Antibiotic Production and Growth Inhibiting Activities on Fruit Rot Fungus of Antagonistic *Bacillus subtilis* in Citrus

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ชื่อวิทยานิพนธ์	การผลิตสารปฏิชีวนะและการยับยั้งการเจริญของเชื้อราโรคผลเน่าของส้มโดยจุลินทรีย์ปฏิปักษ์แบซิลลัส ซับทิลิส
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บทคัดย่อ

การควบคุมโรคพืชด้วยชีววิธีโดยใช้เชื้อ *Bacillus subtilis* ได้รับการยืนยันและพิสูจน์อย่างต่อเนื่อง ทั้งยังมีการนำไปใช้ในเชิงการค้าอย่างกว้างขวาง งานวิจัยครั้งนี้มุ่งเน้นที่จะศึกษาและประเมินศักยภาพของเชื้อปฏิปักษ์ *B. subtilis* ในการยับยั้งเชื้อราโรคผลเน่าในส้ม (*Penicillium digitatum*) และยังประเมินถึงคุณสมบัติในการกระตุ้นการต้านทานโรคต่อผลส้มด้วย จากการศึกษาพบว่าเชื้อ *B. subtilis* ทั้งสองสายพันธุ์คือ ABS-S14 และ 155 สามารถยับยั้งการเจริญของเส้นใยเชื้อราก่อโรคในระบบจานอาหารทดลองได้ดี รวมทั้งสารสกัดหยาบจากน้ำเลี้ยงเชื้อ เมื่อวิเคราะห์สารสกัดหยาบด้วยเทคนิค TLC, HPLC และ MALDI-TOF พบว่าเชื้อ *B. subtilis* ทั้งสองสายพันธุ์ผลิตสารกลุ่ม lipopeptides ซึ่งประกอบด้วย fengycin, iturin และ surfactin เมื่อนำไปแยกกึ่งบริสุทธิ์โดยการขูดออกจากแผ่น PLC แล้วนำมาทดสอบการยับยั้งพบว่าสารกลุ่ม iturin และ fengycin แสดงฤทธิ์ยับยั้งการเจริญของเชื้อราได้ชัดเจน แต่ไม่พบฤทธิ์การยับยั้งจากสารปฏิชีวนะกลุ่ม surfactin นอกจากนี้ *B. subtilis* ทั้งสองสายพันธุ์นี้ยังสามารถผลิตสารระเหยอินทรีย์ (Volatile organic compounds, VOCs) ซึ่งมีฤทธิ์ยับยั้งการเจริญของเชื้อราโดยการกระตุ้นให้โครงสร้างของเส้นใยเกิดความผิดปรกติและยังยับยั้งการสร้างสปอร์ และสมบัติการยับยั้งของสารระเหยขึ้นอยู่กับอาหารที่ใช้เลี้ยงเชื้อโดยอาหาร TSA ให้ผลเปอร์เซ็นต์การยับยั้งได้สูงสุด รองลงมาคือ LA, PDA และ NA ตามลำดับ การทดสอบในระดับ *in vivo* แบคทีเรียปฏิปักษ์ *B. subtilis* สามารถดำรงชีวิตอยู่บนผลส้มโดยไม่มีการเพิ่มจำนวนประชากรนาน 20 วัน และสารสกัดหยาบจากเชื้อ *B. subtilis* สามารถกระตุ้นแอคติวิตี้ของเอนไซม์ peroxidase (POX) และ phenylalanine ammonia-lyase (PAL) ในเปลือกส้มได้อย่างชัดเจน

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ABSTRACT

Biological control by antagonistic *Bacillus subtilis* is a subject that is receiving considerable attention and has now moved from a laboratory scale to patents and evaluations of commercial applications. This study focuses on the determination and interpretation of the potential of *B. subtilis* ABS-S14 and 155 strains to suppress citrus fruit rot disease caused by *Penicillium digitatum*. In addition, the ability of these pathogen antagonists to induce resistance mechanisms in citrus fruit was also examined. Both strains of *B. subtilis* showed strong antagonistic activity to the fungus pathogen *in vitro*. The crude extracts from cell-free cultured broth also showed significant antifungal activities. Upon TLC, HPLC and MALDI-TOF analysis, both strains produced antifungal lipopeptides belonging to the families of fengycin, iturin and surfactin. Partially purified fractions of crude extracts revealed potent antifungal activity related to iturins and fengycins, but not from surfactins. Volatile organic compounds (VOCs) emitted by these two antagonists induced mycelial morphological abnormalities and inhibited the formation of spores. Moreover, fungal inhibition property varied with culture media. Bacterial culture in TSA medium showed the highest level of mycelial inhibition, followed by LA, PDA and NA, respectively. *In vivo* experiment, antagonistic *B. subtilis* survived on the surface of citrus fruit over 20 days and their crude extracts were clearly induced activity of peroxidase (POX) and phenylalanine ammonia-lyase (PAL) enzymes in the citrus rind.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	=	percentage
°C	=	degree Celsius
µg	=	microgram
µL	=	microliter
µm	=	micrometer
A_{470}	=	light absorbance at 470 nanometer
CFU	=	colony forming unit
cm	=	centimeter
e.g.	=	exempli gratia (for example)
Fig	=	figure
g	=	gram
gAP	=	gram acetone power
GC	=	gas chromatography
h	=	hour
H_2O_2	=	hydrogen peroxide
HCl	=	hydrochloric acid
HPLC	=	high performance liquid chromatography
i.d.	=	inner diameter
kDa	=	kilodalton
LA	=	Luria-Bertani agar
LB	=	Luria-Bertani broth
LP	=	lipopeptides
M	=	molar
MALDI-TOF	=	Matrix-assisted laser desorption/ionization-time of flight
mg	=	milligram
min	=	minute
mL	=	milliliter
mM	=	milimolar
mm	=	millimeter

LIST OF ABBREVIATIONS AND SYMBOLS

(Continued)

MS	=	mass spectrometry
N	=	normal
NA	=	nutrient agar
Nm	=	nanometer
nmol	=	nanomol
p	=	para
PAGE	=	polyacrylamide gel electrophoresis
PAL	=	phenylalanine ammonia-lyase
PDA	=	potato dextrose agar
PDMS/DVB	=	polydimethylsiloxane/Divinylbenzene
PLC	=	preparative thin-layer chromatography
POX	=	peroxidase
rpm	=	revolution per minute
sec	=	second
SPME	=	solid phase microextraction
TEMED	=	tetramethylethylenediamine
TFA	=	trifluoroacetic acid
TLC	=	thin-layer chromatography
TSA	=	tryptic soy agar
TSB	=	tryptic soy broth
UV	=	ultraviolet
VOCs	=	volatile organic compounds
α	=	alpha
β	=	beta

Chapter 1

INTRODUCTION

Introduction

Citrus is a very ancient crop known for over 4,000 years and is now only second to grapes as the most widely cultivated crop in the world (Mukhopadhyay, 2004). Citrus fruit is widely consumed both as fresh fruit or juice and is now recognized as being a principal source of vitamin C, a highly effective antioxidant. In addition, citrus flavonoids, concentrated in the citrus peel, have the potential for use as cancer-preventing agents (Pan *et al.*, 2002), for improvements to the LDL/HDL ratio (James *et al.*, 2007) and potential for neuroprotective activity (Hwang and Yen, 2008). Thus, these therapeutic and nutritive values together with its taste and flavor have placed citrus fruits on the essential dietary lists for humans.

Unfortunately, citrus is attacked by several plant pathogens that can affect the fruit quality. In developing countries, where protection and proper handling of fresh fruit is inadequate, losses during transit and storage can represent in excess of 50% of the harvest crop (Eckert and Ogawa, 1985; Wisniewski and Wilson, 1992). Therefore, the reduced production due to yield losses by pathogenic fungal infections after harvesting and during storage is an enormous problem. Worldwide the major cause of postharvest decay of citrus fruit is the green and blue mold caused by *Penicillium* spp. The spores of these two wound pathogens initiate infections through fruit surface injuries that are inflicted during harvesting and/or in careless handling in the packing-house and presents opportunities for citrus fruit rot disease. The disease progression leads to release of spores that will consecutively contaminate other fruits from the packinghouse equipments, storage room, transit containers, and retail marketplace. Therefore, postharvest management to the fruit during harvest and subsequent handling processes, sanitation practices and the maintainance of the natural resistance of the fruit with the aid of abiotic and biotic elicitors becomes a strategic tool to control citrus fruit losses. These beneficial practices, however, are usually not sufficient to completely protect the fruit from development of disease, so agricultural

fungicides such as imazalil and thiabendazole have now been accepted as effective tools to maintain and control postharvest diseases of citrus fruits. However, the fungicide residues on the product are harmful to human health, natural food chains and also have encouraged the development of fungicide-resistant pathogens. Thus, as the world is now increasingly embracing the concepts of "environmentally friendly actions", control of disease by using natural strategies such as the use of antagonistic microorganisms and natural compounds to replace the application of fungicides has attracted considerable attention. Using these strategies to induce resistance to pathogens could provide interesting alternative methods to reduce the application of fungicides and protect against disease.

In recent years, the biological control of postharvest disease and spoilage has gained considerable attention and moved from a laboratory scale to patents and evaluations of commercial applications. The control of disease by using antagonistic bacteria, particularly *B. subtilis* has been shown to be effective as it can outcompete various plant pathogens, such as *Rhizoctonia*, *Fusarium*, *Aspergillus*, *Alternaria* and *Penicillium* (Reviewed in Nagórska *et al.*, 2007) and is now registered under the trade names of Kodiak® (*B. subtilis* GB03), Histick® N/T (*B. subtilis* MBI600), Rhapsody® and Serenade® (*B. subtilis* QST 713). Moreover, *B. subtilis* also has an ability to induce plant immune systems (Ongena *et al.*, 2005, Hanafi *et al.*, 2007, Ongena *et al.*, 2007).

The purposes of this study are to determine and interpret the potential of *B. subtilis* to suppress citrus fruit rot disease caused by *P. digitatum* Sac. with an emphasis on the isolation and identification of secondary metabolites including lipopeptides (LPs), antibiotics and volatile organic compounds (VOCs) that might be involved in the antagonistic mechanisms. The ability of the antagonist and its secondary metabolites to induce disease resistance in citrus fruit was also examined by determining the activity levels of Peroxidase (POX) and phenylalanine ammonia-lyase (PAL) enzymes after treatment with *B. subtilis*. Finally, the population dynamics of the microbe under *in vivo* conditions were investigated to try to understand the inhibitory mechanisms of antagonist on fruit.

1. Review of literatures

1.1 *Citrus* sp.

Citrus, belongs to the *Rutaceae* family, and its origins are from south-east Asia, China and the east Indian Archipelago from at least 2000 BC (Webber *et al.*, 1967; Gmitter and Hu, 1990). Later, citrus fruits were disseminated by Arab traders to the new world via the trade routes of Africa to the Mediterranean and extended to Italy, Spain and Portugal (Scora, 1975). Nowadays, citrus is distributed throughout the world and has become one of the most economically important crops in the world.

Citrus plants are large shrubs or small trees, with thorny shoots and evergreen leaves. The flowers are solitary or in small corymbs with five white petals and numerous stamens and they have a very strong fragrance. The blossoms are therefore sources of essential oils used by perfume manufacturers and in other aromatic products. The fruit of these plants is a hesperidium, globose to elongate, with a leathery rind surrounding segments filled with pulp vesicles. The rind of the fruit consists of a colored outer layer (flavedo) and white inner layer (albedo), as shown in Fig.1.1. Flavedo consists mainly of a cellulosic component but also has oil sacs (storage of essential oils), plastids that provide the rind color and enzymes. The outer cuticle layer of flavedo is covered with wax but has a few stomata, which are closed when the fruit is ripe. In addition, citrus fruit is "tree ripe" meaning that they pass from immaturity to maturity to over-maturity while still on the tree. They do not increase their sweetness or continue to ripen but changes often occur after harvesting and the fruit will finally start to decay. Moreover, the rind color of the fruit depends upon the climatic conditions, in cool winter regions; the rind color changes when ripening, while in the tropical regions; the citrus fruit remains in the green color until maturity, and are greenish oranges.

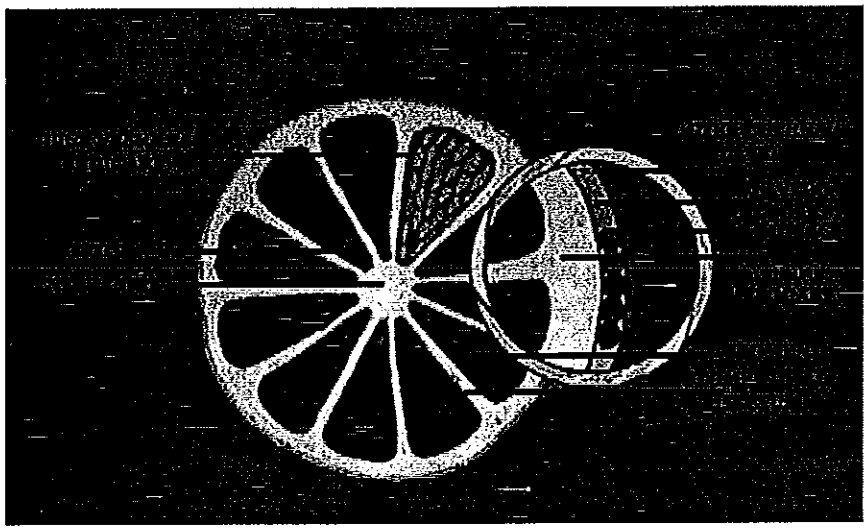

Fig. 1.1. Schematic of the cross section of a citrus fruit

The *Citrus* family is comprised of many varieties because it easily hybridizes. Originally there were three different kinds of citrus including; pomelo, citron and mandarin (*C. grandis*, *C. medica* and *C. reticulata*, respectively). Later, hybrids of these three genera appeared. In the first instance, Lemon (*C. limon*) is a cross between pomelo and citron, Lime (*C. aurantiifolia*) is a hybrid of lemon and citron, Sour orange (*C. aurantium*) and Sweet orange (*C. sinensis*) are hybrids of pomelo and mandarin, while Grapefruit (*C. paradisi*) is a backcross of sweet orange with pomelo (http://users.kymp.net/citruspages/introduction.html#citrus, accessed 25/09/09) These hybrids are being continuously increased by man-made or natural processes. Arising from these abundant variety of citrus fruits, are those of commercial importance; oranges, mandarins, limes, lemons, pomelos and grapefruits.

The Mandarin orange (*C. reticulata* Blanco.) occurs naturally in Asia. Tangerine is an alternative name for mandarin, but the trend, is usually towards a deeper orange to reddish-orange and is smaller in size than the common mandarin orange (http://www.answers.com/topic/tangerine, accessed 25/09/09). The remarkable characteristic of this variety are its easy-peeling, attractive taste and seedlessness. This is why buyers add this fruit to their dietary list. Many varieties of mandarin oranges derived from either human or natural processes are cultivated. In Thailand,

the mandarin orange is also called "Khieo Waan" (*C. reticulata* Blanco cv. Khieo Wann) which refers to its green color rind and sweet to sour-sweet taste. There are many cultivars of mandarin oranges that depend on their region of cultivation. Shogun (*C. reticulata*, Blanco cv Shogun) and Sai nam phuend (*C. reticulata*, Blanco cv Sai Nam Pung) oranges are the variety of Khieo Wann orange that is largely cultivated in the south and north part of Thailand. Nowadays, mandarin oranges are probably the best known edible citrus; they are commercially cultivated and have become of great economic importance.

1.2 Green mold disease of postharvest citrus fruit

Unfortunately, due to their nutritive value, citrus are not only a favorite attraction to humans but also to pathogenic microorganisms. Citrus can be invaded by many pathogens both pre and post-harvesting and these pathogens affect the quality of the fruit. Pre-harvest diseases are mainly caused by fungal pathogens such as *Phytophthora* spp. that initiate infection during wet periods before harvest while *Botrytis cinerea* can infect in plantations and during storage. On the other hand, Post-harvest infections by wound pathogens such as green and blue molds are caused by *Penicillium* spp. (Droby *et al.*, 1989), anthracnose is caused by *Colletotrichum gloeosporioides* Penz (Davies and Albrigo, 1994) and sour rot is caused by *Geotrichum candidum* Link ex Pers (Chalutz and Wilson, 1990). These are all of importance and cause yield losses worldwide.

Green mold disease caused by *Penicillium digitatum* is the major postharvest disease of citrus fruit. The pathogenic fungus, *P. digitatum* is the only one representative species in the genus *Digitata* that can be found in soil air and storage rooms. The name *Penicillium* comes from penicillus that mean brush appearance, which is based on the production of typical brush-like head structures (Fig 1.2A). The stripe structure of *P. digitatum* is called the conidiophores. The conidiophores are biverticillate branches and irregular in shape. At the end of each branchlet is a cluster of spore-producing cells called phialides. The phialide of *P. digitatum* has a long cylindrical shape. A chain of spores is formed from each phialide. The spore, called a conidium, is ellipsoidal to cylindrical, smooth-walled and contains green pigments

that give the colonies their characteristic color and is exceptionally large for the *Penicillium* family. The conidia are formed by blastic phialidic conidiogenesis, where a conidium develops from a distinct bud-like protrusion on the phialide and is ejected from the open end of the phialide tip (Fig 1.2B). The dispersal spore can live up to several weeks at room temperature, then under the appropriate conditions, especially with moisture, a conidium initiates polar growth by producing a germ tube which then becomes the first hyphae. The hyphae of *P. digitatum* are pale and brightly colored and also have a septate structure. The septate structure has a pore region to allow the cytoplasm and nuclei to pass into another cell compartment and thus limit the loss of cytoplasm if the hyphal wall is ruptured.

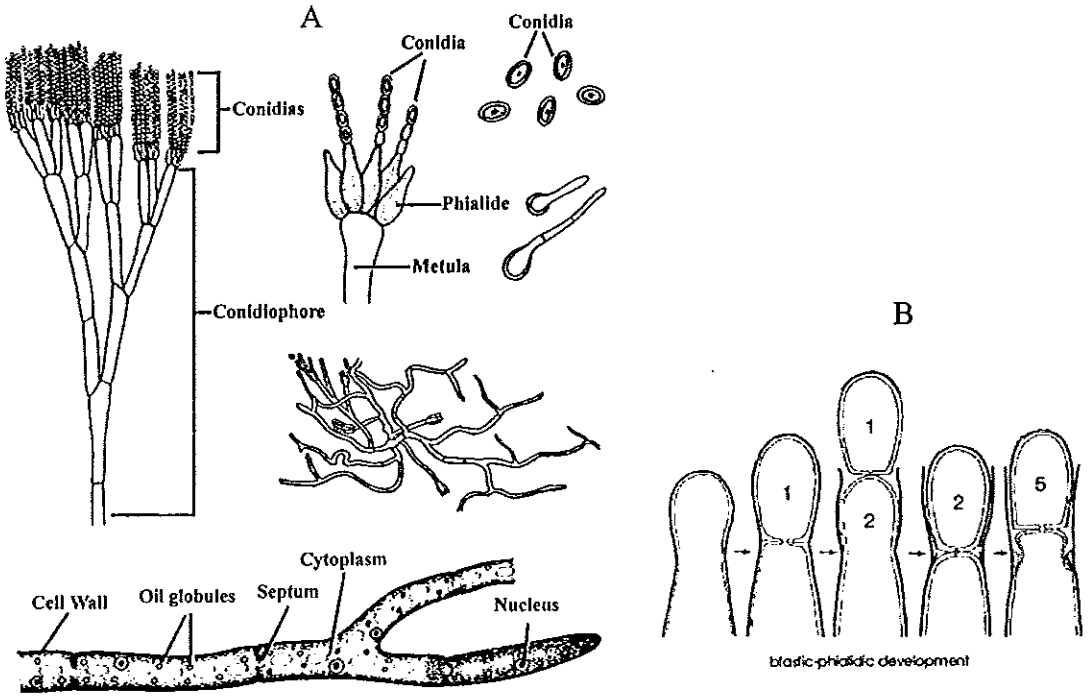

Fig. 1.2. Morphology of the structure (A) and conidia formation (B), of *P. digitatum* (www.mycolog.com /CHAP4a.htm)

The *P. digitatum* grows poorly at low water activity and higher temperatures and is the only species in the *Penicillium* family that grows poorly on Czapek agar and cannot use nitrate as sole nitrogen source (Fergus, 1952). This species has only been found on rotting citrus fruits and causes an enormous problem in the postharvest management of citrus fruit production. The *P. digitatum* causes a rapid breakdown of fruit by entering through a wound that occurs during harvesting and packing operations. This fungus can emit ethylene to accelerate fruit ripening and produces an arsenal of enzymes that share in the severity of the disease symptoms. After a few days following infection, it covers the fruit with a white mycelium and then later from 3-6 days turns to the green of conidia on the fruit (Fig 1.3). The decaying fruit becomes soft and shrinks in size.

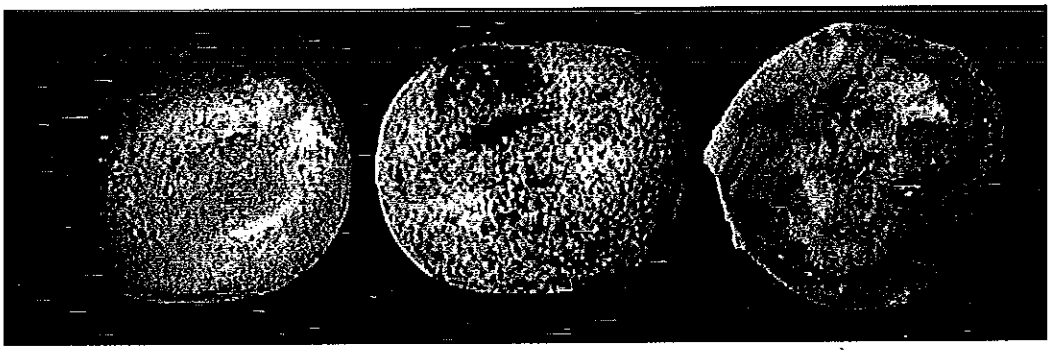

Fig. 1.3. The decay of citrus fruits after *P. digitatum* infection of a wound.

Moreover, *P. digitatum* can also produce mycotoxins during colonization of the fruit. The mycotoxin is dangerously toxic and is produced to supplement the pathogens survival by inhibiting the growth of both Gram positive and negative bacteria as well as other fungi. The production of antibiotics will inhibit competition and protect the substrate for the exclusive use of the fungus. Therefore, if bulk quantities of fresh contaminated-fruit are squeezed for juice production making it impossible to control the quality of individual fruit, the juice will most likely be contaminated with mycotoxin.

1.3 Postharvest control of citrus disease

In order to control plant disease and spoilage all over the world, the first choice of agricultural management is to apply chemical fungicides to protect crops (Tripati and Dubey, 2004). The commercial fungicides routinely used to treat citrus after harvesting are sodium *o*-phenylphenate (*o*-phenylphenol), imazalil and thiabendazole. However the repeated use of fungicides has encouraged the selection of resistance strains. The first resistant biotypes of *P. digitatum* were reported in 1970 (Harding, 1962; Harding, 1972) and in 1987 (Eckert, 1987) for *o*-phenylphenol and thiabendazole and imazalil. Nowadays humans are becoming more aware of environmental hazards and the pressure to reduce fungicide residues in foods has been increasing.

Among the practices now used to control the use of fungicides to prevent plant disease, there have been many attempts to develop alternative biological control methods for example by using microorganisms that are antagonistic to the pathogens. Many species of bacteria, yeasts and fungus have been used for controlling diseases of pome fruit (Mercier and Wilson, 1994; Janisiewicz *et al.*, 2000), apple (Vero *et al.*, 2002; Spadaro *et al.*, 2002; Batta, 2004), grape (Droby *et al.*, 2002), avocado (Korsten and De-Jager, 1995; Demoz and Korsten, 2006), pear (Zhang *et al.*, 2005) and mango (Korsten *et al.*, 1991; Koomen and Jeffries, 1993; Govender and Korsten, 2006) and some have been registered under trademarks (reviewed in Fravel, 2005). Control of disease by antagonistic microorganisms has featured the production of narrow spectrum antibiotics (Fravel, 1988), competition for nutrition and space (Janisiewicz *et al.*, 2000), direct contact with the pathogen, inducing plant resistant mechanisms (Poppe *et al.*, 2003; reviewed on Ongena and Jacque, 2008) and/or production of volatile antagonists (Fravel, 1988; Fernando *et al.*, 2006 and Kai *et al.*, 2007). Although their modes of action may have been described, it is essential to fully elucidate the mechanism of antagonist to the pathogen. Antibiotic production and inducing plant resistance are the action modes for an antagonist that have been demonstrated by many research groups and have been used as critical points to screen for new biocontrol agents for postharvest application.

1.4 Biocontrol of plant disease with antagonistic *Bacillus subtilis*

Bacillus represents a family of Gram positive, motile and spore-forming bacteria that are ubiquitous in nature (soil, water, and airborne dust). *Thus,* their endospore-forming characteristic allows these bacteria to survive and tolerate many different conditions. In addition, the *Bacillus* genus is well known and made use of as cell factories for the commercial production of such biologically active compound as cellulase and protease enzymes, antibiotics and amino acids.

In citrus, *Bacillus* spp. especially *B. subtilis* has been reported to reduce postharvest decay (Obagwu and Korsten, 2003, Leelasuphakul *et al.*, 2008). The ability of *B. subtilis* to control plant pathogen is by producing active compounds such as LP antibiotics and VOCs that inhibit plant pathogens and some strains can also catalyse systemic induced resistance (ISR) in plants (Ping and Boland, 2004; Stein, 2005; Chaurasia *et al.*, 2005; Farag *et al.*, 2006; Ongena and Jacque, 2008).

1.5 The function of lipopeptides against plant pathogens

The potential of *B. subtilis* to produce antibiotics has been recognized for 50 years and approximately 4-5% of their genomes can be devoted to antibiotic synthesis with a potential to produce a range of structurally diverse antimicrobial compounds (Stein, 2005). During the sporulation stage, it can synthesize three distinct families of LP including surfactins, iturins and fengycin to minimize the growth of other bacteria by competition for nutrients. The LPs belonging to the three families are amphiphilic cyclic peptides composed of ten amino acids for fengycins or seven amino acids for iturins and surfactins (Fig 1.4). This peptidyl part is linked to a β-amino- in the case of iturin and a β-hydroxy-fatty acid for surfactins and fengycins.

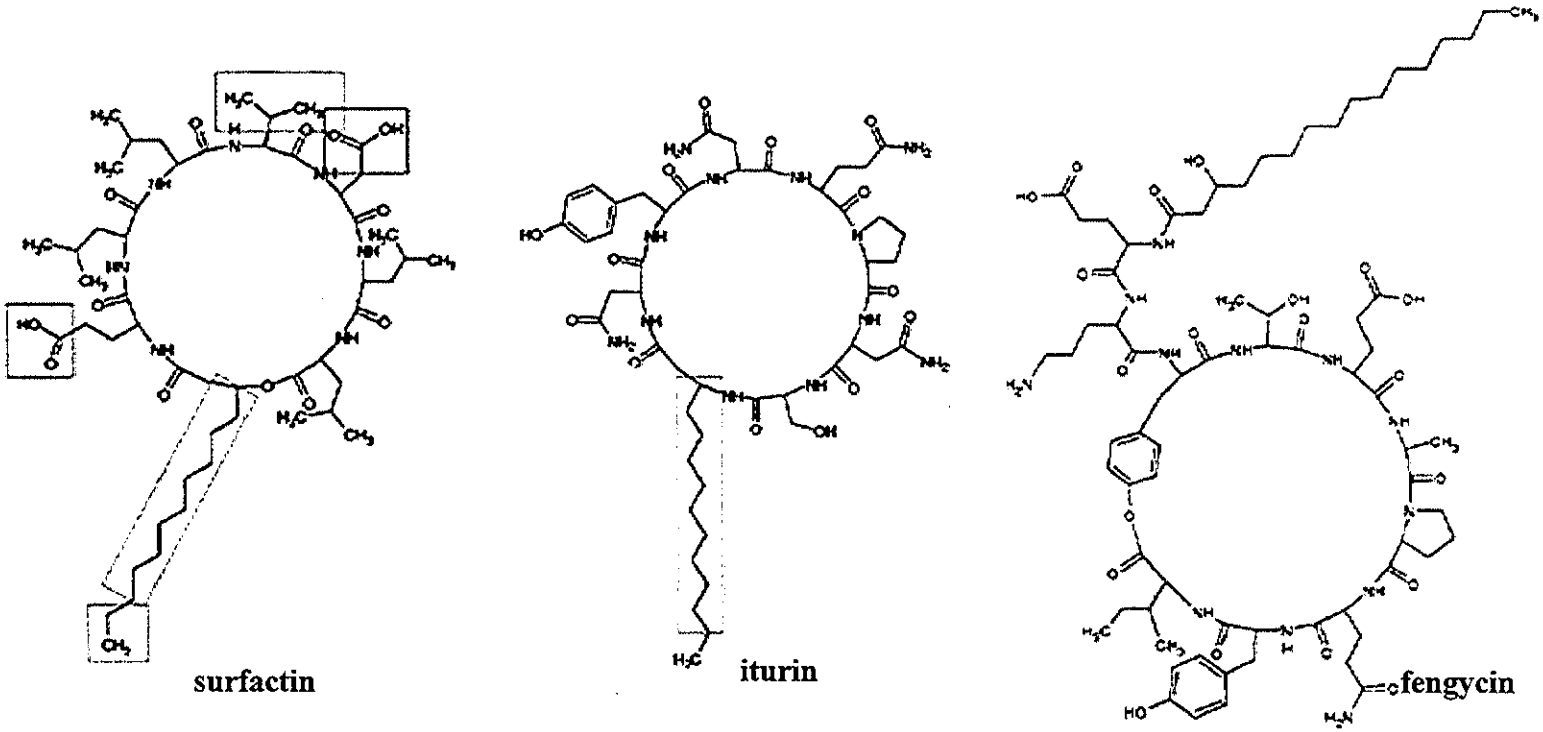

Fig. 1.4. The structures of representative members within the three lipopeptide families produced by *Bacillus* species. The boxed alphabet corresponds to enzymes involved in the biosynthesis of each compound (Ongena and Jacque, 2008).

The fatty acid chain can be of various lengths from C13 to C16, C14 to C17 or C14 to C18 for surfactins, iturins and fengycins, respectively, and there are homologous compounds in each family. LPs are synthesized by a cluster of enzymes via nonribosomal peptide synthetases (NRPSs) or hybrid polyketide synthases and nonribosomal peptide synthetases (PKSs/NRPSs) system. The biosynthesis of surfactin and fengycin homologues consisted of three (SrfA, SrfB and C) and five (FenA-E) enzyme modules, respectively, and are synthesized via NRPS system while iturin homologues are synthesized via a hybrid system (PKS-NRPS) and consisting of three enzyme modules (ItuA-D) involved in the biosynthetic steps (Ongena and Jacques, 2008). The biosynthesis progresses by an enzyme complex that is organized so that each module introduces one amino acid to form a linear peptidyl intermediate. In the final step of biosynthesis (Fig. 1.5), the linear full-length peptide is cleaved off from NRPS by a dedicated thioesterase domain (TE), that usually forms the C-terminal domain of the biosynthetic enzyme cluster and transforms the peptide into a cyclic compound (Samel *et al.*, 2006).

The activities of LPs produced by *B. subtilis* differ due to their different structures. The surfactin family consists of several homologues all of them are heptapeptides (L-Glu, L-Leu, D-Leu, L-Val, L-Asp, D-Leu and L-Leu) linked by a β-hydroxy fatty acid to form a cyclic structure. Surfactin is the most powerful lipopeptide-type biosurfactant with a potential application in petroleum recovery, as an emulsifier-de-emulsifier in pharmaceutical, cosmetics and the food industries (Wei *et al.*, 2003). Surfactin was also found to cause lysis of erythrocytes, inhibits fibril formation and exhibits antibacterial activity but intriguingly has no marked toxicity for fungi. Due to their *in vitro* antibacterial properties, surfactins may play important roles in biocontrol functions. Bai *et al.* (2004) demonstrated that the production of surfactin not only reduced the infection by *Pseudomonas syringae* on *Arabidopsis* plants, but was also necessary for root colonization and biofilm formation. The iturin is a heptapeptide linked to a β-amino fatty acid and has strong haemolytic activity and displays a strong *in vitro* antifungal property against a wide variety of yeasts and

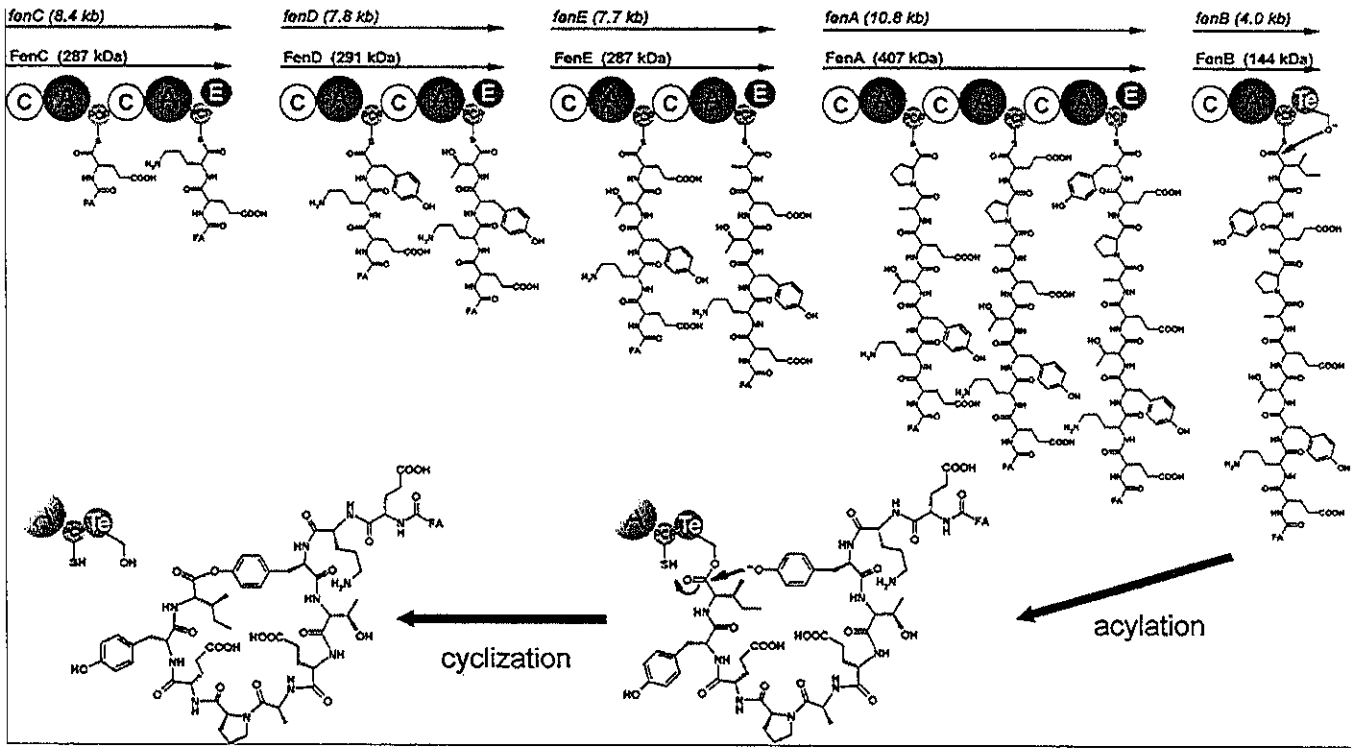

Fig. 1.5. A schematic of some nonribosomal peptide biosynthetic systems. Fengycin biosynthesis consists of five synthetase enzymes modules, FenA-E and a TE domain located at the end of the cluster. Each enzyme module is involved in the introduction of one amino acid to form a linear peptide. Subsequently, the full-length peptide is cleaved from NRPS cluster by the TE domain to form a cyclic structure (Samel *et al.*, 2006).

fungi but has only limited antibacterial and no antiviral activities. The toxicity of iturin to fungal pathogens almost certainly relies on their membrane permeabilization properties (Aranda *et al.*, 2005). The fengycin family is a series of decapeptides linked to a β-hydroxy fatty acid chain. This LP is composed of closely related variants that differ both in the length of the fatty acid chain and the amino acid in position 6 of the peptide moiety (D-Ala or D-Val for fengycin A and fengycin B, respectively).

The involvement of iturins and fengycins in the antibiosis-based biocontrol activity of *Bacillus strains* against various pathogens and in different plant species was shown in many reports. In the case of soil-borne diseases, iturin A produced by *B. subtilis* RB14 is involved in restricting damping-off of tomato (a seedling disease) caused by *Rhizoctonia solani* (Asaka and Shoda, 1996). Overexpression of the closely related mycosubtilin by *B. subtilis* ATCC 6633 also led to a significant reduction of seedling infection by *Pythium aphanidermatum* (Leclere *et al.*, 2005). In the control of phyllosphere diseases, a contribution of both iturins and fengycins was recently shown by their ability to antagonize *Podosphaera fusca* that infected melon leaves (Romero *et al.*, 2007). This was demonstrated by identifying iturins and fengycins as the main antibiotic products excreted by the particular strain of *B. subtilis* that inhibited, the germination of *P. fusca* conidia and by recovering LPs from bacterial-treated leaves and comparisons with the use of LP-deficient transformants. *B. subtilis* S499 efficiently produces LPs from the three families, and notably produces a wide variety of fengycins (Jacques *et al.*, 1999; Ongena *et al.*, 2005). Direct evidence for a role of fengycins in disease reduction derived from experiments showing the ability of strain S499 to protect wounded apple fruits against gray mold disease caused by *Botrytis cinerea*. This role was demonstrated by the effective disease control provided by treatment of fruits with lipopeptide-enriched extracts and by *in situ* detection of fengycins in inhibitory amounts.

1.6 The potential of volatile organic compounds (VOCs) in control of plant pathogen

The antifungal nature of organic volatiles has been demonstrated in several pathogen systems, such as inhibition of hyphal extension and formation of arthrospores in *G. candidum* by trimethylamine (Robinson *et al.*, 1989), inhibition of

carpogenic germination of sclerotia of *S. sclerotiorum* in bean by allyl alcohol (Huang *et al.*, 1997), and control of root rot of tobacco by hydrogen cyanide produced by pseudomonads (Voisard *et al.*, 1989). Other than organic volatiles, inorganic volatiles such as ammonia, produced by *Enterobacter cloacae*, appear to be one of many mechanisms that bacteria use in the biocontrol of pre-emergence damping-off caused by *Pythium* spp. (Howell *et al.*, 1988). Fungal response to bacterial volatiles appears to be species-, environment-, and age-specific (Mackie and Wheatley, 1999). Allyl alcohol inhibits carpogenic germination of sclerotia of *S. sclerotiorum*, but at the same time stimulates growth and enhances sclerotial colonization by *Trichoderma* spp. (Huang *et al.*, 1997). Allyl alcohol also increases populations of beneficial bacteria such as *P. fluorescens* and *P. putida* (Altman and Lawlor, 1966). The chemical nature of the organic volatiles appears to determine their antifungal activity. Aliphatic aldehydes were more effective in the post-harvest control of gray mould caused by *B. cinerea* in strawberry, blackberry and grape (Archibold *et al.*, 1997), and aliphatic aldehydes and ketones were more effective than alcohols in the inhibition of germ tube formation of *Alternaria alternata* (Andersen *et al.*, 1994). The result has been continuously confirmed by Fernando *et al.* (2006) by testing the synthetic volatile chemicals that correspond to the GC-MS profiles from the bacterial culture and showed high fungicidal activity. The two compounds were aldehydes (nonanal and n-decanal), and the other two were alcohols (cyclohexanol and 2-ethyl, 1-hexanol). Alcohol such as 1-hexanol, a relative of 2-ethyl 1-hexanol also have antifungal activity and prevent diseases (Archibold et al., 1997). The sulfur-based compounds, such as benzothiazole and dimethyl trisulfide that was found in the bacteria also showed high antifungal activity. The antifungal of nonanal and decanal also demonstrated toward *Colletotrichum* sp. (Kobaisy *et al.*, 2001). Moreover, Arrebola *et al.*, (2009) found that 3-hydroxy-2-butanone (acetoin) was a major compound produced by *B. subtilis* PPCB001 and it contributed to antifungal activity by reducing the disease incidence and severity in fruit inoculated with *P. crustosum* (Table 1.1).

Table 1.1. VOCs profile of *B. subtilis* strains (Farag *et al.*, 2006)

Type	Compounds
Alcohols	Ethanol, 1-propanol-2-methyl, 1-Butanol, 1-Pentanol, 1-Butanol-3methyl, 1-Butanol-2-methyl, 2,3-Butanediol, 2-Phenyl-2propanol
Aldehydes	Butanal-3-methyl, Butanal-2-methyl, 2,4-Hexadienal, Benzaldehyde
Acids	Glyoxylic acid, Butanoic acid-3-methyl, Acetic acid diethyl
Esters	Ethyl acetate, Acetic acid butyl ester, Butanol-3-methyl-acetate, 2-Butene-1ol-3-methyl-acetate
Ethers	Furan-2-methyl, Furan-2-ethyl, Butane-1-methoxy-3-methyl, Furan-tetrahydro-2,5-dimethyl, Furan-2-pentyl, Hexadecane
Hydrocarbons	Isoprene, Acetylene, Cyclohexane, 1-Undecene, 1-Undecane, Dodecane
Ketones	Acetone, 2,3-Butanedione, 2-Butanone, 3-Hydroxy-2butanone (acetoin), 2-hydroxy-3-pentanone
S-containing compounds	Methanethiol, Dimethyl disulfide, Dimethyl trisulfide
N-containing compounds (Arrebola *et al.*, 2009)	Pyrrole, 2-Methyl-1*H*-pyrrole, *n*-(Diphenylmethylene)aminoacetonitrile

1.7 The induction of plant resistance

Plants possess both preformed and inducible mechanisms to resist infections by pathogens. The inducible defense mechanisms against pathogen attacks can trigger a systemic resistance reaction that renders the host less susceptible to subsequent infections in distal tissues. This phenomenon was termed systemic

acquired resistance (SAR). However, some non-pathogenic bacteria can also induce the resistance in plants to make them more resistant to a new infection. The plant immunity induced by non-pathogenic microorganism such as rhizobacteria is called induced systemic resistance (ISR). The general ISR process includes three main steps; elicitor recognition, systemic signal transduction and the defense response (Ongena and Jacque, 2008). The inductions of plant defense response were presented in Fig 1.6. Fengycin- and surfactin-type LPs secreted by *B. subtilis* act as elicitors with some plant cells and can initiate an immune response through the stimulation of the induced systemic resistance phenomenon. In tomato and bean, purified surfactins and fengycins have been shown to play crucial roles in inducing plant defense mechanisms and provide similar protective activity to that of the bacterial source strain (Ongena *et al.*, 2007). More conclusively, a significant protective effect was produced by plants treated with various LP-overproducing bacterial strains. The macroscopic disease reduction was related to metabolic changes associated with the plant defense responses (Ongena *et al.*, 2007). Ongena *et al.* (2005) demonstrated that potato tuber cells treated with purified fengycins accumulated plant phenolics involved in, or derived from, phenylpropanoid metabolism. This pathway is also well known to be stimulated concomitantly with the activation of plant defense reactions (Dixon *et al.*, 2002). Major changes in the early events of defense related responses after treatment with surfactins, such as increases in phenolics and reactive oxygen species production were observed in tobacco cells as well as there being a significant accumulation of PAL and lipoxygenase activities (Jourdan *et al.*, 2009). *B. subtilis* S499 can also stimulate a systemic defense response in cucumber leading to protection against *C. lagenarium* (Ongena *et al.*, 2005). However, semi-purified LP extracts enriched in fengycins, surfactins and iturins failed to confer protection, to indicate that they might not be involved in the resistance triggering process in that plant. Most of the biological activity of LPs is probably related to their effect on the lipid part of their biological membranes. However, the presence of LPs was not associated with any phytotoxicity or adverse effects on the integrity and growth potential of plant cells. This indicates that these molecules could interact without creating irreversible pore formation but in a way capable of inducing a disturbance or transient channelling in the plasma membrane that can in turn activate a biochemical

cascade of molecular events leading to defensive responses (Ongena and Jacque, 2008). The molecular pattern of ISR induced by LPs is unclear. However, it does seem that each family retains a specific ability to stimulate different plant cells. Surfactins interact with bean, tomato and tobacco but not potato; whereas fengycins can elicit a response in the three solanaceae species and none of the LPs tested so far have any effect on cucumber (Ongena and Jacque, 2008). Intriguingly, ISR-inducing activity of iturins is not observed after treatment of tomato plants, potato tuber slices or tobacco cells. The activity of iturins against fungi and yeasts depends on the presence of ergosterol in their plasma membranes (Bonmatin *et al.*, 2003) The lack of effect on plant cells could be due to their different compositions as ergosterol is replaced by phytosterols mainly composed of sitosterol, stigmasterol, campesterol and in some species cholesterol, and this might lead to an attenuated membrane-disruptive property by iturins (Ongena and Jacque, 2008).

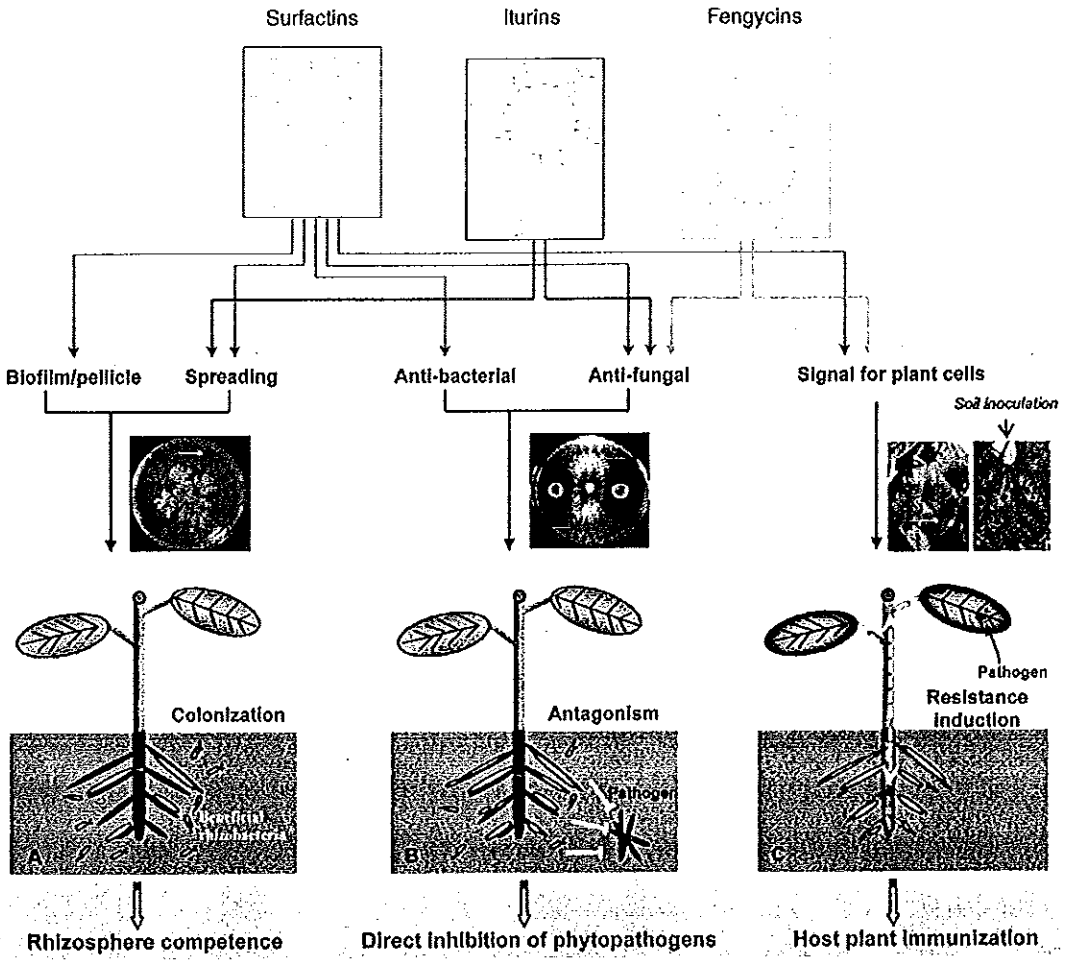

Fig. 1.6. Overview of *Bacillus* lipopeptide interactions in the context of biological control of plant diseases. The three photographs show bacterial spreading, and fungal growth inhibition through the production of fungitoxic compounds by blue bacterial cells and leaf disease reduction following inoculation of the beneficial bacterium onto roots. The phenomena schematically represented in (A), (B) and (C) is the biofilm formation of the rhizobacterium, *in vitro* antibiosis by the biocontrol strain towards pathogens and the arrows illustrate the emission of a signal following detection of the rhizobacterium at the root level, respectively (Ongena and Jacque, 2008).

Besides LPs, the VOCs emitted by rhizobacteria can also regulate plant growth and development. Ryu *et al.* (2003) has found that volatiles from bacteria can trigger promotion of growth and ISR in *Arabidopsis* seedlings when continuously exposed to volatiles for as short as 4 days derived from *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a and showed a significant reduction in symptomatic leaf inoculated with the softrot-causing pathogen, *Erwinia carotovora.* The volatile compounds, 2,3-butanediol and 3-hydroxy-2-butanone (acetoin) were isolated from the headspace of these two plant growth promoting rhizosphere (PGPR), while these metabolite were not detected from non-growth promoting bacteria (Farag *et al.*, 2006). However, several other VOCs were also observed including dodecane, 2-undecanone, 2-tridecanone, 2-tridecanol and tetramethylpyrazine from complex bacterial volatiles that did not exhibit ISR priming activity. To confirm the induced resistance activity of the compound, *Bacillus* strains that were genetically blocked for the production of 2, 3-butanediol were used. The result showed negative phenomena with no disease protection observed in plants. Bacteria employ different mechanisms to produce VOCs e.g., in *Bacillus* spp. strain GB03 and IN937a, 2, 3-butanediol and acetoin were produced under low atmospheric O_2 partial pressure to provide an alternative electronsink for the regeneration of NAD^+ when usual respiration was impossible (Ryu *et al.*, 2004a, Ryu *et al.*, 2004b). The involvement of a known signaling pathway was screened by exposing genetically modified plants to these volatile compounds (Ping and Boland, 2004). Plant resistance induced by exposing them to volatiles from *Bacillus* sp. strain GB03 was independent of salicylic acid, jasmonic acid and Npr1, but it appeared to be mediated by ethylene. In contrast, induction of ISR elicited by volatiles obtained from strain IN937 was independent upon the ethylene signaling pathway.

Finally, *B. subtilis* that efficiently produce a vast array of biologically active compounds (especially LPs and VOC) is unlikely to act only on phytopathogens. These compounds might also affect other microbial population that allows the *Bacillus spp.* to compete effectively with other organisms within the upper- or underground levels of the plant. Moreover, these secondary metabolites could also act as inducers of plant resistance to also prevent attack by pathogens.

1.8 Phenylalanine ammonia-lyase (PAL) a key enzyme in plant defense mechanism

Several defense mechanisms are induced in the plant in response to pathogen infection, such as the induction of phytoalexin biosynthetic pathways (, the intensification of lignifications,the accumulation of cell wall-bound phenolics, and the increased accumulation soluble phenolics, which represents the principal fungitoxic compounds (Dixon *et al.*, 2002). All these biochemical mechanisms induced in the plant depend on the PAL activity. PAL represents the key enzyme for the metabolism of phenolic compounds, phytoalexins, and lignin precursors in plant to fight the invasion of the pathogen. It catalyses the non-oxidative deamination of L-phenylalanine to trans-cinnamic acid and into the cinnamic acid polymers to provide a starting point for cinnamate-based metabolism (Fig 1.7). Several simple phenylpropanoids (with the basic C6-C3 carbon skeleton of phenylalanine) are produced from cinnamate via a series of hydroxylation, methylation, and dehydration reactions. These compounds include *p*-coumaric, caffeic, ferulic, and sinapic acids and simple coumarins. The free acids rarely accumulate to high levels inside plant cells; instead, they are usually conjugated to sugars (e.g., salicylate-glucose conjugates), cell wall carbohydrates (e.g., ferulate esters), or organic acids (e.g., sinapate esters, chlorogenic acid). Although, Salicylic, benzoic and phydroxybenzoic acids are not strictly phenylpropanoids themselves, since they lack the three carbon side chain, they are originated from the phenylpropanoids cinnamate and *p*-coumarate. Moreover, monolignol biosynthetic grid leading to polymeric lignin, oligomeric lignans, and monomeric phenylpropenes which are complex polymers formed from a mixture of simple phenylpropanoids and their composition varies from species to species. The flavonoid pathway leads to anthocyanin pigments, isoflavonoid 'phytoestrogens', polymeric phlobaphenes and condensed tannins, and various antimicrobial phytoalexins. (Reviewed in Dixon and Palva, 1995)

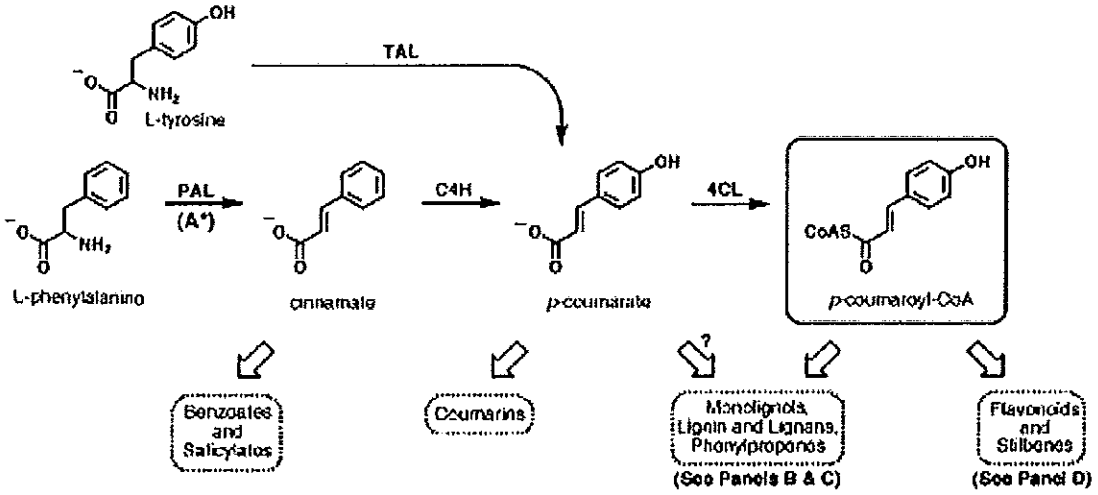

Fig. 1.7. Biosynthesis of phenylpropanoids pathway. The general phenylpropanoid pathway leading from phenylalanine to p-coumaroyl-CoA, the entry point to each major downstream pathway (Dixon and Palva, 1995).

Product diversity emanating from the phenylpropanoids pathway is brought about by a variety of modifications, including regiospecific hydroxylation, glycosylation, acylation, prenylation, sulfation, and methylation. Figure 1.8 is a summary of the types of phenylpropanoid compounds induced in plants by various biotic and abiotic stresses. Many stress-induced phenylpropanoids are classified as phytoalexins. These are antimicrobial compounds synthesized in response to pathogen attack. They include pterocarpans (e.g., glyceollin), isoflavans, prenylated isoflavonoids (e.g., kievitone), stilbenes, psoralens, coumarins, 3-deoxyanthocyanidins, flavonols (e.g., quercetin, kaempferol), and aurones (Dixon *et al.*, 1995). The levels of these compounds increases greatly around the site of infection to produce concentrations that are toxic to pathogens in *in vitro* bioassays. Salicylic acid levels increase in tobacco, cucumber, and *Arabidopsis* in response to infection and exposure to UV light and ozone (Rasmussen *et al.*, 1991; Yalpani *et al.*, 1994), but rather than salicylic acid having any antimicrobial activity per se, it is believed to be part of a signaling process that results in SAR activity (Rasmussen *et al.*, 1991; Delaney *et al.*, 1994). It is not known whether other phenolic compounds play similar signaling roles in plants. Many phenylpropanoid compounds are induced in response to wounding or

to feeding by herbivores. Increased levels of coumestrol and coumarin are toxic to potential herbivores, causing estrogenic and anticoagulant effects, and psoralens can cause photo-induced blistering (Smith, 1982). Wound induced chlorogenic acid, alkyl ferulate esters, and cell wall-bound phenolic esters may act directly as defense compounds or may serve as precursors for the synthesis of lignin, suberin, and other wound-induced polyphenolic barriers (Hahlbrock and Scheel, 1989; Bernards and Lewis, 1992). The accumulation of flavonols such as kaempferol and its glycosides was induced by both wounding and pollination in petunia stigmas and appears to be required for normal pollen development (Mo *et al.*, 1992; van der Meer *et al.*, 1992; Vogt *et al.*, 1994). These flavonoids may also serve to prevent microbial infection in an otherwise nutrient-rich environment.

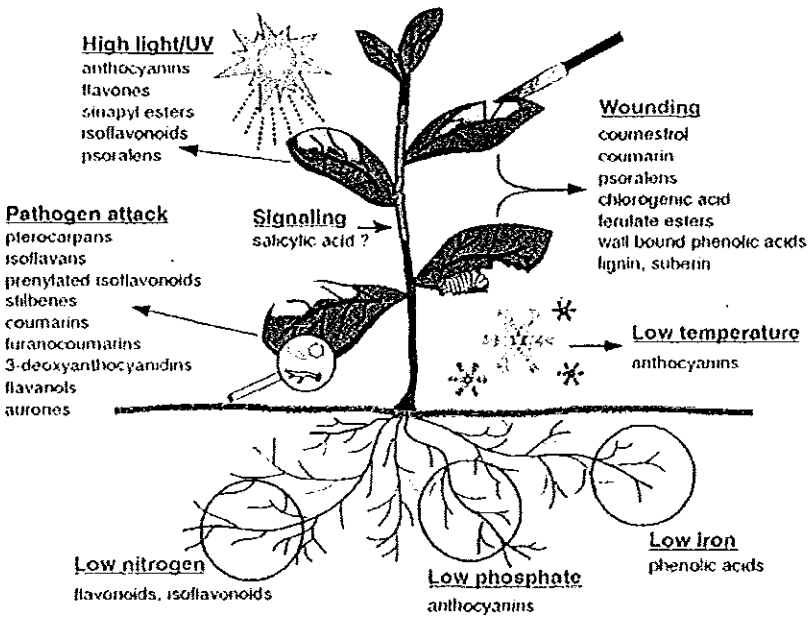

Fig. 1.8. The induction of phenylpropanoid compounds in plant when exposed to various biotic and abiotic stresses. (Dixon and Palva, 1995)

Anthocyanins and flavones increase in response to high visible light levels, and it was thought that these compounds help attenuate the amount of light reaching the photosynthetic cells (Beggs *et al.*, 1987). UV irradiation induces flavonoids (particularly kaempferol derivatives) and sinapate esters in *Arabidopsis* and isoflavonoids and psoralens in other species (Li *et al.*, 1993; Lois, 1994). These UV-absorbing compounds are thought to provide a means of protection against UV-B damage and subsequent cell death by protecting DNA from dimerization and breakage. Other stresses that induce phenylpropanoids have been less well studied. Levels of anthocyanins increase following cold stress (Christie *et al.*, 1994) and nutritional stress (notably phosphate limitation), but the reasons for this increase are unclear. Other nutritional stresses cause increases in the concentrations of phenylpropanoids in roots or root exudates; for example, low nitrogen induces flavonoid and isoflavonoid nod gene inducers and chemo-attractants for nitrogen-fixing symbionts (Graham, 1991; Wojtaszek *et al.*, 1993), whereas low iron levels can cause increased release of phenolic acids, presumably to help solubilize metals and thereby facilitate their uptake (Marschner, 1991).

1.9 Peroxidase is a pathogenesis-related protein associated in plant defense mechanism.

The pathogenesis-related proteins (PRs) are defined as proteins coded for by the host plant but induced especially in pathological or related situations, do not only accumulate locally in the infected tissue , but are also induced systemically. This results in the development of SAR against further infection of the plant by fungi, bacteria and viruses. Induction of PRs has been found in many plant species belonging to various families to indicate that there is probably a general role for these proteins in adaptating to biotic stress conditions (Van Loon and Van Strien, 1999). The PRs have been classified into eleven families (PR-1 to PR-11) based on their grouping into families sharing either amino acid sequences, serological relationships, and enzymatic or biological activities (Table 1.2). Members of the eleven families (PR-1 to PR-11) were recognized and classified from tobacco and tomato, with the families PR-8 and PR-10 being present in cucumber and parsley, respectively (Van Loon *et al.*, 1994). Later three novel new families (PR-12, PR-13 and PR-14) were recognized in radish, *Arabidopsis* and barley, respectively (Van Loon and Van Strien,

1999). Germins and germin-like proteins (GLPs) were classified as PR-15 and PR-16. PR-16 was isolated from hot pepper during its resistance response to bacterial and viral infections (Park *et al.*, 2004 b).

Table 1.2. Classification of the families of pathogenesis-related proteins (Van loon and Van strien, 1999)

Family	Type member	Properties
PR-1	Tobacco PR-1a	unknown
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	chitinase type I, II, IV, V, VI, VII
PR-4	Tobacco "R"	chitinase type I, II
PR-5	Tobacco S	thaumatin-like
PR-6	Tomato Inhibitor I	proteinase-inhibitor
PR-7	Tomato P_{6g}	endoproteinase
PR-8	Cucumber chitinase	chitinase type III
PR-9	Tobacco "lignin-forming peroxidase"	peroxidase
PR-10	Parsley "PR1"	"ribonuclease-like"
PR-11	Tobacco class V chitinase	chitinase type I
PR-12	Radish Rs-AFP3	defensin
PR-13	*Arabidopsis* THI2.1	thionin
PR-14	Barley LTP4	lipid-transfer protein

Peroxidases (POXs) are among the PR-protein member that exists as isoenzymes with diverse expression profiles. Plants contain two classes of heme Peroxidases, class I and class III that usually contain ferriprotoporphyrin IX as a prosthetic group and are able to oxidize various substrates via the reduction of hydrogen peroxide. They participate in various physiological processes, such as lignification, suberization, auxin catabolism, wound healing and defense mechanisms against pathogen infections (Hiraga *et al.* 2001). The plant POXs in the apoplectic space are considered to catalyze the generation of aromatic oxyl radicals from several aromatic compounds (Takahama and Yoshitama 1998), and the POX-dependent production of such organic radicals often results in the generation of reactive oxygen species (ROS) (Kagan, 1988). Currently, a growing number of studies on POX-dependent ROS have been reported, all repeatedly examining and confirming the roles of POX in oxidative signal transduction leading to controls in redox and Ca^{2+} homeostasis, stomatal movements, expression of defense-related genes, gravitropic

responses, cell-wall plasticity and cell elongation in plants. Also due to their involvement in the production or modulation of active oxygen species, then, they may play various roles directly or indirectly in reducing pathogen viability and spread (Lamb and Dixon, 1997)

In addition, several studies have shown that POX can also be induced by non-pathogenic microorganisms associated with an ISR cascade (Podile and Laxmi, 1998, Silva *et al.,* 2004). Nandakumar (1998) found that two POXs and one chitinase (35 kDa) isoform were induced in respectively, and Plant Growth Promoting Rhizobacteria (PGPR)-treated plants inoculated with the rice sheath blight pathogen, *R. solani*. Viswanathan and Samiyappan (1999a) showed that the PGPR-mediated ISR against *C. falcatum*, enhanced levels of chitinase and POX in sugarcane and Zdor and Anderson (1992) also reported a POX activity as well as an increase in the level of mRNAs encoding for the PAL enzyme and chalcone synthase, that led to the synthesis of phytoalexin in the early stages of interaction between bean roots and various bacterial endophytes. Similarly, Podile and Laxmi (1998) observed a peak in peroxidase activity in pea plants treated with *B. subtilis*, seven days after inoculation with *Fusarium udum* and this corresponded to the report by Silva *et al.* (2004) that tomato seeds treated with non-pathogenic rhizobacteria had an extremely high POX activity when compared to other treatments.

OBJECTIVES

1) To isolate and identify the secondary metabolites including LPs, antibiotics and volatile organic compounds (VOCs) produced by *B. subtilis* and their involvement in the antagonistic mechanisms of bacteria toward the plant pathogens.
2) To evaluate the surviving capability of the *B. subtilis* on the citrus fruit surface that necessitated for the antagonistic exhibition.
3) To investigate of the ability of the antagonistic *B. subtilis* and its secondary metabolites to induce disease resistance in citrus fruit by determining the activity levels of the POX and PAL enzymes

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments and Equipments

Instrument	Model	Company
Autoclave	ES-315	Tomy
Bacteria incubator		Memmert
Bioharzard carbinet	BSB 3A	Gelaire
Centrifuge	J2-21	Beckman
	J-30I	Beckman
	EBA 12R	Hettichzentrifugen
Gas chromatography	5890 II Plus	Hewlett packard
Gel documentation	Bio chemi system	UVP
Gel electrophoresis apparatus	Miniprotean	Bio-rad
Haemacytometer		Boeco
Heat block		VWR Scientific
Hot air oven		Binder
Homogenizer		Kinematica
HPLC column	Lichrosphere C18e	Merck
HPLC System	1200	Agilent
Micropipet	P20	Gilson
	P200	Gilson
	P1000	Gilson
Microscope	compound, YS2	Nikon
	stereo	Meiji
Orbital shaker		Gallenkamp
pH meter		Metrohm
Rotary evaporator	R-210	Buchi
Sonicator		Delta
Spectrophotometer	UV-Vis 8435	Hewlett-Packard
SPME apparatus	SPME Holder	Supelco
	PDMS/DVB fibers	Supelco
TLC plate	Silica gel 60 F_{254}	Merck
Water bath		Memmert

2.1.2 Chemicals

Chemicals	Grade	Company
Absolute ethanol	AR	J.T. Baker
Acetic acid	AR	J.T. Baker
Acetone	AR	J.T. Baker
Acetonitrile	HPLC	Lab-scan
Acrylamide	AR	Fluka
Agar	Microbiology	Merck
Ammonium sulfate	AR	Lab-Scan
Ammoniumpersulfate	AR	Merck
Bis-acrylamide	AR	Sigma
Bovine serum albumin (BSA)	Molecular biology	Sigma
Cinnamic acid	AR	Sigma
Coomassie brilliant blue G-250	AR	Fluka
Coomassie brilliant blue R-250	AR	Sigma
D-glucose	AR	Univar
Glycine	AR	Fisher Scientific
Guaiacol	AR	Sigma
Hydrochloric acid	AR	J.T. Baker
Hydrogen peroxide	AR	Merck
Iturin A	HPLC standard	Sigma
L-phenylalanine	AR	Sigma
Methanol	AR	Lab-scan
Methanol	HPLC	Lab-scan
Phosphoric acid	AR	BDH
Sodium chloride	AR	Lab-Scan
Sodium dodecyl sulfate	AR	Sigma
Surfactin	TLC standard	Sigma
Trifluoroacetic acid	AR	Merck
Tris base	AR	Fisher Scientific

*** AR = Analytical Reagent grade**

2.1.3 Bacterial and fungal isolate

- Two strains of *B. subtilis*, ABS-S14 and 155 (Kuyyogsuy et al., 2006 and Leelasuphakul *et al.*, 2008) were used. For experimental use, bacteria were streaked onto nutrient agar (NA) and incubated at 35°C for 18 h. For long-term storage, bacterial spores were maintained at -80°C in Tryptic soy broth (TSB) containing 15% glycerol.

- The fungus, *Penicillium digitatum* Sacc., (Leelasuphakul *et al.*, 2008) was routinely grown on potato dextrose agar (PDA) and incubated at 25°C. In addition, this pathogenic fungus was occasionally inoculated onto citrus fruits for maintenance its virulence.

2.2 Method

2.2.1 *In vitro* study of antagonistic activity using the dual-culture plate method

The ability of *B. subtillis* to inhibit the growth of *P. digitatum* Sacc. was tested by the dual-culture assay. An actively growing mycelium plug (0.1 cm) of fungus were placed in the center of a 9 cm-Petri dish containing PDA medium and incubated at 25°C for 24 h prior to the experiment. The *B. subtilis* was cultured in Luria-Bertani (LB) broth and incubated at 35°C, 200 rpm for 24 h and the cells were harvested by centrifugation at 8,000 rpm for 30 min. The cell pellet was streaked 1 cm away from the growing edge of the mycelium. Dual-inoculated plates, with fungus alone as control, were incubated at 25°C for 2 days. Any inhibition zone between the two cultures was measured and the percentage of mycelium growth inhibition was calculated using the formula: {100-[(radius of treatment^2/radius of control^2) x 100]}. The tests were carried out with three replicates (Gamliel *et al.*, 1989).

2.2.2 *In vitro* study of antagonistic activity by the sealed-plate method

The ability of *B. subtilis* ABS-S14 and 155 to inhibit the growth of *P. digitatum* Sacc. via volatile organic compounds (VOCs) was tested by the sealed-plate assay. The bacteria were harvested from the culture medium (LB medium, 200 rpm 35°C for 24 h), were spreaded on 9 cm-Petri dishes containing NA, LA, PDA and Tryptic soy agar (TSA) medium and incubated for 24 h prior to the experiment. After that, the plates containing bacteria had their lids removed and were then sealed together open face to open face with a PDA plate containing a fungal plug using Parafilm® and incubated at 25°C for 3 days. The percentage of mycelium growth inhibition was calculated as previously described (section 2.2.1). The tests were carried out with three replicates.

2.2.3 Production and extraction of antifungal compounds

The *B. subtilis* strain was grown in LB medium with shaking speed of 200 rpm, 24 h at 35°C for preparation of the inoculums. The seed culture at 10% (v/v) was used to inoculate 400 mL of LB medium in a 1000 mL Erlenmeyer flask. The cultures were incubated on a temperature-controlled shaker incubator at 200 rpm

35 °C for 72 h. After cultivating, the cells were removed by centrifugation at 8,000 rpm for 30 min. The cell-free supernatant was precipitated by adding 6 N HCl to a final pH of 2.0. The precipitates were collected by centrifugation at 10,000 rpm for 30 min at 4 °C, and extracted twice with 80% aqueous ethanol. The culture supernatant was dried using a rotary vacuum evaporator, weighed and re-dissolved in 80% aqueous ethanol to make a 50 mg/mL stock solution.

2.2.4 Determination of antifungal activity of crude ethanolic extract by the paper disc method

The antifungal activity of the crude ethanolic extracts from the *B. subtillis* strains against the fungal pathogen, *P. digitatum* Sacc. was determined by a paper disc assay. An actively growing mycelium plug (0.1 cm) of fungus was placed in the center of a Petri dish containing PDA medium and incubated at 25°C for 24 h prior to the experiment. The paper discs (dia. = 6 mm) containing of crude ethanolic extract were laid on the agar surface 0.5 cm away from the growing edge of the fungal colony. The results were observed after 48 h of incubation at 25°C and the antifungal activity was evaluated by the presence of an inhibition zone. The percentage of mycelium growth inhibition was calculated as previously described (section 2.2.1). The tests were carried out with three replicates.

2.2.5 Thin-layer chromatography (TLC) analysis

The crude ethanolic extracts were analyzed by thin-layer chromatography (TLC) on silica gel 60 F_{254} (Merck, Germany) using chloroform-methanol-water (v/v =65:25:4) as the mobile phase. Spots were detected under UV light and subsequently sprayed with water and subsequently sprayed with 1% ninhydrin (1 g ninhydrin in100 mL methanol).

To determine antifungal activity of each fraction, preparative thin-layer chromatograph (PLC) was used to collect the quantities of compounds. The PLC plate (1 mm thickness, 20x20 cm, Merck, Germany) was streak-loaded with 50 mg of crude ethanolic extract and developed with chromatographic system as mentioned above. After development, the bands on PLC plate were fractionated by scraping-out after visualized with water spraying. The compounds adsorbed onto silica matrices were

then extracted with ethanol, precipitated by centrifugation at 6,000 rpm for 20 min and evaporated to dryness. The resulting compounds were re-dissolved in 80% ethanol and then were tested for their antifungal activities by paper disc assay as previously described (section 2.2.4).

2.2.6 Determination of the EC_{50} of the antifungal activity of the crude ethanolic extracts.

The EC_{50} of the crude ethanolic extract was tested on 2 wells of concavity slides (Picman *et al.*, 1990). The slides were placed on a moistened semicircle of filter paper in a 15 cm. Petri dish plate (4 slides/plate). Each well of the slides contained PDA (Difco) medium previously mixed in a 9:1 (V/V) proportion with different concentrations of the crude ethanolic extracts and 80% aqueous ethanol used as a control treatment. An actively growing mycelium plug (0.1 cm) of the fungus was deposited in the center of the well and incubated in 25°C for 2 days. The radius of the fungal colonies was measured using a stereo-microscope and the percentage inhibition of crude ethanolic extract was calculated as previously described (section 2.2.1). The EC_{50} value was analyzed using the linear regression equation with the Microsoft Excel program.

2.2.7 High performance liquid chromatography (HPLC) analysis

The crude ethanolic extract from the *B. subtilis* strains were fractionated with a Agilent HPLC system model 1200 consisting of quatenary pumps and photodiode array detector (Central Equipment Unit, Faculty of Science, Prince of Songkla University, Thailand). The compounds were separated on a LiChrospher® 100 RP-18e column (dimensions, 0.4 x 125 mm, particle size, 5 µm, Merck, Germany) with the gradient elution mode. The system was operated at a flow rate of 1.0 mL/min, with the mobile phase: acetonitrile (A) and 0.1% (v/v) TFA in MiliQ water (B).The gradient program: 35% (A) for 0 - 5 min; 35% - 60% (A) for 6 - 35 min and 60% - 90% (A) for 36 - 60 min. Peaks in the effluent were detected at 210 nm.

2.2.8 MALDI-TOF mass spectrometry analysis.

To determine the molecular mass of compounds, matrix-assisted laser desorption/ionization-time of fight (MALDI-TOF) mass spectrometry was used. MALDI-TOF mass spectra were recorded using a Bruker Daltonic Ultraflex III MALDI-TOF/TOF instrument (Bioservice Unit, National Center for Genetic Engineering and Biotechnology, Thailand).

2.2.9 Headspace-Solid-phase microextraction coupled with Gas chromatography-Mass spectrometry (HS-SPME-GC-MS) analysis of volatile compounds

2.2.9.1 HS-SPME procedure

To analysis of volatile organic compounds (VOCs) emitted by *B. subtilis*, SPME technique coupled with GC-MS was used. SPME is a sensitive, pre-concentrated technique which routinely applied to extract gas phase sample with ideally limited the interference from the matrices. The principle of SPME technique depends on the coating-material of a fiber that function to absorb the analyses and this technique relies upon two steps including 1) extraction, adsorbed analyses via the fiber from the head-space (gas-phase) of the sample and 2) desorption which analyses were released from SPME fiber to the GC column for analysis (Fig. 2.1).

For sample extraction step, a polydimethylsiloxane-divinylbenzene (PDMS/DVB, 65μm thickness) was choosing according to their property for analysis of volatile compounds. The fiber and manual holder were purchased from Sigma-Aldrich. Before analysis, the fiber was conditioned in the injector of the GC system following the instruction manual provided by the manufacturer (30 min at 250°C).

Samples were prepared by streaking one-day old bacteria cell cultured in LB medium into 20 mL screw-top vial containing TSA medium, and then the vial was hermetically sealed with a polypropylene hole cap and silicone septa. Samples were incubated at 35°C for 2 days. Next, each sample was put into thermostatic water bath for an equilibrium time after with or without sonication.

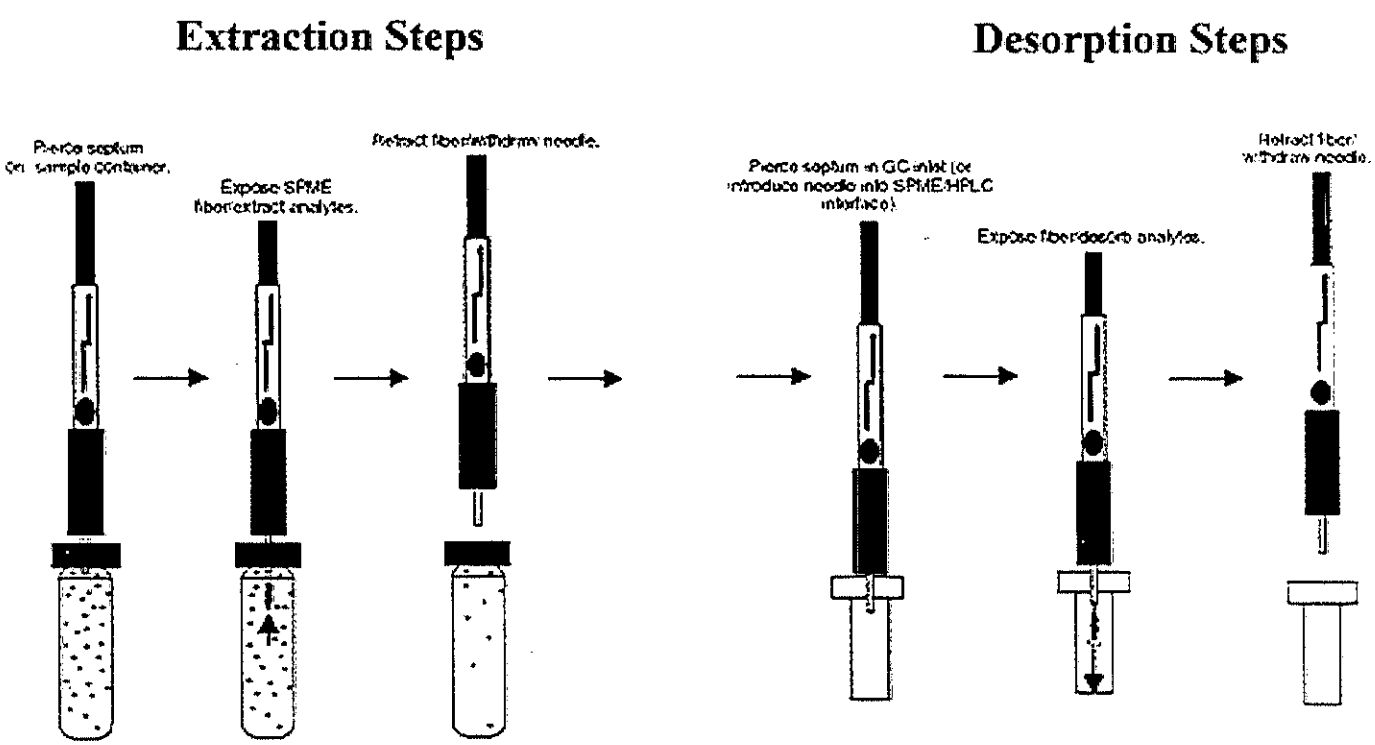

Fig. 2.1. SPME extraction procedures. SPME fiber holder was punctured to the sample vial and fiber was extended for collect compounds from the headspace and then the compounds were released by thermally desorption in GC injector port.

Then, the SPME fiber was inserted into the sealed vial by manually penetrating the septum, and it was exposed to the sample headspace during an extraction time. After the sampling, the SPME fiber was immediately inserted into the GC injector and thermally desorbed.

2.2.9.2 GC analysis

For GC analysis of extracted sample, a Hewlett Packard 5890 Series II Plus gas chromatography system (Department of Biochemistry, Faculty of Scince, Prince of Songkla University, Thailand) equipped with an HP-innowax (60 m x 0.25 mm I.D., 0.5 μm) capillary column (Agilent J&W, USA) and a flame ionization detector (FID) system was employed. To optimize the GC conditions, various injection temperatures and temperature programs were been tested. The final analysis condition was followed: the column temperature was initially maintained at 50 °C for 4 min after injection, increased at 8 °C/min to 200 °C, held for 30 sec, then programmed at 20 °C/min to 230 °C and kept for 1 min. Injections were done using the splitless system. Helium was used as carrier gas. Injector and detector were maintained at 250 °C. A fiber desorption time of 10 min at 250 °C was used. To identify the compounds, the sample was further analysed by GC-MS that consisted of Hewlett Packard 5890 Series II GC instrument and Hewlett Packard 5972A mass detector with condition mentioned above (serviced by Scientific Equipment Center, Prince of Songkla University, Thailand). Compounds were analyzed on Stabilwax® (30 m x 0.25 mm I.D., 0.5 μm) capillary column (Restek, USA). Detector was maintained at 250 °C and was programmed for mass scanning in the range of 50 -500 m/z.

2.2.10 *In vivo* study of the inhibitory activity of *B. subtilis* against *P. digitatum* on citrus fruit

2.2.10.1 Preparation of fruit material

The citrus fruits (*Citrus reticulata* Blanco) were bought at a local market and selected for uniformity of size, ripeness and any fruit with apparent injuries was removed. The fruits were washed with tap water, immersed in 1% sodium hypochorite for 5 min, and finally rinsed with tap water. After air drying at the room temperature, fruits were surface sterilized with 70% ethanol. The sterilized

citrus fruits were pierced by a sterile-needle to make five, 3 mm-depth punctures on the fruit equator. Twenty microliters of solution of each treatment was added onto the wound, T1, distilled water (control); T2, spore suspension of *P. digitatum* (10^4 spores/mL); T3, spore suspension of *B. subtilis* strain ABS-S14 (10^8 CFU/mL); T4, co-inoculated strain ABS-S14 with fungal pathogen; T5, spore suspension of *B. subtilis* strain 155 (10^8 CFU/mL); T6, co-inoculated strain 155 with fungal pathogen; T7, crude extract of strain ABS-S14 (10 mg/mL) and T8, crude extract of ABS-S14 and fungal pathogen. Three replicates of 12 fruits were lined up separately in a plastic box containing a cup of water to maintain a relatively high humidity. All treatments were performed at 25°C for 72 h. Sample tissues (citrus rind) with area of 1.5 diameters concentric to the inoculation site were taken at 0, 24, 48 and 72 h of incubation time. The tissues of each treatment were pooled, frozen in liquid nitrogen and stored at -80°C.

2.2.10.2 Preparation of *B. subtilis* spore suspension

The spore suspension of *B. subtilis* strains was prepared from 3-d-old cultures grown by shaking at 200 rpm 35 °C in LB medium. After the incubation period the cultured medium was centrifuged at 8,000 rpm for 30 min and the pellet was washed twice with sterile distilled water. The spore suspension concentration was adjusted to 10^8 CFU/mL in comparison with the absorbance of McFarland No 0.5 and a viable plate count on NA medium was performed.

2.2.10.3 Preparation of *P. digitatum* conidia spore suspension

Spores of *P. digitatum* were harvested from 7-d old cultures on a PDA plate by flooding the plate with sterile distilled water, gently rubbing surface with bacteriological loop to disperse the spores followed by vortexing the suspension with glass beads. The concentration of spores was determined using a heamacytometer and adjusted to 10^4spores/mL with sterile distilled water.

2.2.10.4 Population study of *B. subtilis* in fruit wounds

The citrus peel with 1.5 x 1.5 cm. square of citrus peel was cut from around a wound with a sterile scalpel and transferred to a 50 mL conical tube containing 10 mL of sterile 0.85% water saline. Bacteria were recovered by shaking at

150 rpm for 5 min. Serial ten-fold dilutions were made and 0.1 mL of each dilution was spread onto NA plates. The plates were incubated at 35 °C for 18 h and the colonies were counted. Population densities of *B. subtilis* were expressed as log CFU/mL.

2.2.10.5 Enzyme activity assays

1. Plant tissue preparation

Tissues were grounded in liquid nitrogen, and 5 g of tissue was taken and extracted with 50 mL acetone, pre-chilled at -20°C. The homogenate were filtered though a What man No.1 filter paper using vacuum suction pump. The sample residues were washed once with cold acetone and the resulting powder was dried at room temperature so called the acetone powdered tissues (AP). They were kept at -20 °C. Protein concentration was determined according to Bradford method (1976) using bovine serum albumin as a standard protein.

2. Assay of peroxidase activity (POX)

POX was extracted from 10 mg of AP with 1 mL of 100 mM phosphate buffer pH 6.0, centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was used for assay of the enzyme activity. The assay mixture (2 mL) consisted of 100 µL of plant extract, 6 µL of guaiacol and 20 µL of H_2O_2. The reaction mixture was recorded every 15 sec for 2 min at absorbency of 470 nm. The maximum slope of the curve was used to calculate the enzyme activity, which is expressed as the increase in absorbance at 470 nm/min/mg protein.

3. Assay of phenyalanine ammonia-lyase activity (PAL)

PAL was extracted from 50 mg of AP with 1 mL of 100 mM borate buffer pH 8.5 containing 20 mM β-mercaptoethanol and centrifuged at 10,000 rpm for 20 min at 4°C to eliminate cell debris. The supernatant was used for the assay of enzyme activity by measuring the absorbance at 290 nm of cinnamic acid over the incubation period for 2 h at 40 °C. The assay mixtures contained 50 µL of enzyme extract and 500 µL of 0.1 M L-phenylalanine in a total volume of 2 mL. The activity of PAL was expressed as nmoles of cinnamic acid /g of AP/ h.

4. Activity staining of peroxidase enzyme on polyacrylamide gel electrophoresis

Plant extracts were separated on discontinuous nondenaturing polyacrylamide gels (10%) prepared in according to Laemmli (1970) and were shown in Table 2.1. After electophoretic separation, the gels were immediately stained for peroxidase activity following the method of Fieldes (1992). The peroxidase stain solution consisting of 10 mM H_2O_2 and 20 mM guaiacol in 200 mL of 100 mM potassium phosphate buffer, pH 6.0 (freshly prepared). The gel was stained with constant shaking for 10-20 min and it was photographed promptly and then dipped in Coomassie fixing solution (150 mL). The fixing solution contained 1% acetic acid, 24% methanol and 3.75 mL of Coomassie stock solution (consisting of 0.75 mg/mL Coomassie Brilliant Blue R-250 in a solution containing 1% acetic acid and 43% methanol). A freshly made solution was used for each polyacrylamide gel. All electrophoretic separations were operated at a constant voltage of 100 V and room temperature.

Table 2.1. Native-polyacrylamide gel electrophoresis (Native-PAGE) recipe.

Composition	Stacking (4%, mL)	Separating (10%, mL)
30% Acrylamide-0.8% Bis-acrylamide	0.67	2.0
0.5 M Tris-HCl, pH 6.8	0.63	-
1.5 M Tris-HCl, pH 8.8	-	3.0
10% Ammonium persulfate	0.05	0.06
TEMED	0.005	0.006
Distrilled water	3.65	0.94
Total volume (mL)	5.0	6.0

2.2.11 Statistical analysis

All data obtained from the experiments were subjected to statistical analysis by the Statistical Package for the Social Sciences (SPSS) version 15.0. The average difference was compared by the Duncan's New Multiple Range Test (DMRT)

Chapter 3

RESULTS

3.1 *In vitro* inhibitory effect of antagonistic *B. subtilis* isolates and their metabolites to citrus fruit rot pathogen, *P. digitatum.*

3.1.1 The inhibitory effect of antagonistic *B. subtilis*

The *in vitro* inhibitory effect of *B. subtilis* isolates against citrus fruit rot pathogen, *P. digitatum* was evaluated by dual-culture plate assay and presented in Fig. 3.1. By this assay, the antagonistic bacteria were grown at temperature charitable to the fungal growth (25°C). The mycelia growth of fungus was inhibited by bacterial-antagonists when compared with the control plate. The mycelia could not cross the line of bacteria and the clear zone of fungal inhibition was shown. The inhibition activity of the antagonists was measured and calculated (section 2.2.1). The *B. subtilis* strains, ABS-S14 and 155 showed similary percentage of inhibition (81.66±0.52 and 80.77±0.84, respectively) (Table 3.1).

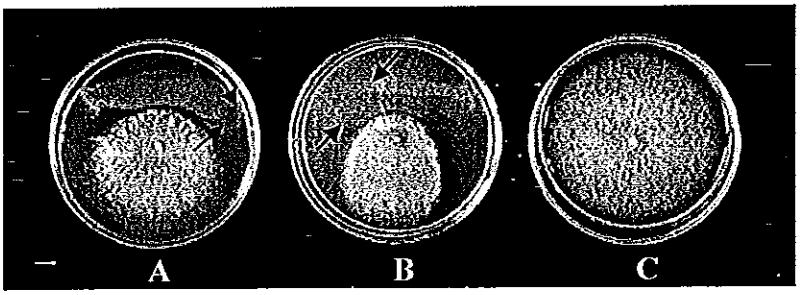

Fig. 3.1. Inhibitory effects of *B. subtilis* on hyphal growth of *P. digitatum.* Inhibition of antagonistic *B. subtilis* strain 155 (A) and ABS-S14 (B) compared with normal morphology of fungal pathogen, *P. digitatum* (C) grown on PDA medium at 25°C for 72 h. The arrows indicated biofilm formation by the antagonistic bacteria.

Table 3.1. The inhibitory percentage of cell and crude extract of *B. subtilis* strains toward *P. digitatum* growth

Strains of *B. subtilis*	% inhibition	
	Dual-culture test	Paper disc test
ABS-S14	81.66±0.52	85.72±0.48
155	80.77±0.84	82.67±0.59

Further investigation of antifungal metabolite production, the *B. subtilis* strains were cultured in LB liquid medium for 72 h at 200 rpm, 35 °C. The antifungal compounds released in cell-free cultured broth were precipitated, collected and extracted with 80% aqueous ethanol and subsequently evaporated to dryness. The resulting crude extract had sticky slimy and brown to dark brown color and then was re-dissolved in 80% aqueous ethanol for further determination of inhibitory effect using the paper disc method. The fungal mycelia were inhibited by the compounds (20 µL of 20 mg/mL) with percentage of inhibition at 85.72±0.48 and 82.67±0.59, respectively for *B. subtilis* ABS-S14 and 155 (Table 3.1), when 80% ethanol was used as control. The result showed the variation depends on the isolate. The crude ethanolic extract from *B. subtilis* strain ABS-S14 gave better inhibition when compared with strain 155 (Fig 3.2). Therefore, the quality of crude ethanolic extract was further determined via the term of half maximal effective concentration (EC_{50}).

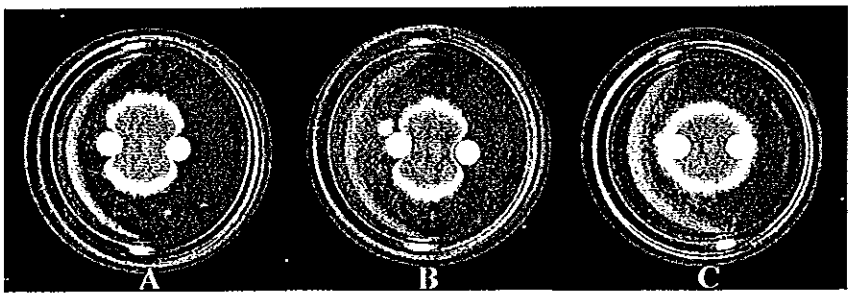

Fig 3.2. Inhibitory effect of crude ethanolic extract from *B. subtilis* culture medium. Each disc containing 10 µL of 5 mg/mL crude extract from *B. subtilis* strain 155 (A), ABS-S14 (B) and ethanol (C) as a control plate.

The EC_{50} is commonly used as a measurement of drug potency and toxicity. It refers to the concentration of compounds which induce a response halfway between the bottom (baseline) and top (maximum) after specified exposure time. In this study, the EC_{50} values of the crude ethanolic extract of *B. subtilis* strains were evaluated from linear regression curves of the concentration of substance (X axis) plot against the inhibition percentage of *P. digitatum* mycelia (Y axis) and calculated using Excel software. The statistical significance was demonstrated when coefficient of determination (R^2) was greater than 0.8. From the experiment, the EC_{50} value of crude ethanolic extract from *B. subtilis* strain ABS-S14 and 155 was 29.71 µg/mL and 82.48 µg/mL, respectively (Table 3.2). The result showed that the efficiency of the extract from *B. subtilis* strain ABS-S14 was much better than strain 155.

Table 3.2. The EC_{50} value of crude ethanolic extract from *B. subtilis* strains against *P. digitatum*

Strains of *B. subtilis*	EC_{50} (µg/mL)	Regression equation	R^2
ABS-S14	29.71	y = 0.2382x + 42.923	0.940
155	82.48	y = 0.2524 + 29.181	0.952

3.1.2 The antifungal activity of volatile compounds from *B. subtilis*

Besides the soluble compounds, the antagonistic *B. subtilis* also produced the volatile organic compounds (VOCs) during their growth. To test the inhibitory effect of volatile compounds produced by antagonistic bacteria, the sealed-plate method was applied. Moreover, the influence of culture medium to volatile production in term of the percentage inhibition was evaluated.

The volatile antifungal activity was observed in all types of culture media. The result indicated that the culture media had influence to volatile production of antagonistic bacteria (Fig 3.3.). TSA medium showed the highest inhibition activity following by LA, PDA and NA medium with the percentage of inhibition at 91.56, 83.27, 59.41, 59.15 for *B. subtilis* strain ABS-S14 and 85.37, 78.53, 49.68, 44.02 for

strain 155, respectively (Fig 3.4). From this result, it appeared that the volatiles produced by the both *B. subtilis* strains showed different degree of inhibitory effect on the tested *P. digitatum* and vary according to the culture media. Interestingly, the fungal pathogen when exposed to VOCs emitted by both bacterial strains could not produce the conidia structure. However, mycelia plug transferred to new PDA plates showed re-growth. The result indicated that the inhibition of volatile from both strains of *B. subtilis* could temporarily inhibit the growth of fungal pathogen.

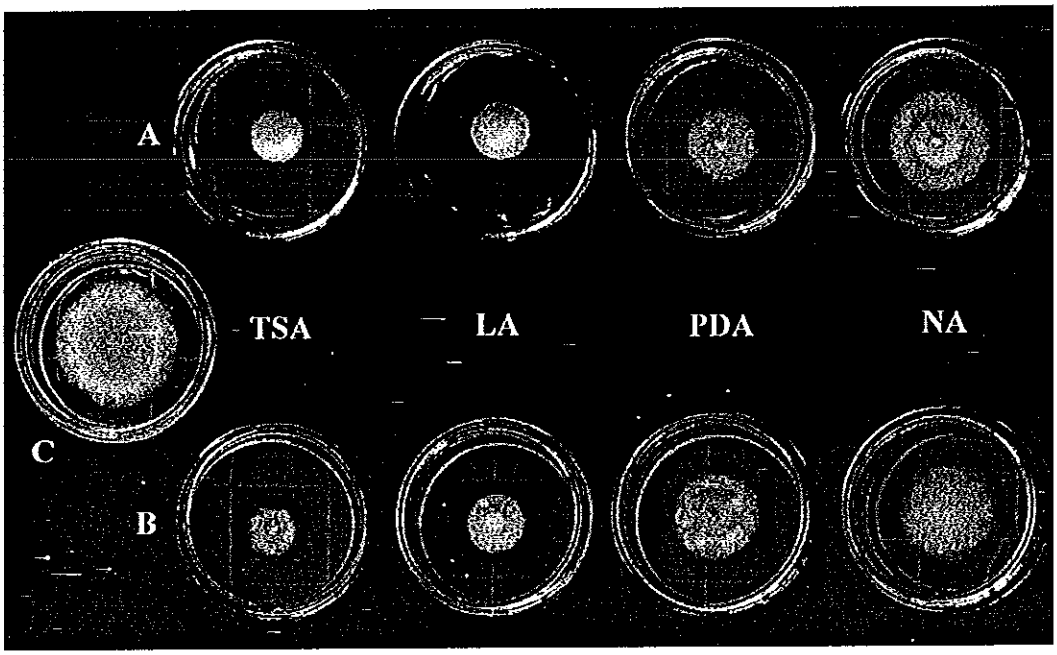

Fig 3.3. Inhibition in radial growth of *P. digitatum* exposed to volatile compounds produced by *B. subtilis* strains grown on various media. (A) *B. subtilis* strain 155, (B) ABS-S14, and (C) control plate.

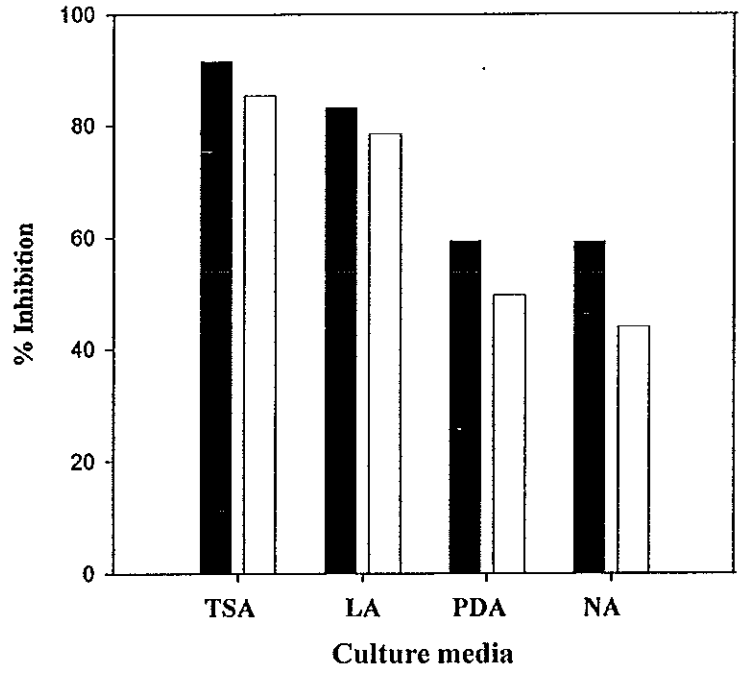

Fig 3.4. The inhibition Percentage of *P. digitatum* mycelium by volatile compound from *B. subtilis* cultured on various culture media after incubation for 72 h. Comparison of *B. subtilis* ABS-S14 (black box) and 155 (white box)

The fungal mycelia from the control- and tested-TSA plates were further observed under light microscopy after staining with lacto-phenol cotton blue dye. As shown in Fig. 3.5, the volatile substances induced morphological abnormalities in fungal structure when compared with the control (Fig 3.5 A-1 and A-2). Deformation in the mycelia and hyphal structure was common after exposure to VOC's from both *B. subtilis* strains. The Fig 3.5B (arrows) showed swelling of hyphae tips and vacuolization in the spore producing structure which caused the fungal failure to form spores. In Fig 3.5C, the fungus mycelium was found to be lysed and vacuolization in the mycelium was observed. However, this fungal pathogen retained its septated structure and this allowed the fungus to re-grow when transferred to a new medium.

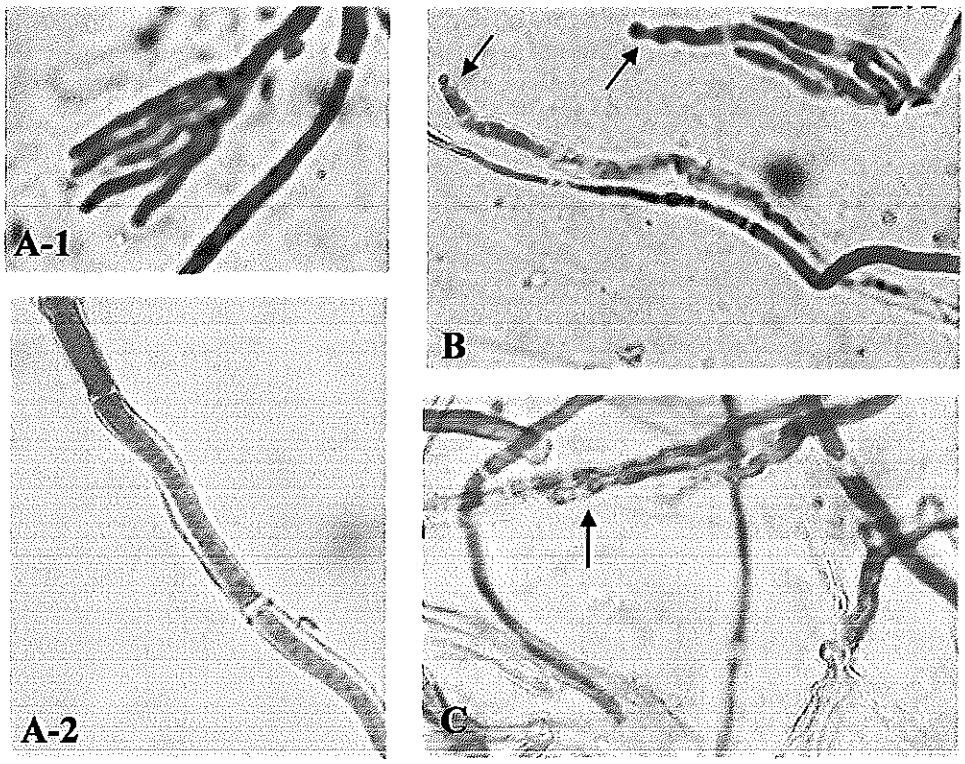

Fig 3.5. Microscopic observation of *P. digitatum* mycelia. The mycelia were stained with lacto-phenol cotton blue dye. A-1 and A-2 were the mycelia obtained from control plates; B and C were the mycelia from tested-TSA plates which were exposed to volatile emitted by *B. subtilis* strains ABS-S14 and 155, respectively.

3.2 Isolation and determination of antifungal compounds from *B. subtilis*

3.2.1 TLC analysis

The crude ethanolic extracts from *B. subtilis* were further analysed by chromatographic techniques. Thin-layer chromatography (TLC) was the preliminary technique used to analyze of the compounds in the extract. The extract was spotted onto commercial TLC plate and developed by using a mixture of $CHCl_3$: MeOH: dH_2O in a ratio 65:25:4 as a mobile phase. Then the TLC plate was visualized by the method described previously (section 2.2.5). The TLC plate (Fig. 3.6) showed three bands with different migration rates at R_f values of 0.09, 0.32 and 0.62, respectively when visualized by spraying with water. These R_f values illustrate the diverse nature

of the compounds present in the crude extracts. Assessed by commercial standards, the compounds at R_f values 0.32 and 0.62 were identified as iturin and surfactin, respectively. When spraying with ninhydrin solution, only one band at the R_f value 0.09 gave a positive reaction to ninhydrin solution. It is known that compounds with free amino acid moieties can react with ninhydrin solution and appear as pink to purple coloured regions (arrow indication).

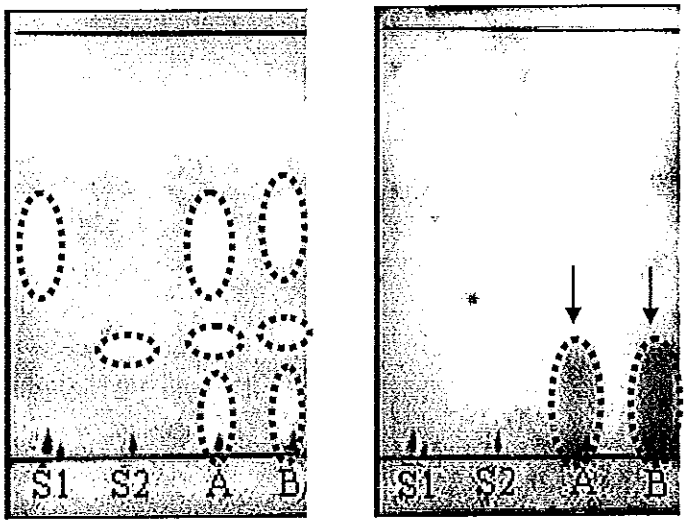

Fig. 3.6. TLC chromatograms of crude ethanolic extract from *B. subtilis* strains. The picture on the left showed the positive bands after spraying with water; Lane S1 and S2 were surfactin and iturin standards and lane A and B were crude extracts obtained from *B. subtilis* ABS-S14 and 155, respectively. The picture on the right showed the positive ninhydrin band.

3.2.2 HPLC analysis

The crude extract was further analyzed by the HPLC technique. The extracts were separated on an RP-C_{18} column and resolved into three clusters (Fig. 3.8 and -3.9). A comparison with the chromatogram of surfactin and iturin standards purchased from Sigma Co. (Fig. 3.7A and B), the peaks eluted between retention time (RT) 7 and 15 min in Fig. 3.8 and 3.9 as well as between RT 48 and 52 min was identified to be those of iturin and surfactin, respectively. The presence of multiple peaks on the chromatograms for commercial standards and crude extracts derived from the existence of several species of surfactin and iturin commonly produced by *B. subtilis*. Since a commercial standard fengycin was not yet available, the peaks with RT between 26 to 30 min that were supposed to be fengycin compounds were tentatively named as LP-x for further analysis.

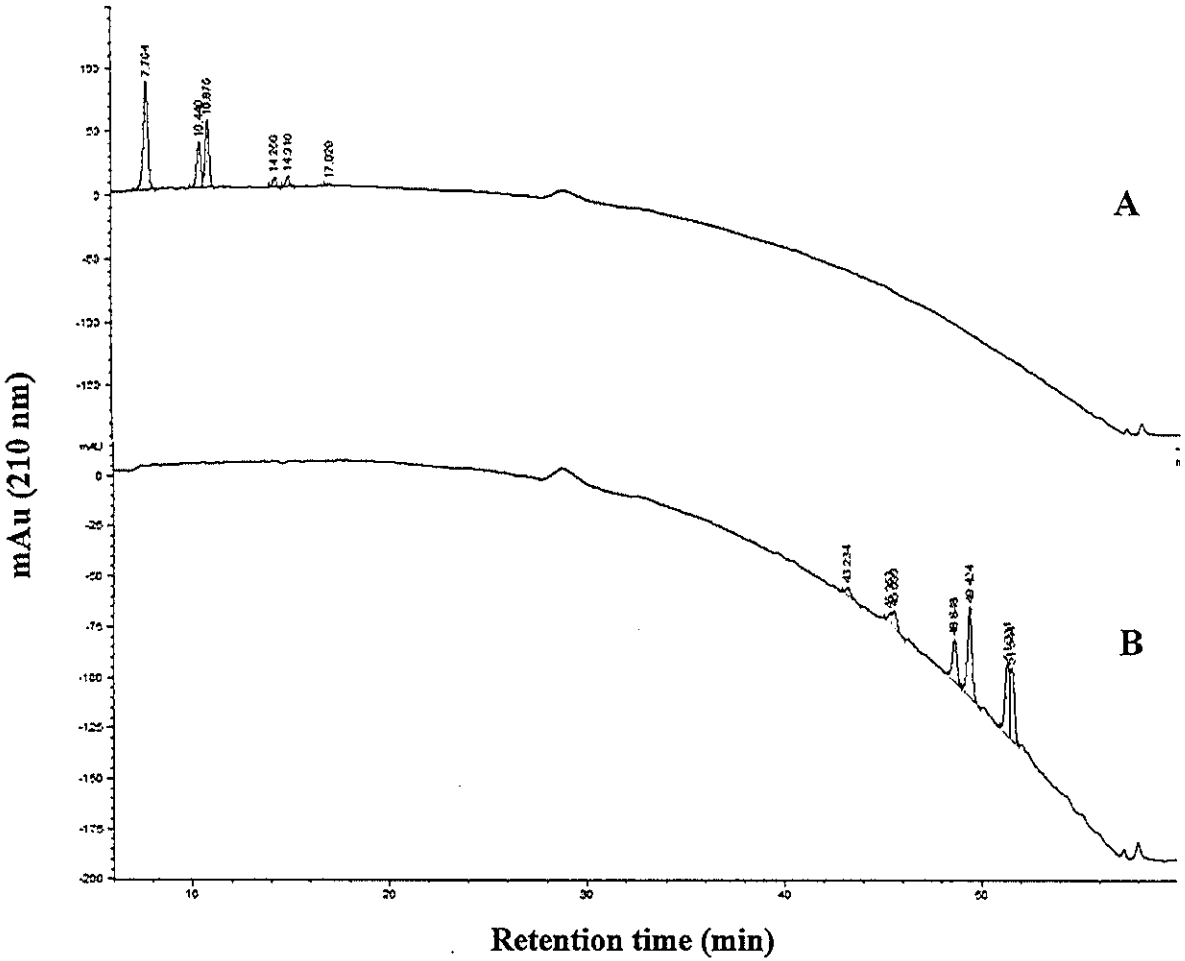

Fig. 3.7. RP-HPLC chromatogram of commercial standard of lipopeptides from *B. subtilis*. (A) iturin A (purity≥97%, HPLC) and (B) surfactin (purity≥98%, TLC) were dissolved in methanol and loaded to 20 µL loop, in order to inject 4 µg (iturin) or 10 µg (surfactin) onto C18 column (LiChrospher® 100 RP18e, 4 x 125 mm, Merck). The elution was carried out with stepwise gradient from 35%-90% of acetonitrile and 0.1% TFA at a flow rate of 1.0 mL/min and peaks were monitored at 210 nm.

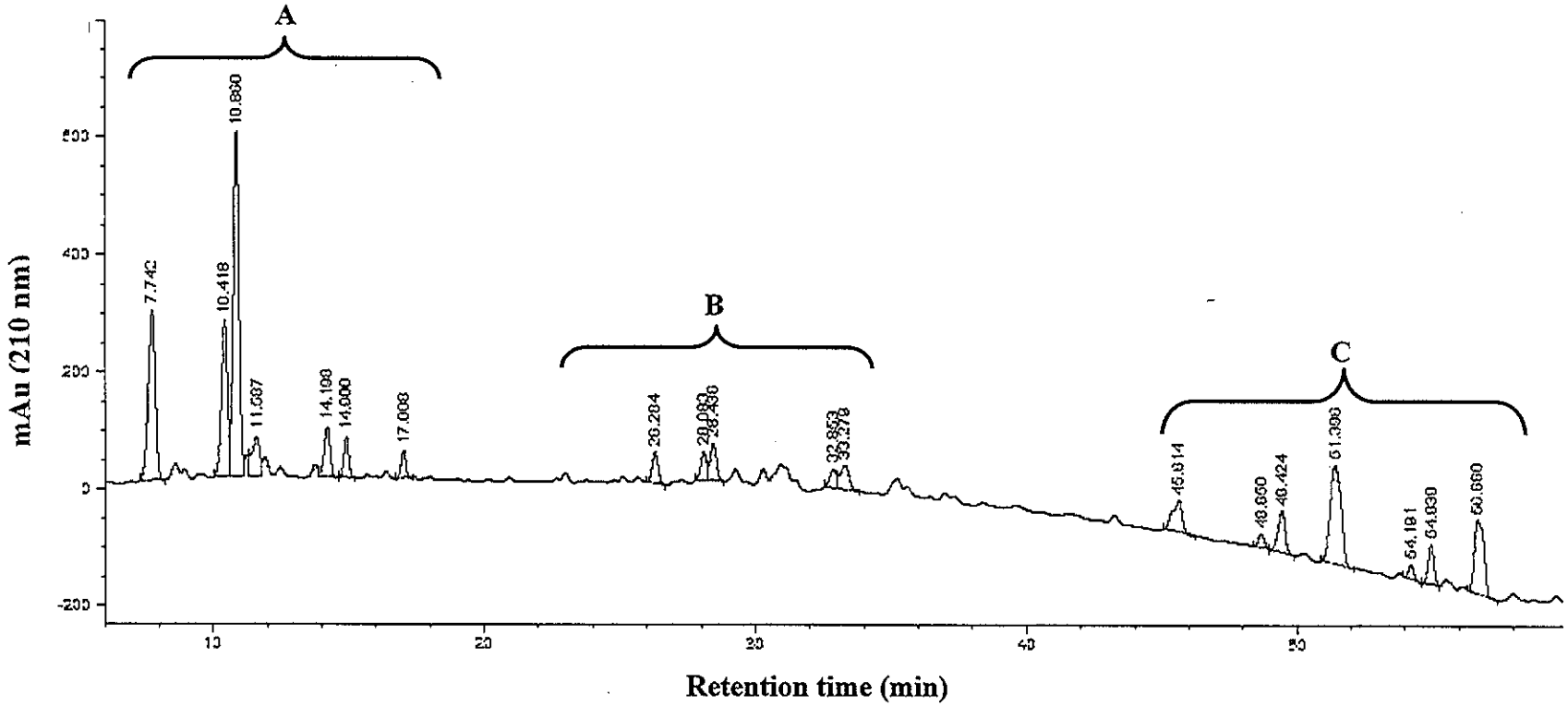

Fig. 3.8. RP-HPLC profiles of crude ethanolic extract from *B. subtilis* ABS-S14 strain cultured in LB medium. The crude ethanolic extract was dissolved in 50% acetonitrile and loaded into 20 µL loop. 20 µg of sample was injected to a LiChrospher® 100 RP-18e column (dimensions, 4 x 125 mm, particle size, 5 µm, Merck, Germany). The elution was carried out with stepwise gradient from 35%-90% of acetonitrile and 0.1% TFA at a flow rate of 1.0 mL/min and peaks were monitored at 210 nm. A= iturin, B= fengycin and C= surfactin families.

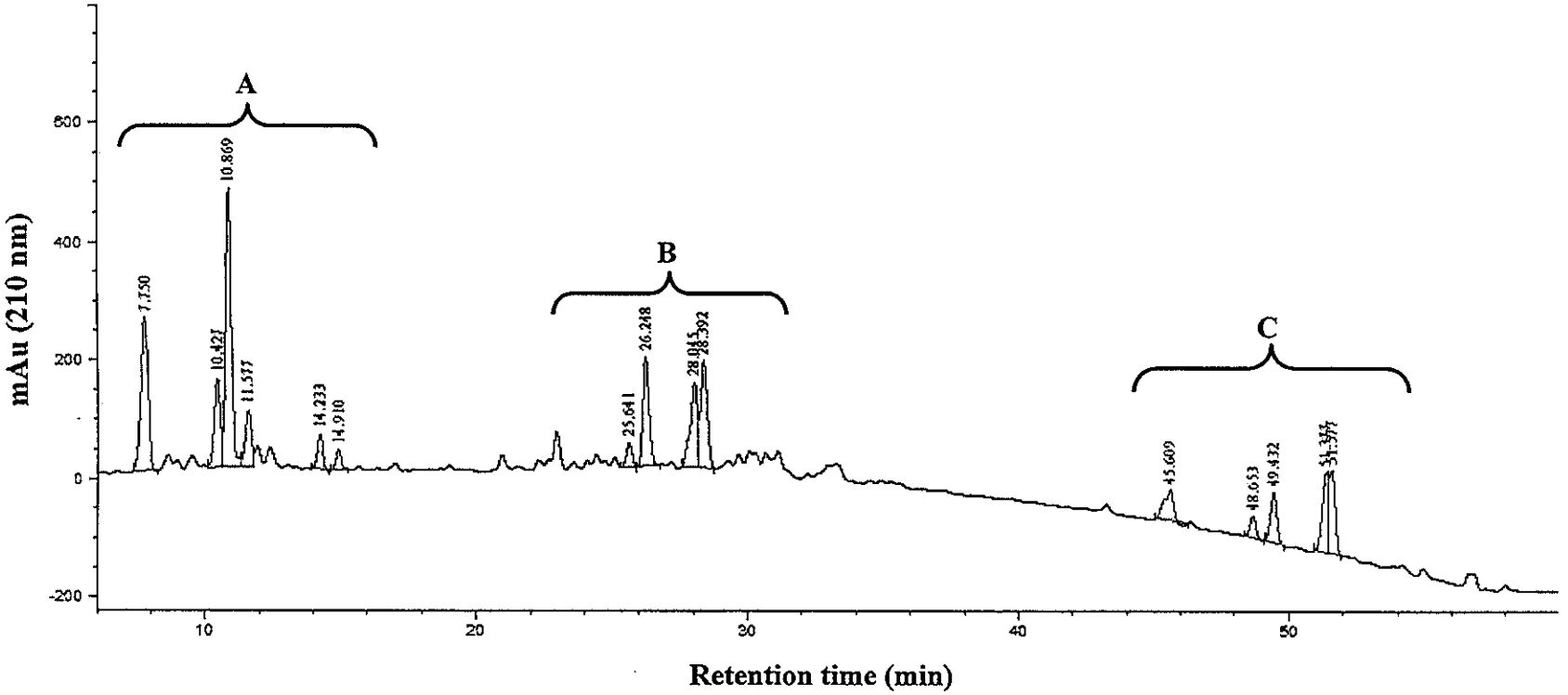

Fig. 3.9. RP-HPLC chromatogram of crude ethanolic extract obtained from *B. subtilis* 155 cultured in LB medium. The crude ethanolic extract was dissolved in 50% acetonitrile and loaded into 20 µL loop. 20 µg of sample was injected to a LiChrospher® 100 RP-18e column (dimensions, 4 x 125 mm, particle size, 5 µm, Merck, Germany). The elution was carried out with stepwise gradient from 35%-90% of acetonitrile and 0.1% TFA at a flow rate of 1.0 mL/min and peaks were monitored at 210 nm. A= iturin , B= fengycin and C= surfactin families.

3.2.3 MALDI-TOF mass spectrometry analysis

The product patterns determined by MALDI-TOF mass spectrometry for crude ethanolic extracts of *B. subtilis* ABS-S14 and 155 were shown (Fig 3.10 and 3.11). The mass spectra of crude ethanolic extract from both *B. subtilis* strains revealed similarity of two clusters of molecular mass. There was a series of mass number of m/z = 1030-1120, 1480-1580 and 1028-1100, 1440-1500, respectively. The groups of peaks represent as isoforms of each compound which corresponding to the well-known LPs families like surfactin, iturin and fengycin produced by *B. subtilis* strains. (Deleu *et al.*, 1999, Leenders *et al.*, 1999 and Vater *et al.*, 2002).

From above result, it can be interpreted that the *B. subtilis* strains ABS-S14 and 155 produced the lipopeptide substances attributed to fengycin, iturin and surfactin with multiple isoforms.

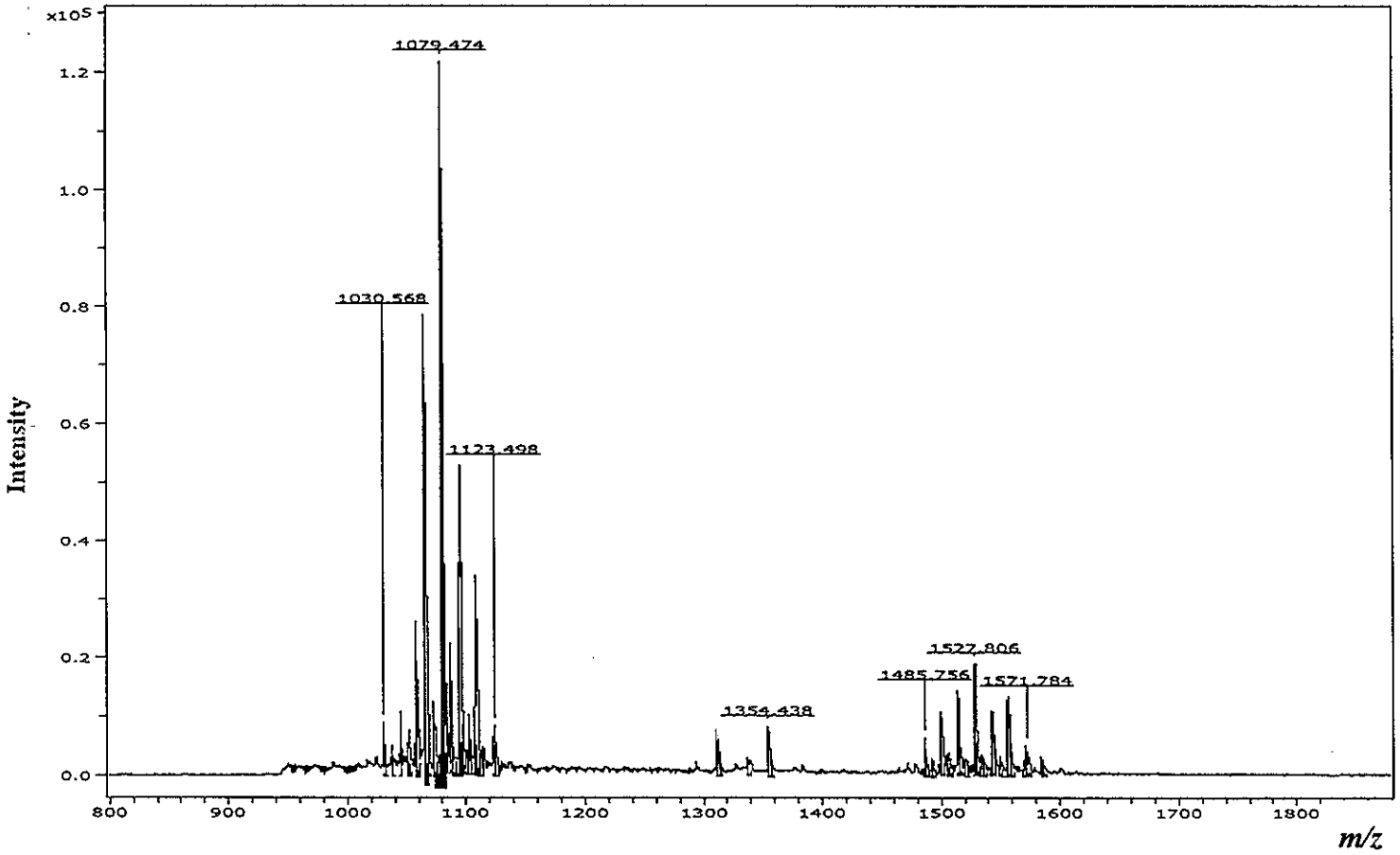

Fig. 3.10. MALDI-TOF mass spectrometric analysis of a crude ethanolic extract from *B. subtilis* ABS-S14. The lipopeptides were detected in the range from m/z 900 to 1800. The analysis was performed on the Bruker Daltonic Ultraflex III MALDI-TOF/TOF instrument.

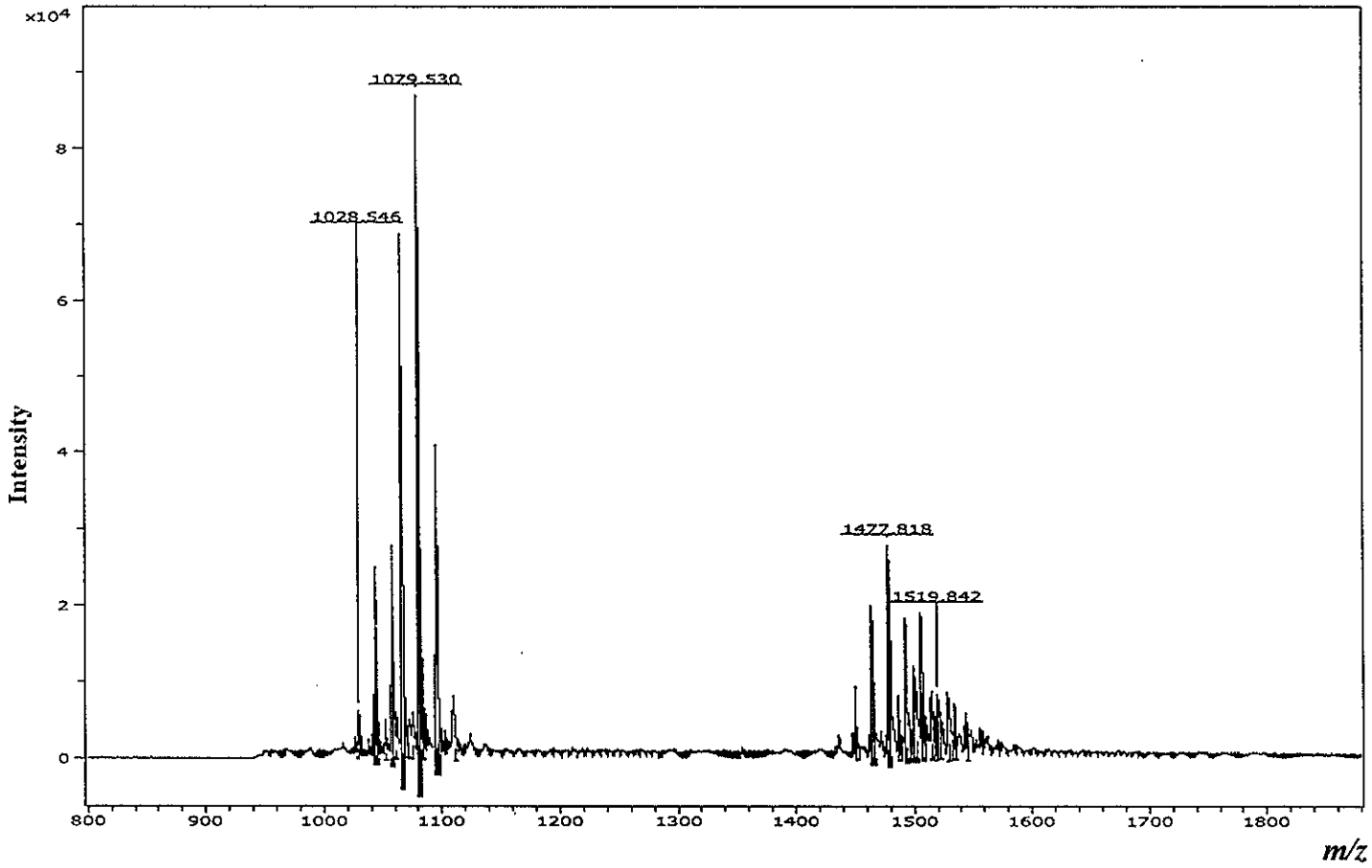

Fig. 3.11. MALDI-TOF mass spectrometric analysis of a crude ethanolic extract from *B. subtilis* 155. The lipopeptides were detected in the range from m/z 900 to 1800. The analysis was performed on the Bruker Daltonic Ultraflex III MALDI-TOF/TOF instrument.

3.2.4 Inhibition activity of partially purified crude extract

The crude ethanolic extract of *B. subtilis* strains was streak-loaded on PLC and the gel containing bands were scraped off the plate after spraying with water. Each band correspondent to a group of compounds was extracted, re-dissolved in ethanol and tested for their inhibitory activities. The result show that inhibition activity toward *P. digitatum* was observed in the bands of R_f values 0.09 and 0.32 (Fig 3.12), which refer to fengycin and iturin compounds. However, no inhibition activity was observed from a band that conferred to surfactin substances ($R_f = 0.62$).

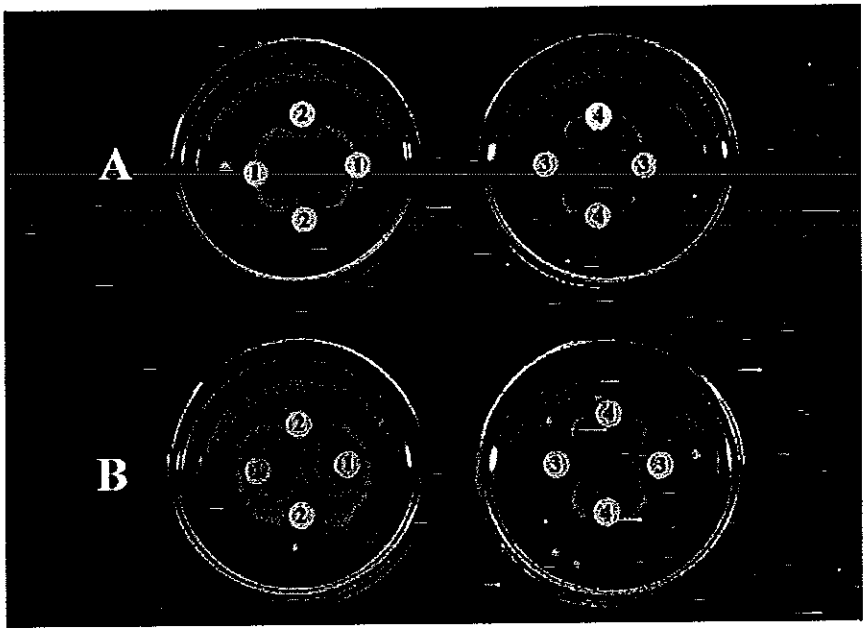

Fig. 3.12. The inhibition of partial purified LPs from PLC plate. A and B were obtained from *B. subtilis* strain ABS-S14 and 155 respectively. Each disc contained 20 µL of 80% ethanol (control, 1) or 1 mg/mL of the partially purified LPs that corresponded to the bands at R_f values = 0.09 (2), 0.32 (3) and 0.62 (4), respectively.

3.2.5 SPME-GC analysis of VOCs

The success of the analysis of VOCs by the SPME technique depends on sample extraction. In this study, the extraction procedure of Farag *et al.* (2006) was used. Moreover, the sonicate-assisted extraction technique was used to determine the influence of sample extraction. The methods for extraction mentioned by this author were that the fiber was added to the vial by puncturing the vial seal, and then placed it in the heating box (50°C) for 30 min to adsorb the VOCs. The procedure was modified using sonication coupled with to the SPME technique and these conditions were compared. The sample containing vial was sonicated for 5 and 15 min before insertion of the SPME fiber. The sample was placed in the heating box and the adsorption time was 5 or 15 min. The sample was prepared using TSA culture medium of *B. subtilis* ABS-S14 according to its highest fungal inhibition percentage. The analysis method was developed initially with the GC-FID instrument. The GC conditions were optimized by testing various injection temperatures and temperature programs. The best results were obtained by the following program, the column temperature was initially maintained at 50 °C for 4 min after injection, then increased at 8 °C/min to 200 °C and held for 30 sec, then programmed at 20 °C/min to 230 °C and kept for 1 min.

SPME-GC chromatogram of VOCs emitted from *B. subtilis* ABS-S14 presented in Fig.3.13 showed one prominent peak in all extraction procedures. This peak was used for indicating of the efficiency of extraction procedures.

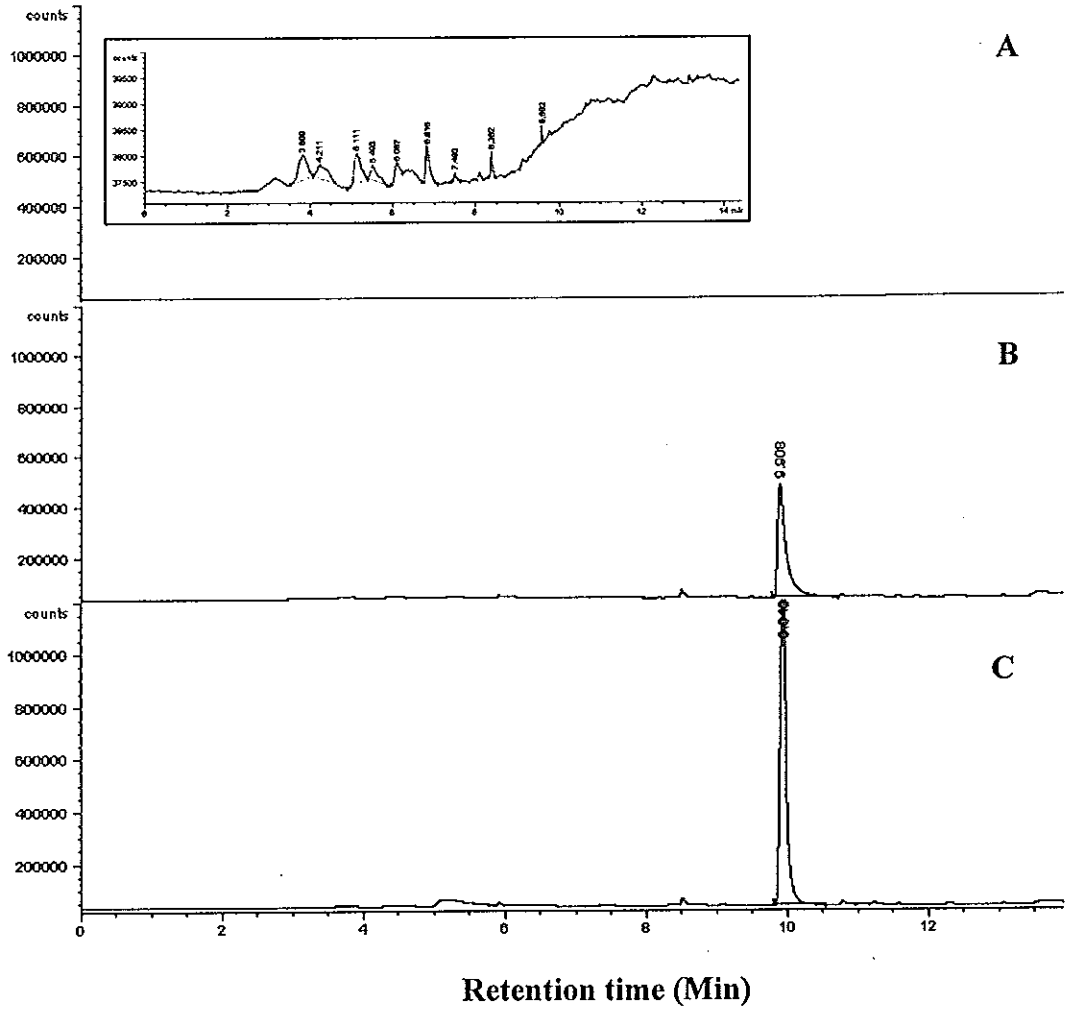

Fig 3.13. GC-FID chromatogram of VOCs emitted from *B. subtilis* ABS-S14 cultured on TSA medium. (A) was a GC chromatogram of TSA medium (control), (B) and (C) were chromatograms of *B. subtilis* inoculated TSA media extracted by heating in a water bath and adsorbed for 30 min at the same time and a extracted by 15 min sonication and adsorbed for 15 min, respectively.

As shown in Fig 3.14, the sample extraction coupled with the sonication method seems to be the most effective. The heating procedure used in this study can help the fibers adsorb the evaporated gas in the headspace phase as well as the dissolve VOCs in the medium under the equilibration state. The equilibration time in the vial was a critical phenomenon for the SPME technique. A liquid form sample reached the equilibrium phase by accelerating with stirring and/or addition of NaCl coupling with heating. On the other hand, the sonication technique was most suitable for the solid medium sample. Sonication is the act of applying sound energy to agitate particles in a sample both liquid and solid form. High energy particles, in this case, volatile compounds could evaporate and be circulated in the vial for adsorption during the equilibration state. Therefore, sonication accelerated the gas phase achieving equilibrium and resulted in an increase in the sensitivity of the SPME technique.

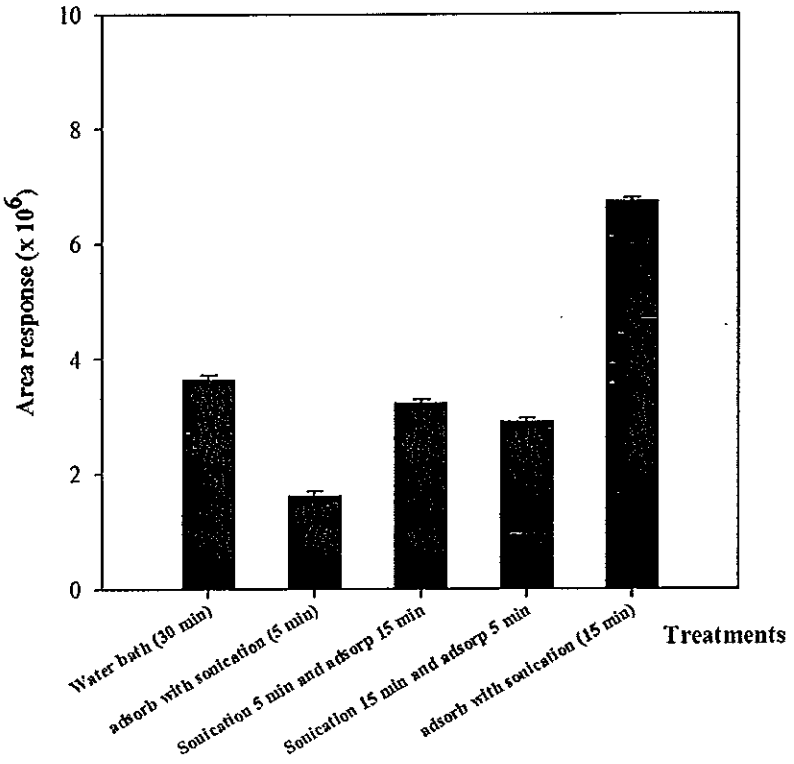

Fig. 3.14. The influence of extraction condition on SPME analysis of VOCs. *B. subtilis* ABS-S14 cultured on TSA medium was used as a model for optimized the condition. The one prominent peak from GC chromatogram was used for comparison of the effect of extraction method.

This analytical method was then transferred to GC-MS. The GC-MS conditions were similarly to those for the GC-FID with mass detector temperature of 250 °C and continuously scanned from *m/z* 40 to 500. Compounds from GC-MS chromatograms were identified by computer matching their mass spectral fragmentation patterns with those of compounds in the library. Unfortunately, the analysis failed because there was either no matching data in the library or the confidence of matching was less than the 80% confidence (Fig 3.15). Then, the sample extraction method needs to be validated for GC-MS analysis or these volatile compounds may not be common VOCs previously identified and recorded in the NIST library.

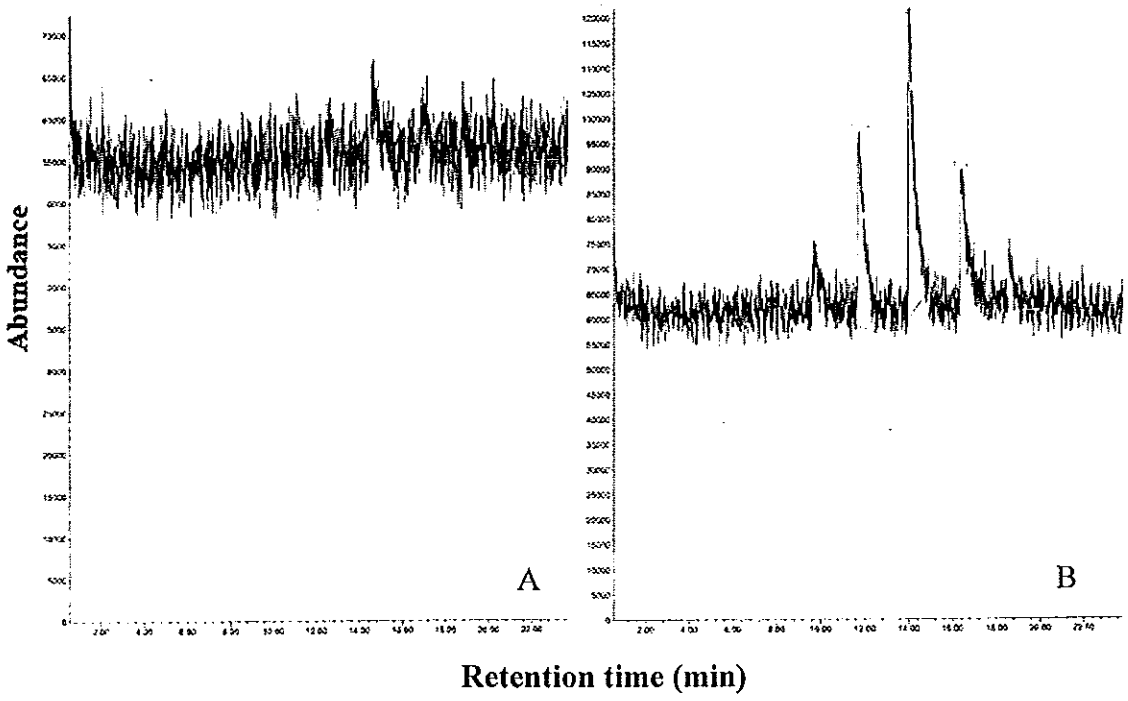

Fig 3.15. GC-MS pattern of volatile compounds (VOCs) from *B. subtilis* ABS-S14. The mass spectra of TSA medium (A) and inoculated TSA with ABS-S14 (B).

3. Population of *B. subtilis* on citrus rind

The antagonist *B. subtilis* ABS-S14 was inoculated onto citrus fruits and recovered during 20 days of incubation time at 25°C. The bacteria were plated onto NA medium and counted for population density. The bacterial morphological and colony forming characteristics were compared with control strain. The colony of *B. subtilis* ABS-S14 recovered from citrus fruit was mucilagenous and creamy. Bacteria recovered from citrus rinds when plated onto NA agar were found similar to control plate and no other bacteria were observed. The population dynamic of ABS-S14 shown in Fig 3.16 demonstrated that the population was gradually decreased from 3.72×10^4 CFU/mL to 2.15×10^4 CFU/mL after 4 days of inoculation. Bacteria were maintaining their numbers for 20 days of inoculation.

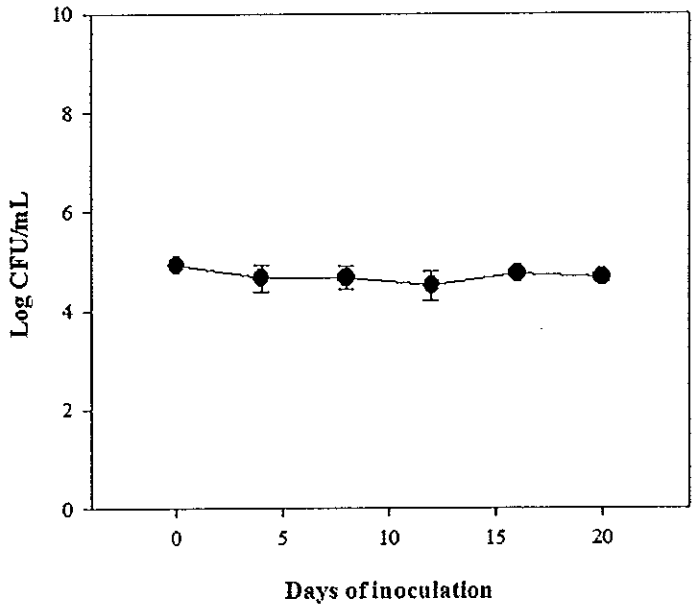

Fig. 3.16. Population dynamic of *B. subtilis* ABS-S14 on surface of citrus fruit. The population of bacterial strain was present in log CFU/mL.

3.4 Peroxidase (POX) activity

The induction of POX enzymes by antagonistic bacteria and their metabolites was evaluated. The POX patterns presented in Fig 3.17 and Table 3.3 revealed that the POX activity in control treatment (T1) was gradually increased after 72 h of inoculation. Disease control treatment (T2) showed the increment of activity at 48 h after inoculation and decreased in 72 h. The highest POX activity was observed in citrus fruit inoculated with 10 mg/mL of crude ethanolic extract of *B. subtilis* ABS-S14 (T7) at first 24 h after inoculation and continuously declined at 48 h. On the contrary, POX activity of citrus inoculated with crude extract combined with fungal pathogen (T8) showed gradually increased and reached its maximum at 48 h of inoculation. Citrus inoculated with *B. subtilis* ABS-S14 (T3) and 155 (T5) showed different induction of POX activity. *B. subtilis* 155 could induce enzyme activity reaching its peak at 24 h after inoculation, but *B. subtilis* ABS-S14 showed gradual increase of POX activity and arrived highest level at 72 h. The induction of POX activity in the treatments that co-inoculated with bacteria and fungal pathogen (ABS-S14, T4 and 155; T6) were similar to their solely inoculated.

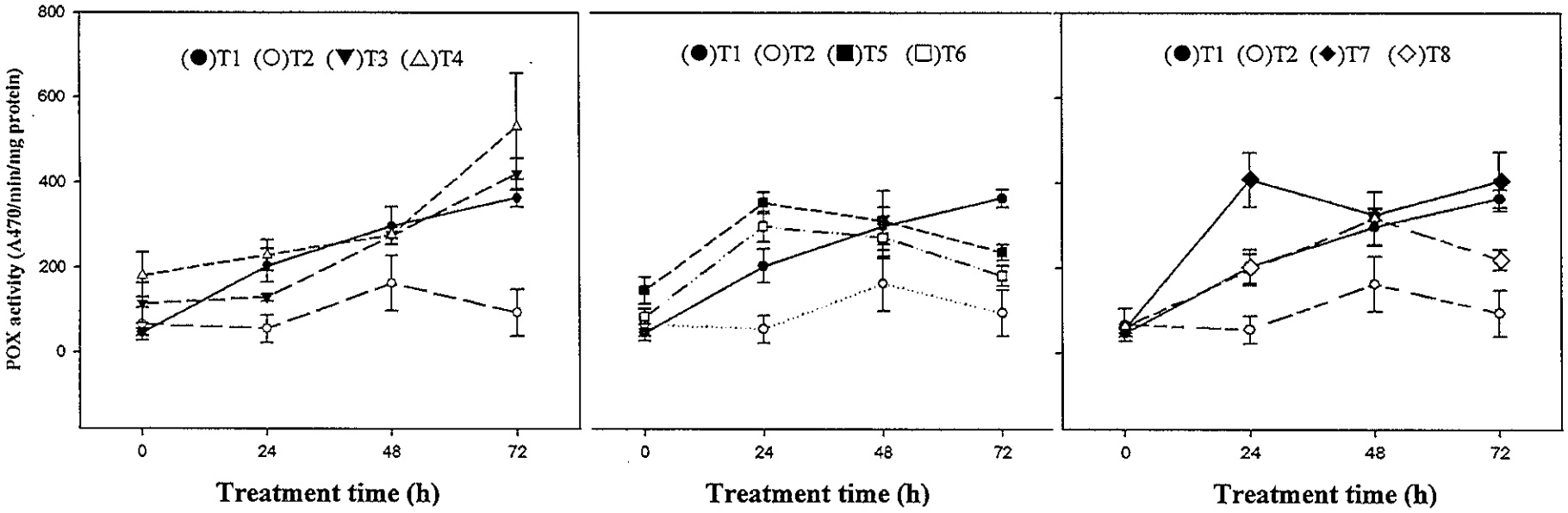

Fig. 3.17. Changes in peroxidase activity in flavedo tissue of citrus fruit of various treatments. POX activity was measured during 72 h of inoculation with various treatments; (●) T1 = control, (distilled water), (○) T2 = disease control, inoculated with spore suspension of *P. digitatum* (10^4 spores/mL, Pd), (▼) T3 = spore suspension of *B. subtilis* strain ABS-S14 (10^8 CFU/mL), (△) T4 = co-inoculated *B. subtilis* ABS-S14 with Pd, (■) T5 = spore suspension of *B. subtilis* 155 (10^8 CFU/mL), (□) T6 = co-inoculated 155 with Pd, (◆) T7 = crude extract of ABS-S14 (10 mg/mL) and (◇) T8 = crude

Table 3.3. Changes in peroxidase (POX) activity of citrus fruit inoculated with various treatments.

Hours	T1	T2	T3	T4	T5	T6	T7	T8
0	47.36±8.43^{c}	66.92±38.63^{b}	115.62±46.67^{ab}	181.73±52.65^{a}	146.89±31.57^{a}	84.51±17.07^{b}	57.2461±10.90^{c}	62.13±6.62^{c}
24	204.68±39.23^{bc}	55.86±32.25^{d}	129.55±8.07^{d}	229.26±36.26^{bc}	351.70±25.16^{b}	296.95±36.19^{b}	407.94±64.19^{a}	197.29±36.99^{bc}
48	297.95±43.25^{b}	162.72±64.50^{c}	274.07±20.64^{b}	276.99±7.82^{b}	311.17±69.24^{ab}	271.86±49.50^{b}	325.62±13.71^{a}	316.81±63.05^{a}
72	363.61±21.56^{ab}	94.41±54.51^{c}	419.17±38.31^{a}	531.86±125.36^{a}	237.01±18.09^{b}	181.65±24.47^{b}	404.22±68.69^{a}	220.46±24.72^{b}

Mean ± S.E. value bearing a different alphabet in the same row show significant differences at p=0.05, whereas T1 = control, (distrilled water), T2 = inoculated with spore suspension of *P. digitatum* (Pd) (10^4 spores/mL), T3 = spore suspension of *B. subtilis* ABS-S14 (10^8 CFU/mL), T4 = co-inoculated ABS-S14 with Pd, T5 = spore suspension of *B. subtilis* 155 (108 CFU/mL), T6 = co-inoculated 155 with Pd, T7 = crude extract of strain ABS-S14 (10 mg/mL) and T8 = crude extract of ABS-S14 and Pd.

Fig. 3.18 (A), demonstrated POX enzyme activity staining after non-denaturing polyacrylamide gel electrophoresis using guaiacol as substrate. The activity staining in the gel was performed in order to investigate the existence of isozyme and variation of POX profile in tissue treated with various treatments. In this study, the stained polyacryalamide gel showed a single band migrated on gel and no difference was observed among treatments. The guaiacol stained gels were further stained with Coomassie brilliant blue solution according to Fieldes (1992) for blocking the guaiacol band. As shown in Fig. 3.18 (B), guaiacol bands turned to blue color of Coomassie and also represented similarly in band intensity when compared with guaiacol stained gel. The POX band expressed approximately molecular weight of 140 kDa.

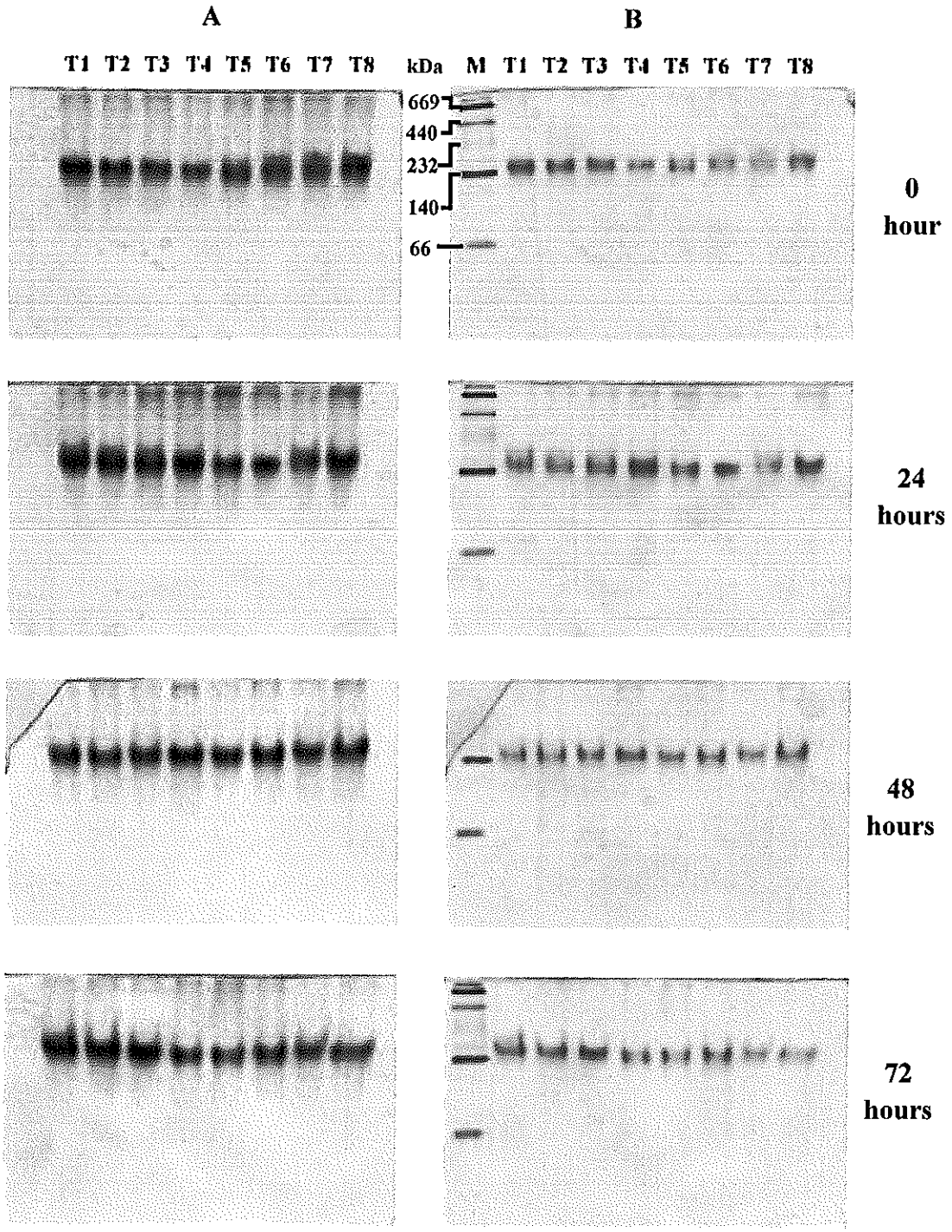
A
B
T1 T2 T3 T4 T5 T6 T7 T8
kDa M T1 T2 T3 T4 T5 T6 T7 T8
669
440
232
140
66
0 hour
24 hours
48 hours
72 hours

Fig. 3.18. Activity staining of Native-PAGE of peroxidase enzyme in plant extract exposed to various treatments. Aliquot of 30 µL of plant extract was electrophoretically separated on 7.5% acrylalmide gel and stained with guaiacol as a substrate (A) and double-staining on the same gel with Coomassie brilliant blue solution (B). M= high molecular weight marker (Amersham), from top; Thyroglobulin (669 kDa), Ferritin (440 kDa), Catalase (232 kDa), Lactate dehydrogenase (140 kDa) and Albumin (66 kDa), T1 = control, (distilled water), T2 = inoculated with spore suspension of *P. digitatum* (10^4 spores/mL, Pd), T3 = spore suspension of *B. subtilis* ABS-S14 (10^8 CFU/mL), T4 = co-inoculated ABS-S14 with Pd, T5 = spore suspension of *B. subtilis* 155 (10^8 CFU/mL), T6 = co-inoculated 155 with Pd, T7 = crude extract of ABS-S14 (10 mg/mL) and T8 = crude extract of ABS-S14 and Pd.

3.5 Phenylalanine ammonia-lyase (PAL) activity

In similar to result of POX activity, the highest PAL activity was found in citrus treated with crude extract from *B. subtilis* ABS-S14 (Fig. 3.19 and Table 3.4). The enzyme activity in this treatment increased and reached its maximum at 48 h. However, it seems to be contrasting to T8 (co-inoculation of crude extract and fungus pathogen) that PAL activity was declined after 24 h of inoculation time. Citrus inoculated with *P. digitatum* (T2) showed the increment of activity from 0 to 48 h and declined at 72 h after inoculation. The induced PAL enzyme pattern of *B. subtilis* 155 treatments (T3) as well as ABS-S14 (T5) which gradually increased from 0 h and reached maximum at 48 h and the pattern of PAL activity was similar to activity induced by crude extract. However, the induction of PAL activity of these treatments dropped at 72 h of incubation period. Antagonist bacteria, however, when added together with *P. digitatum* (T4 and T6) showed a slightly stimulation of PAL activity. At 48 h, PAL activity in T6 was increased and then declined at 72 h. The pattern of PAL activities in all treatments were found to induce and subsequently declined in 72 h during incubation period.

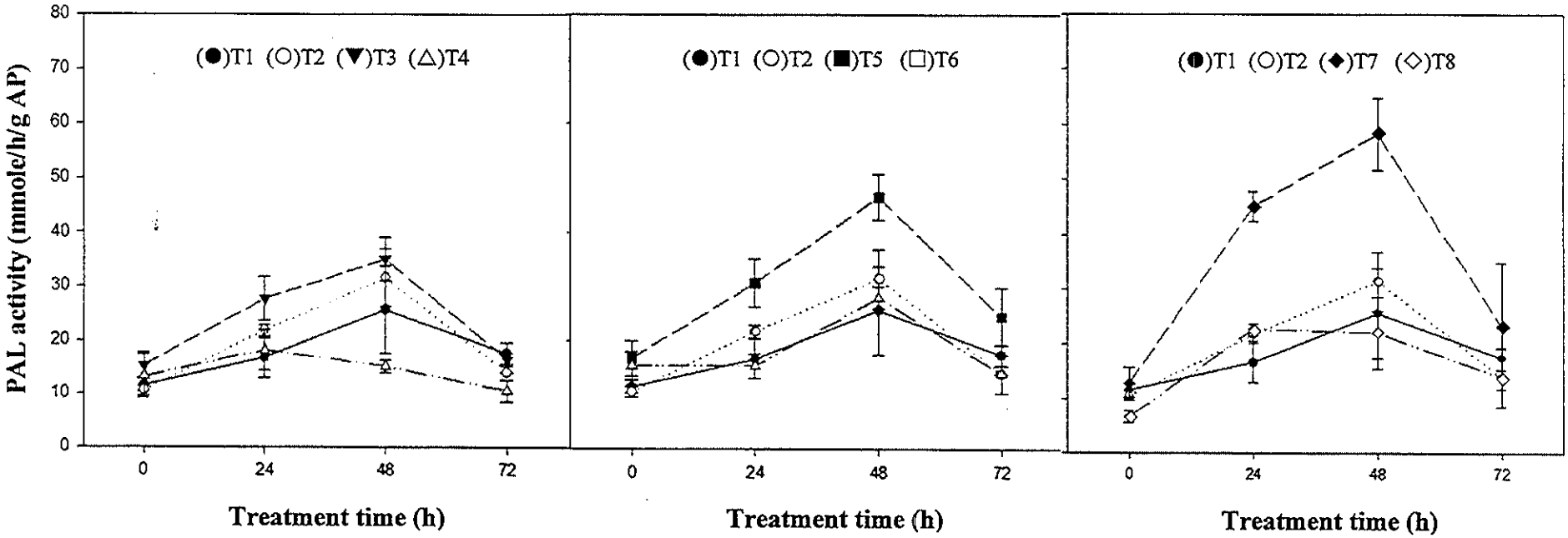

Fig. 3.19. Changes in phenylalanine ammonia-lyase activity in flavedo tissue of citrus fruit of various treatments. PAL activity was measured during 72 h of inoculation with various treatments; (●) T1 = control, (distilled water), (○) T2 = inoculated with spore suspension of *P. digitatum* (10^4 spores/mL, Pd), (▼) T3 = spore suspension of *B. subtilis* strain ABS-S14 (10^8 CFU/mL), (△)T4 = co-inoculated ABS-S14 with Pd, (■)T5 = spore suspension of *B. subtilis* 155 (10^8 CFU/mL), (□)T6 = co-inoculated 155 with Pd, (◆) T7 = crude extract of strain ABS-S14 (10 mg/mL) and (◇) T8 = crude extract of ABS-S14 and Pd.

Table 3.4. Changes in phenylalanine ammonia-lyase (PAL) activity of citrus fruit inoculated with various treatments.

Hours	T1	T2	T3	T4	T5	T6	T7	T8
0	11.67±2.25^{aa}	10.62±1.58^{aa}	15.28±4.43^{aa}	13.30±6.99^{aa}	16.80±5.63^{aa}	15.46±4.59^{aa}	12.95±4.81^{aa}	6.77±1.93^{aa}
24	16.68±6.30^{d}	21.82±1.99^{c}	27.65±7.03^{c}	18.08±6.51^{d}	30.68±7.55^{b}	15.46±3.91^{d}	45.15±4.86^{a}	22.75±1.85^{c}
48	25.67±14.15^{c}	31.50±9.26^{c}	35.00±7.00^{bc}	15.17±2.02^{d}	46.67±7.29^{b}	28.00±3.50^{c}	58.33±11.25^{a}	22.17±11.25^{c}
72	17.50±3.50^{ab}	14.00±0.00^{c}	16.33±2.02^{ab}	10.50±3.50^{d}	24.50±9.26^{a}	14.00±6.06^{c}	23.33±19.90^{a}	14.00±9.26^{c}

Mean ± S.E. value bearing a different alphabet in the same row show significant differences at p=0.05, whereas T1 = control, (distrilled water), T2 = inoculated with spore suspension of *P. digitatum* (Pd) (10^4 spores/mL), T3 = spore suspension of *B. subtilis* ABS-S14 (10^8 CFU/mL), T4 = co-inoculated ABS-S14 with Pd, T5 = spore suspension of *B. subtilis* 155 (108 CFU/mL), T6 = co-inoculated strain 155 with Pd, T7 = crude extract of ABS-S14 (10 mg/mL) and T8 = crude extract of ABS-S14 and Pd.

Chapter 4

DISCUSSIONS

4.1 Antagonistic activity of *B. subtilis* strains toward citrus fruit rot fungus, *P. digitatum*

Many researchers (Walker *et al.* 1998, Yoshida *et al.*, 2001, Leelasuphakul *et al.*, 2008) have used the dual culture technique as an easy way to evaluate the antagonistic activity on agar plate from various groups of bacterial isolates and to compare these selected strains for their fungal growth inhibition capabilities. In this study, two *B. subtilis* strains, ABS-S14 and 155 displayed an antifungal activity. The characteristics of *Bacillus* species as antibiotic producer and inhabitants of phyllosphere are well known (Stein, 2005 and reviewed in Ongena and Jaque, 2008). Moreover, the strains can produced a biofilm (Fig 3.1, black arrow) which is cell community structure containing many biological molecules such as polysaccharides, proteins, nucleic acids (Branda *et al.*, 2005) and lipopeptides that are important for root colonization, antibiosis and induction of plant defense mechanisms (Leclere *et al.*, 2005, Review on Ongena and Jacque, 2009). Bais *et al.* (2004) found that *B. subtilis* 6051 formed an expanded biofilm and secreted surfactin which both act together to protect plants against pathogen attacks. This was similar to other findings (Asaka and Shoda, 1996, Cho *et al.*, 2003, Toure *et al.*, 2004) that *B. subtilis* can suppress plant pathogen by producing secondary metabolites such as surfactin, iturin and fengycin families which proved as the powerful antimicrobial substances. Interestingly, although the fungal growth conditions at a low pH and temperature are not suitable for the growth of *B. subtilis*, however, it could proliferate and also showed their antagonistic activities. Nichols *et al.* (1995) and Holtmann and Bremer (2004) showed that *B. subtilis* was able to sustain growth in the temperature range from approximately 11 °C to 52 °C and can use various carbon sources (Mukherjee and Das, 2005).

4.2 Identification and isolation of antimicrobial lipopeptides produced from *B. subtilis*

The inhibitory effect from ethanol fraction after HCl precipitation (crude extract) was due to their lipopeptide variations. The TLC analysis of crude extract had shown three water positive bands and one ninhydrin positive band. The two bands correspond to commercial standard, iturin and surfactin when compared with those R_f value. The ninhydrin positive band was supposed to be a fengycin because it has free amino acid moiety in their structure that can be reacted with ninhydrin solution. Steller *et al.* (1999) reported that the acid precipitates from *B. subtilis* b213 and A1/3 showed four bands positive to ninhydrin when detected by TLC and had R_f values of 0.07, 0.09, 0.11 and 0.14, respectively. These bands were subsequent to MALDI-TOF analysis of mass spectra and correspond to mass of fengycin. The antifungal activity of each compound blended in crude extract were separated and scraped out of PLC plate. The band corresponding to fengycin and iturin accept surfactin from both strains could inhibit mycelia growth of *P. digitatum*. It is commonly known that the inhibitory effect of the extract was contributed to iturin and fengycin families (reviewed in Ongena and Jaques, 2008). Jacques *et al.* (1999) and Ongena *et al.* (2005) demonstrated by identifying iturins and fengycins as the main antibiotic products excreted by the particular strain of *B. subtilis* that inhibited the germination of *P. fusca* conidia. These LPs were recovered from bacterial-treated leaves and comparisons with the use of LP-deficient transformants. *B. subtilis* S499 efficiently produced LPs of the three families, and notably produced a wide variety of fengycins. Similarly, Romero *et al.* (2007) found a contribution of both iturins and fengycins was recently shown by their abilities to antagonize *P. fusca* that infected melon leaves. On the other hand, surfactin was found to cause lysis of erythrocytes, inhibited fibril formation and exhibited antibacterial activity but had no marked toxicity for fungi (Al-Ajlani *et al.*, 2007; Ongena and Jaque, 2008).

HPLC analysis of crude extracts showed the presence of three clusters of analytes. Each group was eluted at a particular time of the mobile phase composition. The several peaks in each cluster found in HPLC chromatogram usually indicate the multiple forms of LPs which differ only their amino acid compositions and/or fatty

acid moieties (Mukherjee and Das, 2005). The MALDI-TOF analysis of the crude extract from both *B. subtilis* strains (ABS-S14 and 155) showed molecular masses that corresponded to many previously reported (Deleu *et al.*, 1999, Leenders *et al.*, 1999 and Vater *et al.*, 2002). Each group of peaks represents isoforms of one family of compounds that corresponded to the well-known LPs families like surfactin, iturin and fengycin produced by *B. subtilis* strains. Vater *et al.* (2002) demonstrated that the cell-free fermentation broth of *B. subtilis* C-1 showed three clusters of peaks at *m/z* values between 1000 and 1060, between 1070 and 1150, and between 1450 and 1550 and were then further identified as surfactin, iturin and fengycin. MALDI-TOF mass spectrometry is a novel method that can be used to detect LPs in minutes with high sensitivity, precision, and excellent resolution without a requirement for time-consuming isolation and chromatographic separation of the compounds. Thus, in this study, the results of the MALDI-TOF mass spectrometry confirmed the *B. subtilis* strains ABS-S14 and 155 produced the lipopeptide substances belonging to the fengycin, iturin and surfactin families with multiple isoforms.

4.3 Antagonistic activity of volatile organic compounds (VOCs) emitted by *B. subtilis*.

The VOCs are ideal information chemicals because they can act over a wide range of distances and their spheres of activity will extend from proximal interactions, due to aqueous diffusion, to greater distances via diffusion in air, including in soil pores (Kai *et al.*, 2007). The antifungal nature of VOCs has been demonstrated in several pathogen systems (Chaurasia *et al.*, 2005; Fernando *et al.*, 2006). In this study, the antifungal activity was observed in both *B. subtilis* strains and in all media used. Bacteria which grew on TSA plates expressed the highest antifungal volatile activity. TSA was the most nutrient rich medium used in present study. Therefore, it could promote growth of bacteria and also nurture the production of antifungal volatiles. The volatile of these bacteria antagonists caused the abnormalities in fungal structures. Chaurasia *et al.* (2005) demonstrated the effect of volatile antifungal from *B. subtilis* can cause structural deformations in pathogenic fungi under *in vitro* condition. Huang *et al.* (1997) showed that Allyl alcohol inhibits carpogenic germination of sclerotia of *S. sclerotiorum*. Archibold *et al.* (1997) found that aliphatic aldehydes were more effective in the post-harvest control of gray mould

caused by *B. cinerea* in strawberry, blackberry and grape and when combination used with ketone, the inhibition activity was greater than alcohol. By the way, the inhibition activity of volatile compounds found in this study was temporality. After VOCs exposure, the inhibited *P. digitatum* started growing when transferred to new PDA-media. *P. digitatum* has septate structure which limits the loss of cytoplasm during the hyphal wall ruptured. Interestingly, the VOCs produced by antagonistic bacteria could inhibit spore formation of pathogenic fungus and also reduced continuously infection via spores from this pathogen. Although, analysis of VOCs performed on GC-MS was failed, the sample extraction procedure using sonication technique coupled with SPME was satisfied by reducing the sample extraction time and rapidly reaching equilibration time. (Lee *et al.*, 2003).

4.4 *In vivo* population dynamic of *B. subtilis* on citrus fruit.

In order to maintain antagonistic activity, the antagonistic bacteria should be able to survive on the fruit. In this study, the *B. subtilis* ABS-S14 was found to survive on the citrus rind for 20 days. Tokuda *et al.* (1998) reported that spore forming ability of *B. subtilis* NB22-1 was the importance factor to stabilize these bacteria in soil condition. Moreover, this author also found that soil nutrient and maintain temperature to 25°C were effective to stabilize *B. subtilis* NB22-1 and these two factors stimulated spore formation of this bacteria. Kinsella *et al.* (2009) also demonstrated that the population dynamic of *B. subtilis* was no significant increase in rhizosphere. However, the bacteria multiplication was not observed in this study.

4.5 Effect of antagonistic bacteria on the induction of plant defense-related enzyme activities.

The antagonistic *B. subtilis* could induce resistance to pathogens in the plant by their secondary metabolites like LPs and VOC. Results obtained from this study, especially the crude extracts from *B. subtilis* ABS-S14 confirmed that the LPs could induce the activity of the enzymes POX and PAL which are involved in plant defense mechanism. Nandakumar (1998) found that two POXs isoforms and one chitinase (35 kDa) isoform were induced in PGPR-treated plants inoculated with the rice sheath blight pathogen, *R. solani*. Viswanathan and Samiyappan (1999a) showed

that the PGPR-mediated ISR against *C. falcatum*, enhanced levels of chitinase and POX in sugarcane. Zdor and Anderson (1992) also reported a POX activity as well as an increase in the level of mRNAs encoding for the PAL enzyme and chalcone synthase, that led to the synthesis of phytoalexin in the early stages of interaction between bean roots and various bacterial endophytes. Podile and Laxmi (1998) also observed a peak of peroxidase activity in pea plants treated with *B. subtilis*, seven days after inoculation with *F. udum* and this corresponded to the report by Silva *et al.* (2004) that tomato seeds treated with non-pathogenic rhizobacteria had an extremely high POX activity when compared to other treatments. Ongena *et al.* (2005) demonstrated that potato tuber cells treated with purified fengycins accumulated plant phenolics involved in, or derived from, phenylpropanoid metabolism. Ongena *et al.* (2007) showed that purified surfactins and fengycins play crucial roles in inducing plant defense mechanisms and provide similar protective activity to that of the bacterial source strain in tomato and beans. Jourdan *et al.* (2009) found major changes in the early events of defense related responses after treatment with surfactins, such as an increase in phenolics and reactive oxygen species (ROSs) in tobacco cells as well as a significant accumulation of PAL and lipoxygenase activities was revealed. The accumulation of ROSs corresponded to the increment of POX activity. For the induction of plant resistance by LPs, however, it was unclear if different members of the family would stimulate different plant cells (Onegena and Jaques, 2008). Surfactins interacted with beans, tomato and tobacco but not potato, whereas fengycins could elicit a response in the three solanaceae species and none of the LPs tested so far had any effect on cucumber (Ongena and Jacque, 2008).

From global environmental friendly concept, the use of biocontrol agent to protect crops against plant disease is recognized as a promising alternative to limit the widespread and extensive application of synthetic chemicals. Numerous bacterial species have been reported as effective microorganisms for biocontrol application, but in most cases the protective effect is related to one specific mechanism explaining the antagonistic activity developed toward the plant pathogen. Thus, the selection of single strains incidence of disease could be valuable. This study highlights such a multi-faceted biocontrol activity of *B. subtilis* ABS-S14 and 155,

through theirs efficient use for biocontrol of postharvest disease. This activity obviously relies on the direct inhibition of the target pathogen by LPs and VOCs and also on an indirect interaction mediated through the host plant via systemic resistance induction.

For further investigation, the enzyme induction profile of citrus fruit inoculated with each purified LP compound as well as volatile compounds could be investigated for understanding the systemic resistance induction in plant by *B. subtilis*. In addition, the accumulation of defense-related mRNA and phenolic compounds derived from phenylpropanoid biosynthesis could be examined.

Chapter 5

CONCLUSIONS

- *B. subtilis* strains, ABS-S14 and 155 used in this study exhibited antagonistic activity toward the citrus rot fungus, *P. digitatum in vitro*.

- The activity of crude ethanolic extracts obtained from cultured media had an antifungal property with the EC_{50} values of 29.71 and 82.48 µg/mL for ABS-S14 and 155, respectively.

- The chromatographic analyses of the crude ethanolic extracts by TLC, HPLC and MALDI-TOF revealed that both antagonistic strains produced well-known lipopeptide families, fengycin, iturin and surfactin with a variety of isoform and both fengycin and iturin compounds displayed an antifungal activity, but this was not observed on surfactin.

- The production of VOCs by these antagonists showed the antifungal activity which can cause abnormal mycelia and inhibited the spore formation of pathogenic fungus, *P. digitatum* and it was culture media dependent.

- The preliminary analysis of VOCs via GC-FID was performed. A sample extraction prior GC analysis using sonication coupled with SPME was found to be the most effective procedure. However, no promising result was obtained when analyzed with GC-MS.

- The population dynamic of *B. subtilis* ABS-S14 was evaluated and demonstrated its survival on citrus fruit at least 20 days without increment in number.

- The activity of POX and PAL were higher in *B. subtilis*- and/or crude ethanolic-applied treatment. This indicated that these antagonistic bacteria and it LPs could prime the plant before pathogen invasion.

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