Faculty of Resource Science and Technology

Production and Characterization of Bioactive Metabolites Produced by Antagonistic Bacteria Using Different Types of Carbon Sources

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PRODUCTION AND CHARACTERIZATION OF BIOACTIVE METABOLITES PRODUCED BY ANTAGONISTIC BACTERIA USING DIFFERENT TYPES OF CARBON SOURCE

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Declaration

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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LIST OF ABBREVIATIONS

Cm	Centimetre
g/ml	Gram per millitre
MEA	Malt extract agar
mm	Millimetre
rpm	Rotate per minute
w/v	Weight per volume
µl	Microlitre
%	Percent
ºC	Degree Celcius
pH	Potential hydrogen
spp.	species

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PRODUCTION AND CHARACTERIZATION OF BIOACTIVE METABOLITES PRODUCED BY ANTAGONISTIC BACTERIA USING DIFFERENT TYPES OF CARBON SOURCES

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ABSTRACT

The Gram-positive rhizobacterium, *Bacillus amyloliquefaciens* have the ability to produce bioactive metabolite to suppress numerous plant fungal diseases. The present study was performed to find out which carbon source, glucose, maltose and sucrose are able to aids the production of bioactive metabolite by the antagonistic bacteria, *B. amyloliquefaciens in vitro* against three plant pathogenic fungi, *Colletotrichum gloeosporioides, Fusarium solani* and *Phytophthora capsici* in dual culture assay. The results showed that after day 7 of dual culture, maltose is showing the highest percentage of inhibition against C. *gloeosporioides* and *P. capsici* that are 19.05% and 90.91%, respectively while sucrose has the highest percentage of inhibition against *F. solani* at 27.14%. However, when the *B. amyloliquefaciens* with maltose and sucrose was assayed on those enzyme assays such as amylase, cellulose, chitinase and protease, the results showed that *B. amyloliquefaciens* with sucrose as carbon source had more activity as compared to maltose. Therefore, these shows that sucrose is a potential carbon source for the production of bioactive metabolite of *B. amyloliquefaciens* against plant pathogenic fungi.

Keywords: *B. amyloliquefaciens*, bioactive metabolites, carbon source.

ABSTRAK

Bakteria rhizobium yang bergram positif, Bacillus amyloliquefacien *smempunyai keupayaan menghasilkan metabolit bioaktif untuk menghalang pelbagai jenis penyakit kulat tumbuhan. Kajian ini telah dijalankan untuk mengenalpasti sumber karbon yang mana antara glukosa, maltose dan sukrosa yang dapat membantu dalam penghasilan metabolit bioaktif daripada bakteria antagonis iaitu* B. amyloliquefaciens *secara* in vitro *terhadap tiga jenis kulat perosak kepada tumbuhan,* Colletotrichum gloeosporioides, Fusarium solani *dan* Phytophthora capsici *secara pengasaian dual kultur. Keputusan selepas tujuh hari dual kultur menunjukkan bahawa, maltosa mempunyai peratus perencatan yang tertinggi terhadap* C. gloeosporioides *dan* P. capsici *iaitu 19.05% dan 90.91% masing-masing manakala sukrosa mempunyai peratus perrencatan tertinggi terhadap* F. solani *iaitu 27.14%. Walaubagaimanapun, apabila* B. amyloliquefaciens *dengan sumber karbon maltosa dan sukrosa diuji dengan pengasaian enzim iaitu amilase, selulase, kitinase dan protease, pengasaian menunjukkan bahawa* B. amyloliquefaciens *dengan sukrosa sebagai sumber karbon mempunyai lebih banyak aktiviti enzim berbanding maltosa. Oleh sebab itu, kajian ini menunjukkan bahawa sukrosa mempunyai potensi sebagai sumber karbon untuk menghasilkan metabolit bioaktif* B. amyloliquefaciens *dalam merencat pertumbuhan kulat perosak tumbuhan.*

Kata kunci: B. amyloliquefaciens, metabolit bioaktif, sumber karbon.

1.0 INTRODUCTION

The main threat to food production and food storage worldwide are fungi that are pathogenic to crop and post-harvested vegetables. Farmers and producers have become increasingly dependent to chemical fungicides such as metalaxyl and phosphonates. However, continuous use of these chemicals has led to the emergence of pathogen resistance and severe negative ecological impacts (Dinu *et al*, 2007). Through times, the chemical fungicides is becoming less effective in prevent and inhibiting the diseases. Consumers also started to demand for chemical-free food. The use of chemical compounds has failed to control plant disease due to resistance, environmental pollution, and damages human health (Arguelles-Arias *et al*., 2009). This problem is becoming harder to solve and need bigger fund to support the management. There is the need for a new, sustainable, cost effective and environmental friendly agriculture management to supply adequate and healthy food to certain area of the world population (Strobel & Daisy, 2003). Because of these disadvantages, the use of microorganism for pathogen control and for plant growth promotion is becoming more common (Issazadeh *et al*., 2012).

According to Montealegre *et al*. (2003), we are in need to have biological control as for the present and future regulations on the use of chemical fungicides such as methyl bromide, and considering that treatments must prevent environmental pollution. Antagonistic bacteria are being used as the model biological control agents because they are easy to handle, possess rapid growth and mediate aggressive colonization (Karlovsky, 2008). These bacteria may facilitate biological control activities by one or more types of mechanisms of diseases suppression (Weller, 1988). In this project, antagonistic bacteria such *Bacillus amyloliquefaciens* was employed and cultured in a medium containing different types carbon

source namely, sucrose, glucose and maltose to observe the effect of these different types of carbon on the bioactive metabolites production by the bacteria.

Therefore the objectives of the research project are:

i. To produce bioactive metabolite by the selected antagonistic bacteria, *Bacillus amyloliquefaciens* using different types of carbon sources.

ii. To determine the most suitable types of carbon to produce bioactive metabolites.

iii. To test the antifungal activity of *B. amyloliquefaciens* butanol extract.

2.0 LITERATURE REVIEW

2.1 Antagonistic bacteria

Antagonistic bacteria are organism that capable to suppress or inhibit the growth and activity of a certain species. These organisms can be used as biological pest control to inhibit the growth of pathogenic fungi or bacteria. These types of bacteria are important to plant by antagonizing the growth of fungi (Gruntjes, 2013). Antagonists are naturally occurring organisms with the potential to involve in pathogen infection, growth and survival (Chernin & Chet, 2002; Kai *et al.*, 2007).

Some of the antagonistic bacteria that have been studied to have antagonistic activities against phytopathogenic fungi are *Pseudomonas fluorescens*, *Agrobacterium radiobacter*, *Bacillus subtilis*, *Bacillus cereus*, *B. amyloliquefaciens*, *Trichoderma virens*, *Burkholderia cepacia*, *Saccharomyces* sp., *Gliocadium* sp., *Seratia liquefaciens*, *Xanthomonas luminescens,* and *Enterobactera glomerans* (Suprapta, 2012).

The mechanisms of antagonistic bacteria against phytopathogenic fungi are by (i) inhibit the growth of the pathogen using toxins, antibiotics, and surface-active compounds called biosurfactants [antibiosis], (ii) compete for colonization place, the nutrients and minerals, and (iii) parasitism which involves the production of extra-cellular cell wall degrading enzymes such as chitinase and β-1,3glucanase (Chernin & Chet, 2002; Whipps, 2001; De Souza, 2003).

Recently, it has been clear that antagonistic bacteria emit a wide range of volatile organic compounds into the atmosphere. Evidence that has been collected over the past ten

years is showing that these volatiles are not only helps in promoting plant growth, but also to strongly hinder the growth of pathogenic fungi (Weisskopf, 2013).

2.2 *Bacillus* spp.

In this research, *Bacillus* spp is used as the antagonistic bacteria. *Bacillus* spp. are well known rhizosphere residents of many crops and usually show plant growth promoting (PGP) activities that include biocontrol capacity against some phytopathogenic fungi (Calvo *et al*., 2010). The genus *Bacillus* consists of a large number of diverse, rod-shaped Gram-positive bacteria that are motile by peritrichous flagella and are aerobic. Some of the *Bacillus* species are closely related such as *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, and *Bacillus pumilus* (Sansinenea & Ortiz, 2011).

Bacillus spp. is a relatively abundant source of antimicrobials since many species of this genus synthesize antimicrobial peptides. These bacteria in general represent a new and rich source of secondary metabolites that need to be explored. These secondary metabolites exhibit strong antifungal and antibacterial activities and enable the bacterium to survive in its natural environment (Stein, 2005; Calvo *et al.*, 2010). Furthermore, *Bacillus* strains have the advantages of being able to form endospores which confer them high stability as biofungicides or biofertilizers (Calvo *et al*., 2010).However, there are also several species of *Bacillus* species reported to be pathogens such as *Bacillus anthracis*, which is pathogenic to humans and other animals, and also *B. cereus*, which commonly cause of food poisoning (Claus & Berkeley 1986).

2.2.1 *Bacillus subtilis*

The model system for Gram-positive organisms, *B. subtilis* is an endospore-forming rhizobacterium and producing more than two dozen kind of different structures antibiotics. The produced anti-microbial active compounds include predominantly peptides that are either post-translationally modified (lantibiotics and lantibiotic-like peptides) and ribosomally synthesized or non-ribosomally generated, as well as a couple of non-peptidic compounds such as amino sugar, polyketides and a phospholipid (Stein, 2005).

2.2.2 *Bacillus amyloliquefaciens*

Bacillus amyloliquefaciens is a Gram-positive and a spore forming bacteria. It is reported that several strains of *B. amyloliquefaciens* have been reported to have the potential to be used as biological control for several plant diseases (Chiou & Wu, 2003). Other than that, *B. amyloliquefaciens* has been studied extensively as a producer of industrial enzymes, such as a-amylase, subtilisin (a protease which is used as laundry detergents and contact lens cleansers), barnase (a ribonuclease), and antibacterial and antifungal peptide antibiotics (Yu *et al.,* 2002). The rhizosphere colonizing *B. amyloliquefaciens* strain is being distinguished from the related model organism, *B. subtilis* by its ability to stimulate plant growth and to suppress plant pathogenic organisms (Idris *et al.,* 2002; Krebs *et al.,* 1998).

2.3 Pathogenic fungi

Agrios (2005) reported that more than 10, 000 species of fungi are able to cause diseases in plants. There is a significant yield loss of agricultural crops due to diseases caused by pathogenic fungi (Suprapta, 2012). The pathogenic fungi affecting crop and post-harvested vegetables are a main threat to food production and food storage (Arguelles-Arias *et al.*, 2009). Pathogenic fungi also have been responsible for emerging diseases in plants and animal including human beings (Giraud *et al.,* 2009).

2.3.1 *Colletotrichum* sp.

Colletotrichum species interacts with wide range plant species mostly as symptomatic pathogens and sometimes as asymptomatic endophytes. Some kinds of *Colletotrichum* species associated with cacao plant such as *Colletotrichum tropicale* and *Colletotrichum ignotum*, frequently as asymptomatic while *Colletotrichum theobromicola* is associated with foliar and fruit anthracnose lesions of cacao (Rojas *et al.*, 2010). Some known pathogenic fungi that has been reported from a broad range of plant species as hosts are *Colletotrichum siamense* (Prihastuti *et al*. 2009; Yang *et al*. 2009; Phoulivong *et al*. 2010) and *C. gloeosporioides* (Rojas *et al.*, 2010).

2.3.2 *Fusarium* sp.

Fusarium species are being reported as one of the most frequently isolated fungi from soil and plant debris where they act as decomposers and decaying the plant material (Summerbell,

2003). They are ubiquitous in soil and but they are also host-specific pathogens of a number of agriculturally important plants, including pea, sweet potato, and cucurbits (Zhang *et al.*, 2006). An example of disease caused by *Fusarium* species are crown root rot of tomato (Omar *et al.*, 2005).

2.3.3 *Phytophthora* sp.

Phytophthora species commonly known as water moulds, currrently more than 60 *Phytophthora* species have been described and classified in the kingdom Chromista (Yoon *et al.* 2002; Erwin & Ribeiro, 1996). *Phytophthora* species are known that they attacks the basal part of the stem cortex, producing rot and necrosis in tissue of woody species such as olive in either way by root or by air (Del Rio *et al.*, 2003). Some of *Phytophthora* species are also found on commercially important trees, such as pistachio, almond, and fig such as *P. parsiana* (Mostowfizadeh-Ghalamfarsa *et al.*, 2008) and *P. ramorum* has been found to cause oak death in 14 counties of coastal forest in California and in a single county in southern Oregon in the USA (Werres *et al.*, 2001). *P. capsici* is also causing a lot of diseases such as *Phytophthora* blight which is a serious problem in the cultivation Red-peppers (*Capsicum annum L.*), (Akihiri *et al*., 1992).

2.3.4 Mode of actions

Common pathogenic infection methods of fungi (Ma, 2011) involve (i) cell wall degrading enzymes, that these enzymes are used to break down the plant cell wall in order to release the

nutrients inside (ii) toxins, which can be non-host specific to damage all plants, or host specific which cause damage only on a specific host plant and (iii) effector proteins which are secreted into the extracellular environment or directly into the host cell. One method of how the effector protein work is by reducing the plants internal signaling mechanisms or reduction of phytochemicals production (Farber, 2011).

2.4 Bioactive metabolites

Due to the increasing problems associated with the application of fungicides in crops processing such as the emergence of fungicide resistance and the consequent control failures (Fernández-Ortuño *et al.*, 2006), together with public concern over the hazardous effect of chemicals on the environment, growers and farmers had to explore and develop more suitable and environmentally friendly alternatives or complements to chemicals such as biological control. Bacterial strains as biological control agents has received great attention because of the capability of such strains to suppress different plant diseases involving a blend of diverse modes of action (Baehler *et al.*, 2006; Cazorla *et al*., 2006) and the possibilities of these biological control agents with other methods to enhanced the control (Kondoh *et al.*, 2001; Omar *et al.*, 2005; Nofal & Haggag, 2006; Romero *et al.*, 2007).

2.4.1 Volatile organic compounds

It was demonstrated that volatile organic compounds (VOCs) of antagonistic bacteria isolated from soil can influence growth of fungi (Alstrom, 2001; Wheatley, 2002; Kai *et al.*, 2007).

Together, these investigations clearly demonstrated that VOC-mediated interactions between bacteria and fungi occur. Such can be, but it also appears that VOCs of many microorganisms can be species-specific interactions and also can effect on multiple members of the ecological community (Kai *et al.,* 2007). These interactions showed that it inhibited complete mycelium growth to small growth reduction in vitro as well as it lead to mycelial and conidial morphological abnormalities (Chaurasia *et al.,* 2005). The bacteria not only produced the volatiles in vitro but also in the soil, but also as demonstrated in the sealed plates, where soil amended with bacteria was succeed to inhibit the fungal growth (Fernando *et al.*, 2005).

2.4.2 Enzymes

Most alkaliphile bacilli produce various alkaline enzymes, including cellulases, proteases, pullulanases, amylases and xylanases. Numerous species of *Bacillus* have been studied for alkaline protease production, but most potential alkaline protease producers are strains of *B. subtilis, B. amyloliquefaciens, B. mojavensis* and *B. licheniformis* (Rao *et al.* 1998).

Bacillus strains are among the most important industrial enzyme producers due to their capacity to able to produce and secrete large quantities (20–25 g/L) of extracellular enzymes and it is estimated that *Bacillus* spp. enzymes make up about 50% of the total enzyme market (Schallmey*et al.,* 2004). Amylases from *Bacillus* strain have application in a number of industrial processes, such as the food, fermentation, textile, and paper industries (Pandey *et al.* 2000).

2.4.3 Antibiotics

Antibiotics are products of secondary metabolism of group of microorganism during the late growth phase and has low-molecular-mass (>1500kDa) (Demain & Fang, 2000). Among the most frequently produced antibiotics by *Bacillus* are lipopeptide antibiotics that have well-recognized potential biotechnology and biopharmaceutical applications due notably to their surface active properties (Sansinenea & Ortiz, 2011). The potential of *B. subtilis* to produce antibiotics has been recognized for 50 years with peptide antibiotics representing the predominant class.

Bacteriocins are proteins or ribosomal peptides with bactericidal activity towards species that are often closely related to the producer bacteria and display variable molecular weights, biochemical properties, inhibitory spectra, and mechanisms of action. Due to their potential use as natural preservatives, bacteriocins produced by lactic acid bacteria have been the subject of intensive investigation in recent years. Many of antimicrobial peptides such as Bacitracin, tyrocidines, polymyxins, edeined, sirculins, colistins, gramicidins, butirosin complex are being synthesized by *Bacillus spp*. such as *B. licheniformis, B. polymyxa,, B. colistinus,* and *B. circulans* (Cherif *et al.*, 2001; Lisboa *et al.* 2006; Schallmey *et al.*, 2004).

2.5 Production of bioactive metabolites using different types of carbon sources

Figure 1 shows the study done by Ripa *et al.* (2009), the effect of carbon and nitrogen sources on the production of bioactive metabolites. Glucose was proven to be the best carbon source for both cell growth as well as antimicrobial metabolites production by the strain *Streptomyces* sp. RUPA-08PR. Sucrose showed a similar pattern result as the glucose followed by mannose,

fructose, mannitol, rhamnose and xylose respectively. There was no antibiotic was produced when the medium was supplemented with galactose, lactose, raffinose and maltose as a sole carbon source. Carbohydrates such as glycerol, maltose, lactose and some others are known to have interference with the production of secondary metabolites (Demain & Fang, 1995). According to this study, the strain was found to produce high levels of antimicrobial metabolites when the medium is supplemented with 2% glucose as sole carbon source (Ripa *et al*, 2009).

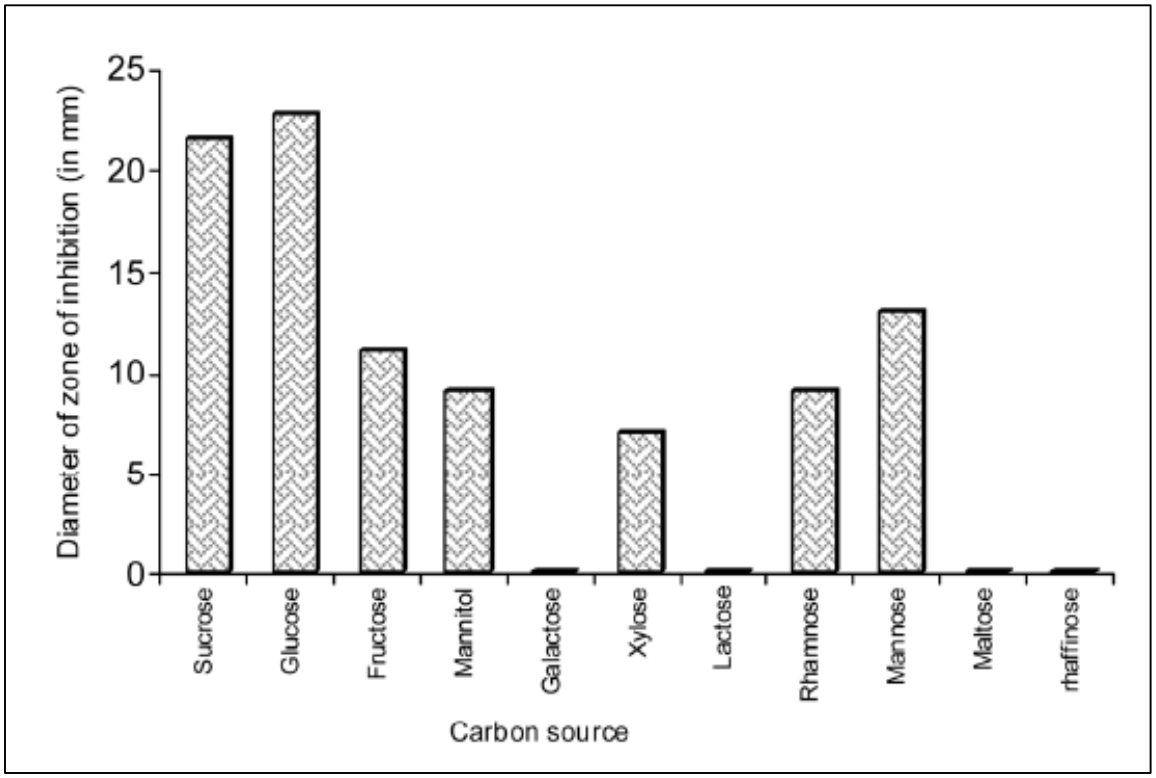

Figure 1. Effect of different carbon sources on the production of antimicrobial metabolites (Ripa*et al*, 2009).

3.0 MATERIALS AND METHODS

3.1 Culture preparation

The bacterium *Bacillus amyloliquefaciens* was used as the antagonistic bacteria tested against pathogenic fungi. The fungal isolates *Colletotrichum gloeosporides, Fusarium solani*, and *Phytophthora capsici* were used as the pathogenic fungi. These fungi and bacteria were obtained from the Molecular Genetic Laboratory, UNIMAS Culture Collection. The bacterium was inoculated into Luria broth (LB) at 37^{o}C overnight, and then was stored in fridge at 4^{o}C before further use. All of the fungal strains were maintained on the V8 agar and MEA for seven days at 27^{o}C until extensive mycelia are seen. This was used as working culture for testing the ability of *Bacillus sp.* to inhibit pathogenic fungi study.

3.2 Stock culture preparation

Bacterial culture was inoculated into 20% (v/v) glycerol stock solution and stored at -20^{o}C. After seven days of cultivation, 5 mm^2 of the agar plug containing fungi was cut and transferred to 20% glycerol stock solution and stored at -20 ^{o}C. These stock cultures must be prepared as a backup preparation of fungal working culture so that inoculums can still be obtained for other sets of experiment.

3.3 Antagonism test using dual culture assay

In the dual plate assay, Luria broth was used to incubate then bacteria prior to be used into the dual plate test. The LB used per liter contains: 20 g of Luria powder. As described in Kai *et*

al,. (2007), *B. amyloliquefaciens* was inoculated into 10ml LB and the bacteria was incubated overnight at 37 ^{o}C and shaken at 160rpm.

The next day, *B. amyloliquefaciens* were inoculated as a streak line on one edge of a 90mm diameter Petri dish and thefungi was cut into 5 mm^2 plug were inoculated onto the M9 salts agar with different carbon sources and incubated at room temperature for 7 days (Ji *et al.*, 2013). The M9 salts agar used per liter contains: 1.36 g of KH_2PO_4, 0.50 g of $(NH_4)_2SO_4$, 0.20 g of $MgSO_4.7H_2O$, 0.01 g of $CaCl_2.2H_2O$, 5 mg of $FeSO_4.7H_2O$, 2.5 mg of $MnSO_4.7H_2O$, 2.5 mg of $Na_2MoO_4.2H_2O$, 2.13 mg of Na_2HPO_4, 15 g of agar and 10ml of 20% (w/v) different carbon sources (Atlas, 2010). The tested carbon sources were glucose, sucrose and maltose. The pH of the M9 salts agar was adjusted to 7.2 ± 2 before autoclaved at 121 ^{o}C for 20 minutes. Controls were prepared by inoculating fungus mycelia plug onto M9 agar without streaking the bacteria. The zone of inhibition was measured by a ruler and recorded at day 7. The percentage of inhibition was calculated based on the formula as shown below (Imran *et. al.*, 2012):

$$\text{Percent inhibition} = \frac{C-T}{C} \text{ X } 100$$

where,

C = The radial growth of fungus in control

T = The radial growth of fungus in treatment

The data were statistically analyzed using statistical software. The means were compared by least significant difference (LSD) test ($P<0.05$).

3.4 Enzyme assay

Hydrolytic enzyme activities (cellulase, amylase, chitinase and protease) were assessed in a qualitative way through the halo zone formation on solid substrate media. To obtain fresh cell populations, the *B. amyloliquefaciens* was inoculated into M9 broth with sucrose as carbon source for a period of 24 hours before being streak onto enzymatic assay medium. This method is being modified from Youcef-Ali *et al*. (2014).

3.4.1 Amylase assay

Amylase agar was prepared. The amylase agar used per liter contains: 10.0 g soluble starch, 10.0 g NaCl and 15.0 g agar (Shanmughapriya *et al.,* 2009). The *B. amyloliquefaciens* was stroked onto the agar and incubated at 30 ^{o}C for 24 to 48 h. Amylase assay plate without inoculating *B. amyloliquefaciens*was prepared as negative control. The halo zone was observed after the agar was flooded with iodine solution for 5 minutes (Youcef-Ali *et al*., 2014).

3.4.2 Celullase assay

Bacillus amyloliquefaciens cultures were tested for cellulose activity using CMC agar. CMC agar contains per liter of 6.0 g Na_2HPO_4, 0.2 g K_2SO_4, 4.0 g NH_4NO_3, 4.0 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.2H_2O$, 0.01 g $FeSO_4.7H_2O$, 15.0 g agar and 1% CMC (Kasana *et. al.,* 2008). The CMC agar plates were then streaked with *B. amyloliquefaciens* and incubated at 37 ^{o}C for five days to allow for the secretion of the cellulase. Cellulose assay plate without

inoculating *B. amyloliquefaciens* was prepared as negative control. The formation of a clear zone of hydrolysis that indicates the cellulose degradation was observed after five days by flooded the agar with iodine solution for 5 min (Ariffin *et al*., 2006).

3.4.3 Chitinase assay

The colloidal chitin was prepared by following Mathur *et al.* (2009) with some modifications. 5 g of standard chitin was grinded then was dissolved in 200 ml 1N HCl by stirring for 2 h at 4 ^{o}C. Two liters of H_2O was added then it was stored at 4 ^{o}C. The precipitate was collected after stored overnight. The colloidal chitin was washed several times with large volume of distilled H_2O until pH 7.0 was obtained. The colloidal chitin was then dissolved in 100 ml dH_2O before being used. The colloidal chitin agar of 1 liter consists of 6.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 1.0 g NH_4Cl, 0.5 g NaCl, 0.05 g yeast extract, 15.0 g agar and 1% w/v colloidal chitin (Kuddus & Ahmad, 2013). The *B. amyloliquefaciens* was streak onto the colloidal chitin agar and incubated at room temperature. Chitinase assay plate without inoculating *B. amyloliquefaciens* was prepared as negative control. The formation of a clear zone of hydrolysis that indicates positive chitinase activity was observed after 2 to 7 days (Youcef-Ali *et al*., 2014).

3.4.4 Protease assay

For protease activity, skimmed milk agar was prepared containing 1.5 % (w/v) agar, 0.5% (w/v) skim milk and 0.5% (w/v) peptone (Katekan *et al.,* 2009). The plates with *B. amyloliquefaciens* were observed after 24 h of incubations at room temperature. Protease