UNIVERSITI PUTRA MALAYSIA

BIOASSAY AND PARTIAL IDENTIFICATION OF NON-VOLATILE BIOACTIVE COMPOUNDS PRODUCED BY *BACILLUS SUBTILIS*

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By

NALISHA ITHNIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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BIOASSAY AND PARTIAL IDENTIFICATION OF NON-VOLATILE BIOACTIVE COMPOUNDS PRODUCED BY *BACILLUS SUBTILIS*

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NALISHA BINTI ITHNIN

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Chairman: **Muskhazli Mustafa, PhD**

Faculty: **Science**

Biological control of plant pathogens is an alternative to the strongly dependence of modern agriculture on chemical fungicides. Extensive applications of chemical control may lead to environmental pollution and development of resistant phytopathogenic fungi strains. It is therefore necessary to develop alternatives to synthetic chemical control to reduce the risks and raise consumer confidence. *Bacillus subtilis* (BS) was used in this study as the biological control agent (BCA) against *Rhizoctonia solani* (RS), *Pythium ultimum* (PU) and *Sclerotium rolfsii* (SR). The first part of this study focus on optimizing BS as BCA by examining application conditions using stability tests and bioassays. The effects of three variables namely temperature (-20^{o}C-100^{o}C), pH (3-11) and light (sunlight, UV and darkness) on the production of bioactive compounds were studied.

From the dual culture bioassay, BS was found to suppress the growth of PU better than RS and SR. Temperature show a considerable effect on BS antifungal activity

with highest inhibition occur on SR at 80^{o}C (58.30%), followed by PU at -20^{o}C treatment (38.68%) and RS at 30^{o}C (35.39%). The optimal pH for antifungal production was pH 3 for RS (51.12%), pH 11 for SR (28.33%) and pH 7 for PU (28.73%). However, neither darkness nor UV treatment altered the antifungal activity. Darkness treatment managed to subdue PU (57.16%), RS (58.30%) and SR (46.24%).

Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used in the second part of this study in order to screen and isolate the bioactive compound produced by BS. Methanolic extracts of BS was found to be the best extraction method from which 2 anticipated peaks with inhibitory activity against PU and *Candida albicans* (CA) were exhibited. However, the activity is more significant when tested against CA compared to PU due to concentration limitation. The TLC profiles of extracts revealed an identical chromatographic mobility to BS iturin A (R_f 0.51) and surfactin (R_f 0.68). Meanwhile through HPLC, homologous compound of fengycin and an iturinic compound were detected.

The final part of this study was to determine the effectiveness of supplementing different carbon sources to BS on its antifungal activity and hydrolytic enzymes production. Bioassay was again applied to record the inhibitory activities. By using 1% (w/v) of three different carbon sources namely oil palm root (OPR), *Ganoderma lucidum* (GL) and ball-milled chitin (CHIT)], respectively, inhibitory activity of BS was induced compared to BS grown in Nutrient Agar (NA). Inhibitory activities (cm ± SD) for each pathogen were as followed: for PU, OPR (3.688 ± 0.01) > CHIT (2.304 ± 0.02) > GL (2.114 ± 0.1); for RS, CHIT (4.171 ± 0.05) > OPR (3.038 ± 0.66)

> GL (2.892 ± 0.06); while for SR, OPR (2.927 ± 0.02) > CHIT (2.854 ± 0.06) > GL (2.843 ± 0.07).

The exposure of selected phytopathogenic fungi to the hydrolytic enzymes such as chitinases, proteases or glucanases was found to degrade the structural matrix of fungal cell walls. BS was found to produce high chitinase in the medium containing CHIT (0.084 U/ml), followed by GL (0.056 U/ml) and OPR (0.051 U/ml), respectively. Meanwhile for β-1,3 and β-1,6-glucanase production, both OPR (1.099 U/ml, 0.716 U/ml) and GL (0.820 U/ml, 1.165 U/ml) showed higher production than CHIT (0.579 U/ml, 0.513 U/ml). The activity of protease was high when BS were cultured with GL (2.579 U/ml), followed by OPR (2.547 U/ml) and CHIT (2.548U/ml).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

BIOASSAI DAN PENGENALPASTIAN SEPARA KOMPONEN BIOAKTIF TIDAK MERUAP DARI *BACILLUS SUBTILIS*

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Kawalan biologi bagi perosak tumbuhan merupakan suatu alternatif bagi mengurangkan penggantungan pertanian moden kini terhadap racun perosak berunsur kimia. Penggunaan bahan kawalan kimia sintetik yang berlebihan menyebabkan pencemaran persekitaran dan penghasilan kulat yang sukar dikawal. Oleh itu adalah satu keperluan untuk menghasilkan alternatif lain selain bahan kawalan kimia sintetik bagi mengurangkan risiko dan meningkatkan kadar keyakinan pengguna. Bagi kajian ini, *Bacillus subtilis* (BS) digunakan sebagai agen kawalan biologi (BCA) terhadap *Rhizoctonia solani* (RS), *Pythium ultimum* (PU) dan *Sclerotium rolfsii* (SR). Bahagian pertama kajian melibatkan penggunaan BS sebagai agen kawalan biologi dengan menguji keadaan aplikasi dengan dalam ujian kestabilan dan bioassay. Kesan 3 parameter iaitu suhu (-20^{o}C-100^{o}C), pH (3-11) dan cahaya (cahaya matahari, UV and gelap) terhadap penghasilan bahan bioaktif adalah dikaji.

Dari ujian bioassay, didapati BS mampu merencat pertumbuhan PU lebih baik dari RS dan SR. Kesan suhu memainkan peranan terhadap aktiviti antikulat BS dengan perencatan terbesar dapat dilihat pada SR dengan suhu 80^{o}C (58.30%), diikuti dengan PU pada -20^{o}C (38.68%) dan RS pada suhu 30^{o}C (35.39%). pH optimum bagi penghasilan aktiviti antikulat adalah pada pH 3 bagi RS (51.12%), pH 11 bagi SR (28.33%) dan pH 7 bagi PU (28.73%). Namun, kesan rawatan cahaya samada gelap atau UV tidak merubah aktiviti antikulat BS secara keseluruhannya. Rawatan gelap merencat PU (57.16%), RS (58.30%) dan SR (46.24%).

'Thin Layer Chromatography' (TLC) dan 'High Performance Liquid Chromatography' (HPLC) telah digunakan pada bahagian kedua kajian ini bagi memperinci dan mengasingkan bahan antikulat yang dihasilkan oleh BS. Didapati ekstrak methanol adalah yang terbaik dimana melalui analisis HPLC, terdapat 2 puncak yang mempunyai aktiviti antikulat terhadap PU dan *Candida albicans* (CA). Namun begitu, aktiviti antikulat ini hanya dapat dilihat dengan jelas terhadap CA berbanding PU akibat kepekatan sampel yang terhad. Profil TLC bagi ekstrak BS ini adalah sama seperti iturin A (Rf 0.51) dan surfactin (Rf 0.68). Manakala dengan menggunakan HPLC, bahan seperti fengycin dan iturin dapat dikesan.

Bahagian terakhir kajian ini adalah bagi mengenalpasti keberkesanan penambahan pelbagai sumber karbon kepada BS terhadap aktiviti antikulat dan penghasilan enzim. Dimana sekali lagi ujian bioassay digunapakai bagi merekod aktiviti perencatan. Dengan menggunakan 1% (mg/L) 3 sumber karbon yang berbeza iaitu akar kelapa sawit (OPR), *Ganoderma lucidum* (GL) dan kitin (CHIT), didapati aktiviti perencatan BS adalah lebih baik berbanding BS yang dikultur didalam Agar Nutrien (NA) sahaja.

Aktiviti perencatan (cm ± r.p) bagi setiap kulat adalah seperti berikut: bagi PU, OPR (3.688 ± 0.01) > CHIT (2.304 ± 0.02) > GL (2.114 ± 0.1); bagi RS, CHIT (4.171 ± 0.05) > OPR (3.038 ± 0.66) > GL (2.892 ± 0.06); manakala bagi SR, OPR (2.927 ± 0.02) > CHIT (2.854 ± 0.06) > GL (2.843 ± 0.07).

Didapati pendedahan kulat perosak tumbuhan kepada enzim seperti chitinase, protease atau glucanase mampu mengurangkan struktur matriks dinding sel kulat. BS didapati menghasilkan enzim chitinase yang tertinggi didalam media mengandungi CHIT (0.084 U/ml), diikuti dengan GL (0.056 U/ml) dan OPR (0.051 U/ml). Manakala bagi penghasilan enzim β-1,3 dan β-1,6-glucanase, kedua-dua OPR (1.099 U/ml, 0.716 U/ml) dan GL (0.820 U/ml, 1.165 U/ml) menunjukkan penghasilan yang lebih berbanding CHIT (0.579 U/ml, 0.513 U/ml). Aktiviti protease adalah tinggi apabila BS dikultur dengan GL (2.579 U/ml), diikuti oleh OPR (2.547 U/ml) dan CHIT (2.548U/ml).

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Above all, I praise Allah for His guidance and continuous blessing upon my life.

THANK YOU

I certify that an Examination Committee has met on 14th March 2007 to conduct the final examination of NALISHA BINTI ITHNIN on her Master of Science thesis entitled "**BIOASSAY AND PARTIAL IDENTIFICATION OF NON VOLATILE BIOACTIVE COMPOUND PRODUCED BY *BACILLUS SUBTILIS***" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date: 17th JULY 2007

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has no been previously or concurrently submitted for any other degree at UPM or other institutions.

NALISHA ITHNIN

Date: 19th JUNE 2007

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LIST OF ABBREVIATIONS

BCA	Biological control agent
NVAF	Non volatile antifungal compound
PDA	Potato Dextrose Agar
NB	Nutrient Broth
UV	Ultra Violet
GlcNac	N-acetylglucosamine
mm	milimeter
cm	centimeter

CHAPTER 1

INTRODUCTION

During the past few years, extensive green house and field trials were conducted to control seed or plant pathogens either by using cultural control or chemical control measures. Crop rotation and cover crop, plowing and seedbed preparation, site selection and planting date, herbicide effects, fertilizer effect, fungicide-treated seed and fungicide soil treatment were examples of many other control measures that have been taken to prevent seed and crop destruction by pathogenic fungi. Even though the cultural control treatments have been an effective traditional procedure in the agricultural practices for a long time, this type of control measure was also found to increase population of the root rot fungi and increase disease severity (Knudsen *et al*., 2002).

Biological control appears to constitute an alternative strategy for controlling diseases, perhaps as part of an integrated control system, thus reducing the use of chemical products and contributing to the preservation of the environment (Souto *et al*., 2004). In biological control, microorganisms or their secretions are biologically used to prevent plant diseases (Wang *et al*., 2002). Biological control of soil borne pathogens by introduced microorganisms has been studied for over 65 years and it is in part a response to public concern about hazards associated with chemical pesticides. It is however, important to know the performance of a biological control in the environment in which it is expected to act upon may lead to improved performance (Collins and Jacobsen, 2003) and wider use of biological control method (Fravel, 1988). However, biological control takes more intensive management and

planning. It is also very often that the results of using biological control are not as dramatic or quick as the results of pesticides used.

Microorganisms are currently considered as biological control agent which offers an environmentally friendly supplement as an alternative to chemical control (Souto *et al*., 2004) with high specificity against the targeted plant pathogens, high degradability after effective usage and low cost of mass production. It may also act as a potent means of reducing the inocula density or disease-producing activities of a pathogen or parasite in organisms (Baker, 1985). Products produced biologically or the microbial cells themselves are called biological control agents (BCAs), which stably inhibit the environment as non-dominant species but still maintain their effectiveness (Shoda, 2000). There have been many studies conducted in order to isolate antifungal agents from a variety of antagonistic microorganisms. The antagonistic activity of these microorganisms may be based on changeable modes of action, including acid and antibiotic production, stimulation of host defense, and direct parasitism of the pathogens or nutrient competition (Whipps, 1997). Therefore, biological control of fungal diseases by applied bacterial inocula has become an important approach to facilitate sustainable agriculture (Lang *et al*., 2002).

Bacillus spp. which showed antagonism are ideal for biological control experiment as they are often soil isolates capable of forming endospores, a characteristic which facilitates long term storage and formulation (Knox *et al*., 2002). *Bacillus* spp. have been used to control a number of leaf spot and post- harvest diseases. They also have shown the capacity to control early leaf spot of peanut, yam leaf spot, post harvest

apple diseases, gray mould of strawberries, and post- bloom fruit drop of citrus (Collins and Jacobsen, 2003).

Bacillus subtilis is a ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. The bacterium produces an endospore that allows it to endure extreme conditions of heat and desiccation in the environment (Knox *et al*., 2002). *B. subtilis* produces a variety of proteases and other enzymes that enable it to effectively degrade a series of biopolymers (i.e proteins, starches, pectins and lipids), thus *B. subtilis* is assumed to contribute to nutrient cycling. However, under most conditions the organism is not biologically active but exists in the spore form (Yu *et al.*, 2002).

It was decided to select for *B. subtilis*, as it is particularly amenable to formulation and long term storage (Powell *et al*., 1990), concerned over inocula viability is less than with gram-negative bacteria (Weller, 1998) and large scale production of bacterial inoculate (Rodgers and Hajjar, 1993). It has been established that specific isolates of *B. subtilis* can operate under subtropical climatic conditions, where the higher ambient temperature favor to bacterial growth and metabolism (Fiddaman and Rossall, 1995). Beside, the potential risk associated with the use of *B. subtilis* in fermentation facilities is low.

The use of *B. subtilis* as a biological control agent of fungal plant pathogens is being investigated because of the effects of antifungal compounds on *Monilinia fructicola* (McKeen *et al*., 1986), *Aspergillus flavus* and *A. parasiticus* (Kimura and Hirano, 1988), and *Rhizoctonia* (Loeffler *et al*., 1986). Lumsden and Locke (1989) also

conclude that effective biological control inhibition of fungal growth in green house and field controls of fungal diseases have been achieved with the application of *B. subtilis*. Yu *et al*. (2002) carried out *in vitro* trials of *B. subtilis* against variety of plant pathogens. They have evaluated *B. subtilis* with several modified antagonistic experiments and identified several compounds produced that inhibit pathogens growth. Alboleutin, bacitracin, botrycidin, clorotetain, dificidin, fengycin, iturins, mycobacilin, rhizocticins and subtilin have been identified as strong causes in pathogens inhibition process (Zuber *et al*., 1993). These antifungal peptides inhibit the growth of a large number of fungi, such as *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. (Munimbazi and Bullerman, 1998), as well as yeasts, i.e. *Candida albicans* and *Saccharomyces cerevisiae* (Thimon *et al*., 1992).

Besides using *B. subtilis* solely in producing bioactive compounds, raw materials that will act as carbon source to *B. subtilis* were also added. These were aimed for obtaining microbial growth and its productions similar to or higher than that attained by routine laboratory media. The raw materials used included agricultural products, i.e. oil palm root and basidiocarp of *Ganoderma lucidum*, and agro-industrial material such as chitin. These fine materials were easy to find and can be obtained at low cost. The ease in propagating *B. subtilis* to obtain high level of bioactive compounds will undoubtedly lead to rapid development of the capability for industrial-scale production. Mahmood *et al*. (1998) have used several additional substances in their study that would support the growth and probably induce the production of extracellular enzymes and other bioactive compounds in *B. subtilis.*

This study was aimed at the potential application of *B. subtilis* as biological control agent against plant pathogenic fungi. Thus the first objective of the study was to determine the antifungal activities produced by *B. subtilis* in culture filtrate. To achieve this, dual culture test were used to test *B. subtilis* antifungal ability against *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotium rolfsii* and the effectiveness of the antifungal produced. Stability test was then carried out to determine the optimum condition for the growth of *B. subtilis* and to evaluate the stability of the bioactive compounds when tested on extreme temperature, pH and light condition.

After establishing the ability of *B. subtilis* to inhibit the growth of all plant pathogens tested, next objective was to isolate the bioactive compound produced by *B. subtilis*. As for this, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) techniques were carried out. By performing TLC, the compounds produced were visualized in terms of spots or bands formed and further characterized using different staining techniques or detectors. Each band was then subjected to bioautography to detect its antifungal antibiotic activity against plant pathogens and *Candida albicans*. HPLC were used to profile and isolate fractions with activity. This will further explain the existence of compounds that were formed by *B. subtilis* in relation to its antifungal antibiotic activity. A comparison to previous studies conducted by others was done in order to get a brief idea on the compound produced.

Assessment on different carbon source amendments was done to evaluate the potential of utilizing bioproduct to induce antifungal production. The hydrolytic enzymes secreted by *B. subtilis*, cultured in the presence of additional carbon source,

were measured and the correlation between antifungal activity and enzyme production was quantified. This is to tackle the third and final objective which was to determine whether the inhibitory activities shown were also caused by enzyme production.