising results in anti-Ganoderma activities [87]. The two new isolates are suggested to be added to the consortia culture to improve the formulation design of anti-Ganoderma mixture.

5.3 Concluding statements

This thesis study aims to isolate indigenous mycolytic producing microorganisms that possessed anti-fungal properties against phytopathogenic fungus, Ganoderma boninense in Sarawak soil. Several assays had been carried out to evaluate the effectiveness of the isolated strains as potential biological control candidates. The aims and objectives of this study have been successfully achieved. The findings obtained in this study have suggested that the isolated strains were potential to act as biological control agents against phytopathogenic fungus, G. boninense in oil palm cultivation.
References


F.W.I. Kuek, Coastal Bacterial Communities: Their Potential Roles in Dimethylsulphide (Dms) Production and Coral Defence, Swinburne Univ. of Technol., 2014.


Appendix

1. Permit for soil samples collection from Sarawak Biodiversity Centre.

AGREEMENT PERTAINING TO ACCESS TO, COLLECTION OF AND RESEARCH ON THE BIOLOGICAL RESOURCES

This AGREEMENT is made this 18th day of November 2015 BETWEEN THE GOVERNMENT OF SARAWAK (hereinafter referred to as "the Government") and for the purpose of this Agreement is represented by the Sarawak Biodiversity Council, KM 20 Jalan Borneo Heights, Semengoh, Locked Bag 3032, 93990 Kuching, Sarawak of the one part

AND

ASSOC. PROF. DR PETER MORIN NISSOM (IC No: 670105-13-5431) of 186, Lorong 5 (2), Jalan Stukan, 93250, Kuching, Sarawak.

AND

MS WEE SHUI SHUI (IC No: 900421-13-7392) of Lot 2609, Lorong 2, Jalan Semaba, 93250, Kuching, Sarawak.

AND

DR. TAN LEE TUNG (IC No: 720722-13-5085) of 1201, Tabuan Jaya Baru 1, Lorong 13E, Jalan Stutong, 93350, Kuching, Sarawak.

(hereinafter referred to as "the Researcher") of the other part.
2. The ten-fold serial dilution performed in screening assay.
3. **Recipe of chitin agar used:**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount needed for 1 litre CMC agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry colloidal chitin powder</td>
<td>4.0 gram</td>
</tr>
<tr>
<td>(R&amp;M, Edmonton, Canada)</td>
<td></td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>0.7 gram</td>
</tr>
<tr>
<td>(Bendosen, Selangor, Malaysia)</td>
<td></td>
</tr>
<tr>
<td>Monopotassium phosphate</td>
<td>0.3 gram</td>
</tr>
<tr>
<td>(Bendosen, Selangor, Malaysia)</td>
<td></td>
</tr>
<tr>
<td>Magnesium sulfate pentahydrate</td>
<td>0.5 gram</td>
</tr>
<tr>
<td>(Bendosen, Selangor, Malaysia)</td>
<td></td>
</tr>
<tr>
<td>Iron (II) sulfate heptahydrate</td>
<td>0.01 gram</td>
</tr>
<tr>
<td>(Sigma-Aldrich, St Louis., USA)</td>
<td></td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>0.001 gram</td>
</tr>
<tr>
<td>(Sigma-Aldrich, St Louis., USA)</td>
<td></td>
</tr>
<tr>
<td>Manganese chloride</td>
<td>0.001 gram</td>
</tr>
<tr>
<td>(Sigma-Aldrich, St Louis., USA)</td>
<td></td>
</tr>
<tr>
<td>Agar-agar powder</td>
<td>20.0 gram</td>
</tr>
<tr>
<td>(Bendosen, Selangor, Malaysia)</td>
<td></td>
</tr>
<tr>
<td>Deionized water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

**Note:** The final pH of the chitin agar was adjusted to pH 8.0 with the used of 5 M sodium hydroxide, NaOH.
4. Pot trial experiment set up