GROWTH SUPPRESSION OF PHYTOPATOGENIC FUNGI BY ANTIFUNGAL METABOLITES FROM ANTAGONISTIC BACTERIA

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GROWTH SUPPRESSION OF PHYTOPATHOGENIC FUNGI BY ANTIFUNGAL METABOLITES FROM ANTAGONISTIC BACTERIA

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ABSTRACT

Phytopathogenic fungi are important problems in agricultural cultivation worldwide. The use of bacterial antagonists for biological control is an important environmentally friendly strategy for controlling phytopathogens. Thus, this study was focused on the isolation and screening of the potential bacterial antagonists from fermented bioextract (FBE) and shrimp shells. Antagonism of isolated bacteria to inhibit the growth of *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* were investigated. Moreover, the efficiency of extracellular antifungal metabolites produced by the selected bacterial antagonists on the fungal growth inhibition and mycelial morphology was also investigated.

A total of 143 bacteria isolated from FBE and shrimp shells were screened for *in vitro* antagonism toward *C. gloeosporioides* and *S. rolfsii* by dual culture technique. The results found that 3 potent antagonistic bacteria, designated SSCHC4, EMC4 and SSE4, showed the high levels of mycelial growth inhibition to both fungi. These 3 bacterial strains were selected and identified by partial 16S rDNA sequence analysis. The strain SSCHC4 showed a high similarity to *Aeromonas salmonicida* while strain SSE4 and EMC4 showed a high similarity to *Bacillus subtilis*. Unfortunately, *A. salmonicida* causes disease in fish, thus it was unsafe to use as biocontrol agent. Cell-free culture filtrates collected from both the exponential and stationary phases of *B. subtilis* SSE4 and *B. subtilis* EMC4 inhibited the growth of both fungi, indicating that growth suppression was associated with the presence of antifungal metabolites in the culture filtrates. Stationary culture filtrate of both antagonists inhibited the submerged growth of *C. gloeosporioides* and *S. rolfsii* better than exponential culture filtrate. Additionally, morphological changes such as hyphal swelling, cytoplasm aggregation and distortion were observed in fungi grown on PDA that contained the culture filtrates. There was no significant decrease in the percentage of fungal growth inhibition by exponential and stationary culture filtrates after boiling for 45 min or treatment with proteinase K. It could be concluded that thermostable antifungal compounds in the culture filtrate of *B. subtilis* SSE4 and *B. subtilis* EMC4 play an important role in the growth suppression of *C. gloeosporioides* and *S. rolfsii*. This finding might lead to the use of both bacterial antagonists for biological control of fungal plant pathogens.

KEY WORDS: ANTIFUNGAL METABOLITES/ ANTAGONICTIC BACTERIA PHYTOPATHOGENIC FUNGI

142 pp.

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บทคัดย่อ

เชื้อราที่ก่อโรคในพืชเป็นศัตรูพืชตัวสำคัญที่ส่งผลกระทบต่อพืชเศรษฐกิจทั่วโลก ดังนั้นวิธีที่ใช้ในการควบคุมโรคจึงมีหลายวิธี แต่วิธีที่มีประสิทธิภาพ ปลอดภัย ควบคุมโรคได้ระยะเวลานาน และเป็นมิตรกับสิ่งแวดล้อม คือการนำวิถีทางธรรมชาติเข้ามาควบคุม ซึ่งหนึ่งในวิถีทางธรรมชาติที่ใช้ คือ การนำแบคทีเรียที่มีประสิทธิภาพในการต่อต้านศัตรูพืชมาใช้ในการควบคุม ดังนั้นในการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อคัดแยกแบคทีเรียที่มีประสิทธิภาพในการต่อต้านหรือยับยั้งการเจริญเติบโตของ *Collectotrichum gloeosporioides* และ *Sclerotium rolfsii* โดยคัดแยกจากน้ำหมักชีวภาพและของเสียจากอุตสาหกรรมอาหารทะเล และนอกจากนั้นยังศึกษาถึงประสิทธิภาพของสารเคมีที่แบคทีเรียผลิตขึ้นเพื่อใช้ในการยับยั้งการเจริญเติบโตของเชื้อรา แบคทีเรีย 143 สายพันธุ์ ซึ่งคัดแยกได้จากน้ำหมักชีวภาพและของเสียจากอุตสาหกรรมอาหารทะเล ได้นำไปทดสอบเพื่อหาประสิทธิภาพในการยับยั้งการเจริญเติบโตของ *Collectotrichum gloeosporioides* และ *Sclerotium rolfsii* ด้วยวิธีการปลูกเลี้ยงเชื้อคู่ พบแบคทีเรียที่มีประสิทธิภาพในการยับยั้งเชื้อราได้ทั้ง 2 สายพันธุ์ จำนวน 3 สายพันธุ์ จากนั้นจึงนำแบคทีเรียทั้ง 3 สายพันธุ์ ซึ่งได้แก่ SSCHC4, EMC4 และ SSE4 ไปวิเคราะห์เพื่อระบุสายพันธุ์ด้วยวิธีการจับคู่ลำดับเบส จากการวิเคราะห์พบว่า แบคทีเรียรหัส SSCHC4 มีความคล้ายคลึงกับ *Aeromonas salmonicida* ซึ่งแบคทีเรียสายพันธุ์นี้เป็นแบคทีเรียที่ก่อให้เกิดโรคในตระกูลปลาแซลมอนจึงไม่เหมาะสมที่จะนำมาใช้ในการใช้ในการควบคุมโรคพืชจึงไม่ได้ศึกษาถึงประสิทธิภาพต่อไป แต่ในส่วนของแบคทีเรียรหัส EMC4 และ SSE4 ซึ่งมีความคล้ายคลึงกับ แบคทีเรียสายพันธุ์ *Bacillus subtilis* จึงได้ศึกษาถึงประสิทธิภาพของสารซึ่งแบคทีเรียผลิตขึ้นตามธรรมชาติเพื่อใช้ในยับยั้งการเจริญเติบโตของเชื้อรา ณ ช่วงระยะที่มีอัตราการเพิ่มของจำนวนประชากรของแบคทีเรีย และช่วงระยะที่อัตราการเจริญเติบโตของประชากรคงที่ ด้วยวิธีเลี้ยงในอาหารเหลว พบว่า สารที่ผลิตขึ้นในช่วงอัตราการเพิ่มของประชากรคงที่ สามารถยับยั้งการเจริญเติบโตของราได้ทั้ง 2 ชนิด นอกจากนี้ยังได้ศึกษาถึงลักษณะทางสัณฐานวิทยาของการเจริญเติบโตของเชื้อราโดยเลี้ยงเชื้อบนอาหารแข็ง (PDA) ซึ่งมีส่วนผสมของสารที่แบคทีเรียผลิตขึ้นต่อเชื้อราทั้ง 2 ชนิด พบว่าลักษณะทางสัณฐานวิทยาเปลี่ยนแปลงไปคือ เกิดการบวม การบิดเบี้ยวของเส้นใย การรวมตัวของไซโต พลาสซึมในเส้นใย และการแตกหักบริเวณรอยแยกของแขนง จากนั้นจึงได้ศึกษาเพิ่มเติมถึงกระบวนการที่ใช้ในการยับยั้งการเจริญเติบโตของเชื้อรา โดยการนำสารที่แบคทีเรียผลิตขึ้นทั้ง 2 ช่วงเวลา ไปทำลายประสิทธิภาพด้วยความร้อน โดยการต้มด้วยน้ำเดือดเป็นเวลา 45 นาที และเอนไซม์โปรตีนเนส พบว่าเมื่อนำสารเคมีที่ผ่านกระบวนข้างต้นไปทดสอบแล้วไม่เกิดการเปลี่ยนแปลงของประสิทธิภาพในการยับยั้งการเจริญเติบโตของเชื้อราทั้ง 2 ชนิด จึงสรุปได้ว่า *B. subtilis* SSE4 และ *B. subtilis* EMC4 ใช้กลไกในการสร้างสารเคมีขึ้นเพื่อใช้ในการยับยั้งการเจริญเติบโตของรา จากการทดลองข้างต้นจึงสามารถนำแบคทีเรียสายพันธุ์ *B. subtilis* SSE4 and *B. subtilis* EMC4 ไปใช้ในการควบคุมโรคพืชด้วยวิธีทางธรรมชาติได้

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LIST OF ABBREVIATIONS AND SYMBOLS

A_{420}	=	absorbance value at the wavelength of 420 nm
A_{595}	=	absorbance value at the wavelength of 595 nm
BSA	=	bovine serum albumin
° C	=	degree celcius
cm	=	centrimetre (s)
DNA	=	deoxyribonucleic acid
g	=	gram (s)
GlcNAc	=	N-acetyl-D-glucosamine
hr	=	hour (s)
mg	=	milligram (s)
min	=	minute (s)
ml	=	millilitre (s)
mM	=	millimolar (s)
mm	=	millimetre (s)
Ø	=	diameter
rpm	=	revolution per minute
U	=	unit (s) of enzyme
µM	=	micromolar (s)
v/v	=	volume per volume
w/v	=	weight per volume

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CHAPTER I
INTRODUCTION

1.1 Statement of Problems

Agricultural plant production in Thailand usually involves maximum inputs of various pesticides. Thai growers spend approximately 15% of total costs for chemicals to control plant diseases (1). At the same time, there is growing interest in Thailand in producing high-quality agricultural products that have a few or no pesticide residue. These factors have prompted the consideration of biological disease control strategies (2).

Pest and plant pathogens of agricultural plant production in Thailand, particularly phytopathogenic fungi are the important problems to the agricultural products. Fungi have around 8,000 strains that caused the disease of plant and agriculture products (3). Phytopathogenic fungi are important can built the damage severely with the plant, for instance *Collectrotrichum gloeosporioides* (4, 5) and *Sclerotium rolfsii* (6, 7) were fungus phytopathogens which cause the anthracnose and stem rot disease respectively.

Fungus disease prevention in plant can do in many ways but the most popular method among agriculturists was the widely uses of chemicals fungicides. However, the cost of the chemical fungicide is high and questionable of the safe for human health (8). Moreover the use of fungicides was also of stimulus resistance in pests and plant pathogens (9). These chemical substances could cause adversely health effect and contaminate into the soil, air, water as well as products.

Due to these problems, many researchers have been tried to apply the concept of organic farming are utilized the activity of microorganisms to replace chemical pesticides. Organic farming is usually use any biomaterial that possess the following three criteria namely high specificity against the targeted plant pathogens, easy degradability after effective usage, and low cost for mass production. Biological

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control is the alternative method for control pests and plant disease that is environmental friendly, save on money and to be effective protect perennial. The use of living organisms to suppress the population of a specific pest organism, making it less abundant or less damaging that it would otherwise be (10) this sentence refers to biological control definition. Development of biological control methods really blossomed after synthetic chemical pesticide application became the dominant method of pest control. Use of biological control grew due to practical needs to find a solution to pest problems when chemical pesticides did not appropriate for controlling specific pests. Another major impetus for using biological control has been the fact that chemical pesticides can cause negative side-effects, leading to concerns about human health and preservation of the environment. Biological control no leaves chemical residues and are usually quite host specific, specific in comparison to synthetic chemical pesticides (11).

Biological control, using microorganisms to suppress plant disease, offers a powerful alternative to the use of synthetic chemicals. The rich diversity of the microbial world provides a seemingly endless resource for this purpose. Increasing the abundance of a particular strain in the vicinity of a plant can suppress disease without producing lasting effects on the rest of the microbial community or other organisms in the ecosystem (12, 13, 14).

Mechanisms of bacteria able to reduce fungal plant diseases have also been reported in many investigation including competition, antibiosis, parasitism and lysis. Thus, *Bacillus* spp. strains show good control of oilseed Brassicas caused by *Sclerotinia sclerotiorum* (15). *Bacillus* has also been observed to inhibit a few *Collectotrichum* spp. *in vitro* (16, 17) and *in vivo* (16). As such, *Bacillus subtilis* have been used successfully to control a diverse selection of plant pathogenic fungi and bacteria including *Botrytis cinerea* (18) *Fusarium graminearum* (19) *S. sclerotiorum* (20) *Xanthomonas oryzae* (21) and *Pseudomonas solanacearum* (22).

Fermented bioextract (FBE) is one kind of biological substances that agriculturist used to in the farm. FBE consisted of many microorganisms group. These microbes digest complex organic matter into simple molecule for use as the carbon and energy sources under anaerobic condition. However, type of microorganisms and their quantity in FBE depend on a kind of the organic substances that applied in the

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fermentation process as well as the environmental factors including pH, aeration, light, and temperature.

Thus, this study was focused on the isolation and screening of bacteria antagonists to plants pathogenic fungi, *Collectrotrichum gloeosporioides and Sclerotium rolfsii*. Bacteria were isolated from FBE and shrimp shells waste and determined the antifungal potential for use as biocontrol agent against fungal phytopathogens. In addition, the efficiency of extracellular antifungal metabolites produced by selected antagonistic bacteria on the fungal growth inhibition was evaluated. This application might provide an alternative in reducing or substituting of agricultural chemical uses, and minimizing of toxic contamination into the environment as well.

1.2 Objectives of the Study

The objectives of this study were as follows:

1.2.1 To isolate the chitinolytic and non-chitiniolytic bacteria from fermented bioextract (FBE) and shrimp shell waste.

1.2.2 To screen the antagonistic bacteria against phytopathogenic fungi namely *Collectrotrichum gloeosporioides and Sclerotium rolfsii*.

1.2.3 To determine the morphological characteristic and identify bacteria antagonists.

1.2.4 To investigate the efficientcy of selected antagonistic bacteria on the growth suppression of phytopathogenic fungi.

1.3 Study Hypothesis

Selected bacteria from FBE and shrimp shell waste could suppress the growth suppression of, *C. gloeosporioides* and *S. rolfsii* in laboratory scale.

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1.4 Scope of Study

In order to provide a better understanding in biocontrol of fungal plant disease, the efficiency of antagonistic bacteria as a growth suppressor of, *C. gloeosporioides* and *S. rofsii* was studied. The ability of selected bacteria in the production of chitinase and inhibition the growth of *C. gloeosporioides* and *S. rofsii* were performed in the laboratory scale.

1.5 Expected Outputs

1.5.1 The beneficial antagonistic bacteria to *C. gloeosporioides* and *S. rofsii* were isolated.

1.5.2 The antagonistic bacteria caused the growth suppression and destruction of fungal hyphal morphology of *C. gloeosporioides* and *S. rofsii*.

1.6 Conceptual Framework

In order to study the effects of chitinolytic bacteria as a growth suppressor of *C. gloeosporioides* and *S. rolfsii*, the conceptual framework of this study was illustrated in Figure 1-1.

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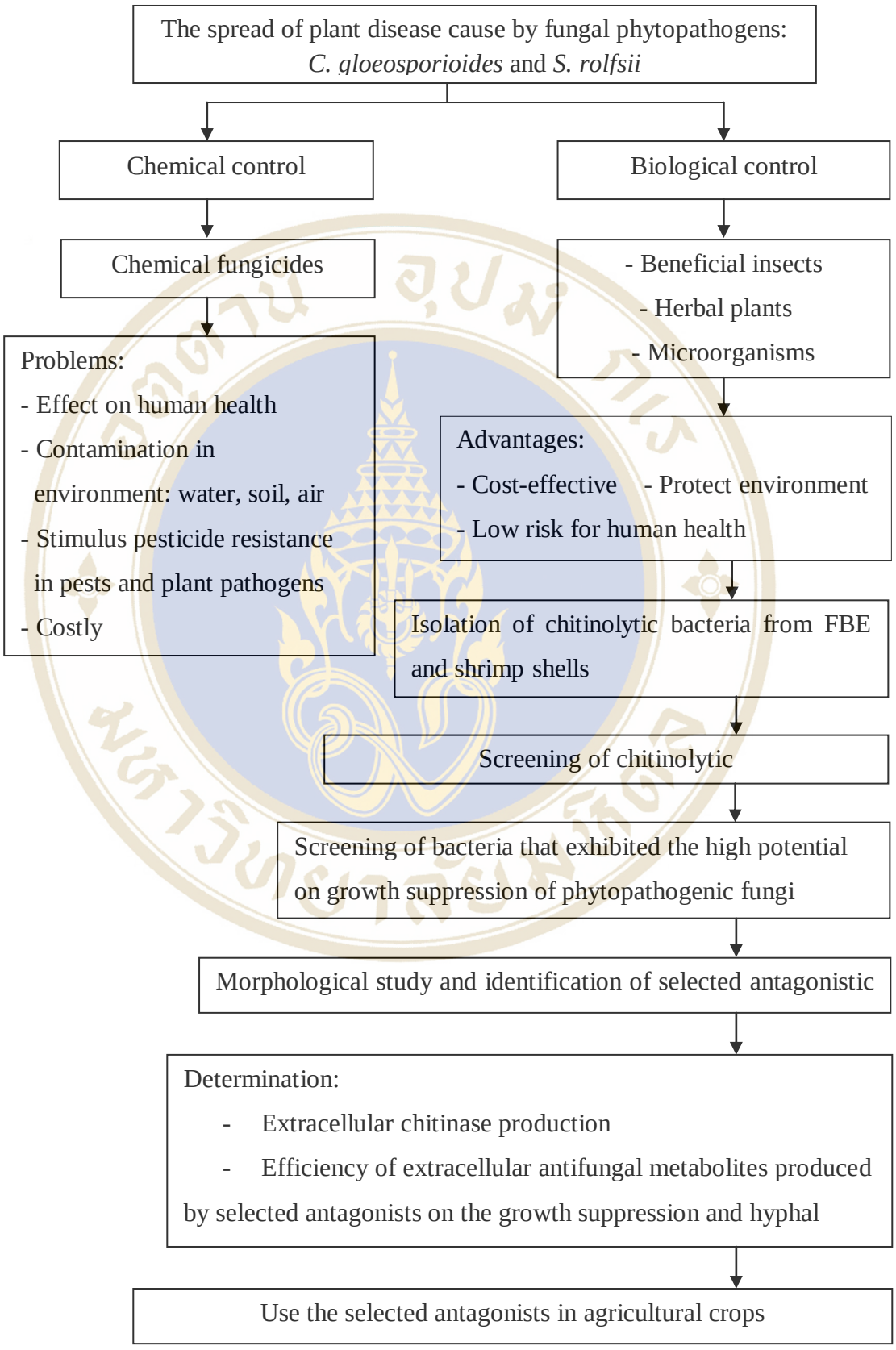

Figure 1-1 Conceptual Framework

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CHAPTER II
LITERATURE REVIEWS

2.1 Defining Biological Control

Populations of all living organism are, to some degree, reduced by the natural actions of their predators, parasites, antagonists and disease. This process has been referred to as "natural control," but when pests are control (sometimes shortened to biocontrol) and the agents that exert the control are frequently called natural enemies. Humans can exploit biological control in various ways to suppress pest populations. The varied approaches for manipulating the activity of natural enemies to control pest differ in how much effort is required, who is involved, and the suitability of the approach for commercial development (11).

Biological control has been defined many times but a commonly accepted definition is provided below.

"The use of living organism to suppress the population of a specific pest organism, making it less abundant or less damaging than it would otherwise be (10)"

As years have passed and scientific research has advanced, the types of approaches available for pest control have also increased in number and complexity. Within the field of biological control, a diversity of natural enemies can be used in many different ways. Other advances have been the ability to synthesize the active compounds used by pests for communication (pheromones), which are then used for controlling those same pests. An example of a relatively new type of control involves the fungus *Myrothecium verrucaria*, which produces multiple compounds that negatively affect plant parasitic nematodes. The fungus is mass-produced and then killed, The active compounds produced by the fungus are then applied to the soil to create an inhospitable environment for the nematodes (11).

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Based on our definition, use of only the compounds produced by natural enemies would not be call biological control. Use of these applications could instead be includes in the larger categories of biologically based pest management or biorational pest control. However disagreements over use of this terminology are far from resolved. Controversy centers around whether the organism used for “biological control” must be living or just the source of compounds and genes. Te following discussion describes the rational underlying the definition of biological control as exploiting living organisms for the control of pests (11).

2.2 History of Biological Control

The first records of biological control describe habitat manipulation to in, crease natural enemy populations. As early as 324 BC, people in China encouraged populations of the ants *Oecophylla smaragdina* in citrus tree to control caterpillars and large boring beetles. This species of ant builds large paper nests in tree resulting in legions of ants inhabiting the trees. Colonies could be purchased or were moved from wild trees into orchards. In addition, to foster movement of ants within the orchard, bamboo runways were placed between trees. Surprisingly, these practices were still seen in the Shan States of North Burma in the 1950s. In 1775, a similar practice was reported from date growers in Yemen, who moved colonies of predatory ants from the mountains to date groves to control pest insects (25).

These earliest uses of natural enemies to control pests involved manipulations of pre-existing natural enemies visible to the naked eye that were generalist predators feeding on many types of pray. With scientific advances, other groups of natural enemies that were smaller began to be investigated and then considered as control agents. The fact that smaller invertebrates live as parasites of larger invertebrates was first reported in the 1600s. With the invention of the microscope by van Leewenhoke in the late 1600s it became possible to learn more about these ever-smaller natural enemies. Although microorganisms had been seen previously, it was until 1835 that microorganisms were first shown to be the cause of disease by Agostino Bassi, working with the fungal pathogen *Beauveria bassiana* infecting silkworm *Bombyx mori*, larvae. In 1874, W. Roberts, working with the

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fungus *Penicillum* and bacteria, first demonstrate that microorganisms could inhibit one another and, in 1908, M. C. Potter first demonstrated such inhibition among plant pathogenic microorganisms (11).

As practices in agriculture and forestry for producing crops improved, single cultivars were grown in ever-larger monocultures. These changes were accompanied by greater pest problems due to both native and introduced pests. With such pest problems, the world was ripe for accepting synthetic chemical pesticides when they were developed. The synthetic chemical insecticide DDT and the synthetic chemical herbicide 2,4-D first began and use for pest control around 1942, and development and use of great diversity of pesticides followed. Although natural enemies had been discovered and described much earlier, developments in the use of natural in the use of natural enemies for control only seriously diversified and escalated after problems with DDT became evident.

Of course scientists had been thinking of using natural enemies for pests control long before the advent of synthetic chemical pesticides. Even Linnaeus suggested using predatory insects to control insect pests in 1752 (US National Research Council, 1996 in (11). The term “biological control” was coined in relation to plant pathogens by C. F. von Tubeuf in 1914 and then applied to insects by H.S. Smith in 1919 (Baker, 1987 in (11). While similar basic principle underlie much of biological control, of different groups of pests evolved quite separately. Scientists working these different groups of pests and different groups of natural enemies need specific training. Scientists trained as entomologists generally specialized either in predators and parasitoids for controlling arthropods in plant science and entomology, plant science, and plant pathology in necessary for plant pathologists working to control plant pathogens or to control weeds with microbes and knowledge of both microbiology and entomology is required to work on pathogens for control of arthropods. As biological control grew, it became evident that the diverse array of pest control problems would require a variety of biological control strategies. Scientist working to control arthropods, weed or plant pathogens historically had few opportunities for interchange although they certainly communicated results within each subdiscipline. The different subdiscipline thus developed their own definition and practices (11). Due to the independent growth of the difference subdisciplines, the

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specific histories of each will be presented separately.

2.2.1 Controlling arthropod pests

Before the advent of restrictions on movement of organisms around the world, pest introductions were numerous and frequently caused dramatic outbreaks. The cottony cushion scale (*Icerya purchasi*), an insects attacking citrus, was introduced to southern California where caused enough damage in the mid-late 1800s to threaten the existence of the California citrus industry. A predator lady beetle (*Rodolia cardinalis*) and a parasitic fly (*Crytochaetum iceryae*) were introduced from Australia, the original home of scale insect. These introductions led to phenomenal success and brought public attention to biological control. For a period following this success, there were many introductions of predator and parasitic insects around the world to control introduced pests, particularly lady beetles to control aphids and scale insect, but no programs successful. This period seemingly haphazard introductions following the cottony cushion scale success was considered a little too enthusiastic by some, who later called this period the “lady bird (lady beetle) fantasy” (26).

Work with pathogens to control arthropods began in earnest later than work with predators and parasitoids, in part because scientific advances were necessary to be able to work easily with microbes. While biological control introductions with arthropod natural enemies were made in North America against cottony cushion scale as early as 1886-87, it was not until the twentieth century that scientists understood how virus worked. Pathogens began developed to be used as formulated biopesticides so that the numbers of organisms released could overcome the lack of dispersal by most pathogens. In 1948, bacteria pathogens for control of Japanese beetles was the first insect pathogen registered for control in the USA. As will be described, the numbers of arthropod pathogens used has increase to fulfill the specific needs of different systems (11).

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2.2.2 Controlling weeds

As stated by Goeden & Andrés (27), “Like so many other aspects of science, (the study of biological control of weeds) began by accident”. In 1975, a scale insect called cochineal that was cultured commercially as a source of carmine dye was introduced from Brazil to northern India for dye production. However, the species that was introduced was not the superb dye-produced *Dactylopius coccus*. Instead of reproducing well on the spineless prickly pear grown specifically for the dye production, *D. ceylonicus* moved onto its natural host plant the prickly pear *Opuntia vulgaris* that had been introduced to northern India and had become a problematic weed. The value of *D. ceylonicus* as a control agent was realized and from 1836 to 1838, this species was introduced to southern India and the in the 1860s to Sri Lanka. In both areas, *D. ceylonicus* provided successful control of the weedy *O. vulgaris*.

2.2.3 Controlling plant pathogens and plant parasitic nematodes

Biological control of plant pathogens got its start much later. Because this field is based totally on microorganisms, more technically advance techniques were required for its growth. The first biological control strategy that was used extensively against arthropods, classical biological control, was not appropriate against plant pathogens. Early in 1900s, plant pathologists recognized that microorganisms could suppress plant disease and this activity could be manipulated through cultural and management practices. The first trial attempting to suppress plant disease by adding beneficial microorganisms to soil occurred in the 1920s. It was not until 1950s that the first biological control organisms was commercial used to control infection or cut tree stumps by *Heterobasidion annosum*, a fungal pathogen that has the potential to spread through root grafts to healthy tress nearby. A second highly successfully product was developed for control of crown gall in the 1970s. Biological control of plant pathogens and plant parasitic nematodes was destined to continue to grow through development of biopesticides, especially against pathogens in the soil environment. By 1995, 30 different biological control organisms were available as commercial formulation for suppression of plan disease. By 2000, the number of

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biological control organisms had not changed substantially but they were being marketed under 80 different product names (28).

2.3 Classical Biological Control

The term "classical biological control" has also been used to refer to this same strategy, describe below

'The intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control (10)*'*

This definition is clear and concise and in agreement with other definitions, e.g., FAO (29)), Coombs and Hall (30). This was the main strategy on which early growth of this field was based, after the great success of releases of the vedalia beetle (*Rodolia (Vedalia) cardinalis* Mulsant (Coleoptera: Coccinellidae) against the cottony cushion scale, *Icerya purchase* Mask. (Homoptera: Margarodidae), in California in the late 1800s. Because of the early development and use of this strategy, it has been called 'classical' (26). The bulk of the literature on classical biological control introductions describes releases of insect parasitoids and predators to control other insect pests and insect herbivores to control weeds. This strategy is also applicable to the deliberate release of micro-organisms, and has been used less frequently to control arthropods (e.g., Hajek et al., 2000) and weeds but is not used for control of plant pathogens. The principle focus in plant pathology is on agriculture and therefore, both plant pathogens and their associated biological control agents are largely considered to have extremely broad, if not world-wide, distributions, thus negating the primary classical biological control requirement for exotic biological control agents.

This same strategy has been called 'importation' by Nordlund (31), but agents used for other strategies, such as inundation and inoculation biological control, can also be imported, so the term importation could lead to confusion. This practice is also referred to as 'introduction of natural enemies' by Van Driesche and Bellows (32) or 'initial augmentation', but again such terminology does not distinguish this strategy from others. The goal of classical biological control, permanent establishment of a

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biological control agent for self-sustained long term control, distinguishes clearly this strategy from inundation and inoculation biological control, thus requiring a distinct name for this practice.

The definition of classical biological control does not depend on achieving complete control. Thus the term ‘semi-classical biological control’ used to describe a programme using an exotic virus to control palm rhinoceros beetle, *Oryctes rhinoceros* L. (Coleoptera: Scarabaeidae) in the South Pacific (33) is not necessary. This virus has been released on individual islands but must be released again after a number of years because its effect diminishes with time. We feel that this additional term, based on the result of the programme, is not needed. Instead, the strategy for the initial releases should correctly be named classical biological control. However, because the virus only provides control temporarily from initial classical biological control releases, this would then be followed by inoculation biological control.

Classical biological control depends on finding an appropriate biological control agent that is not native to the area where the pest needs to be controlled. Thus, classical biological control requires the introduction of an ‘exotic’ organism. In practice, for some natural enemies different races or biotypes within the same species can have vastly different attributes, complicating determination of whether an agent is exotic or not. Whether an organism is ‘exotic’ can also be complicated when the control operation is taking place anywhere other than on an island. In the case of large countries with many differing ecozones, authorities often consider an organism from a different ecozone as exotic. In the case of small countries in the same ecozone, national authorities generally consider any importation across a national border as an exotic introduction. More recently, use of molecular characterisation has been making determination of the area of origin of both biological control agents and pests possible, enabling scientists to determine whether a natural enemy is exotic or not (10).

2.4 Interactions with the Pathogen

Biocontrol involves harnessing disease-suppressive microorganisms to improve plant health. Disease suppression by biocontrol agents is the sustained manifestation of interactions among the plant, the pathogen, the biocontrol agent, the

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microbial community on and around the plant, and the physical environment. Even in model laboratory systems, the study of biocontrol involves interactions among a minimum of three organisms. Therefore, despite its potential in agricultura1 applications, biocontrol is one of the most poorly understood areas of plant-microbe interactions (39).

The complexity of these systems has influenced the acceptance of biocontrol as a means of controlling plant diseases in two ways. First, practical results with biocontrol have been variable. Thus, despite some stunning successes with biocontrol agents in agriculture, there remains a general skepticism born of past failures. Second, progress in understanding an entire system has been slow. Recently, however, substantial progress has been made in a number of biocontrol systems through the application of genetic and mathematical approaches that accommodate the complexity (39).

Modern methods for analysing microbial community structures may prove particularly valuable to help define the key organisms or groups of organisms responsible for spread and impact of introduction of specific biocontrol agents or other management practices on natural microbial populations (34, 35). At present, greatest resides with the development and application of specific biocontrol agents for the control of disease on seeds and roots and the interaction of these with pathogens and hosts. Mode of action include: inhibition of the pathogen by antimicrobial compounds (antibiosis); competition for iron though production of siderophores; competition for colonization sites and nutrients supplied by seeds and roots; induction of plant resistance mechanism; inactivation of pathogen germination factors present in seed or root exudates; degradation of pathogenicity factors of the pathogen such as toxins; parasitism that may involve production of extracellular cell wall-degrading enzyme, for example chitinase and β-1,3 glucanase that can lyses pathogen cell walls (36, 37, 38). None of the mechanisms are necessarily mutually exclusive and frequently several modes of action are exhibited by a single biocontrol agent. Indeed, for some biocontrol agents different mechanisms or combinations of mechanisms may be involved in the suppression of different plant disease.

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2.4.1 Antibiosis

Biocontrol is often attributed to antibiosis. In many biocontrol systems that have been studied, one or more antibiotics have been shown to play a role in disease suppression. The fact that antibiosis is a common mechanism of biocontrol may be due to a bias in choice of organisms for study. Alternatively it may be due to the attractiveness of the antibiosis hypothesis, or antibiosis may be simply a highly effective mechanism for suppressing pathogens in the rhizosphere (39).

A variety of antibiotics have been identified, including compounds such as amphisin, 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and cyclic lipopeptides produced by pseudomonads (40, 41, 42, 43) and oligomycin A, kanosamine, zwittermicin A, and xanthobaccin produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* spp. (44, 45, 46).

Antibiotic synthesis is tightly linked to the overall metabolic status of the cell, which in turn is dictated by nutrient availability and other environmental stimuli, such as major and minor minerals, type of carbon source and supply, pH, temperature, and other parameters (47). Trace elements particularly zinc, and carbon source levels influence the genetic stability/instability of bacteria, affecting their ability to produce secondary metabolites (48). It is important to note that many strains produce pallet of secondary antimicrobial metabolites and that conditions favoring one compound may not favor another. Thus, the varied arsenal of biocontrol strains may enable antagonists to perform their ultimate objective of pathogen suppression under the widest range of environmental conditions. For example, in *P. fluorescens* CHA0 biosynthesis of DAPG is stimulated and pyoluteorin is repressed in the presence of glucose as a carbon source. As glucose is depleted, however, pyoluteorin becomes the more abundantly antimicrobial compound produced by this strain (49). This ensures a degree of flexibility for the antagonist when confronted with a different or a changeable environment. Biotic conditions can also influence antibiotic biosynthesis (50, 51, 52, 53).

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2.4 2 Competition for iron

Biocontrol can involve suppression of the pathogen by depriving it of nutrients. The best understood example of this mechanism is iron competition. Iron is abundant in Earths crust, but most of it is found in the highly insoluble form of ferric hydroxide; thus, iron is only available to organisms at concentrations at or below 10^{18} M in soil solutions at neutra1 pH. This presents a challenge for bacteria, which require iron at micromolar concentrations for growth. Bacteria have evolved high-affinity iron uptake systems to shuttle iron into the cell. The typical system involves a siderophore, which is an iron-binding ligand, and an uptake protein, which transports the siderophore into the cell. The fluorescent pseudomonads produce a class of siderophores known as the pseudobactins, which are structurally complex iron-binding molecules. Analyses of mutants lacking the ability to produce siderophores suggest that they contribute to suppression of certain funga1 and oomycete diseases (24, 39, 47).

2.4.3 Parasitism and production of extracellular enzymes

In addition to antibiosis and iron deprivation, certain biocontrol agents also reduce plant disease by parasitizing pathogens. The ability of bacteria, especially actinomycites, to parasitize and degrade spores of fungal plant pathogens is well established. Assuming that nutrients pass form the plant pathogen to bacteria, and that fungal growth is inhibited, the spectrum of parasitism could range from simple attachment of cells to hyphae as with the *Entorobactor cloacae* (Jordan) Hormaeche & Edwards-*Pythium ultimum* interaction (55). If fungal cells are lysed and cell walls are degraded then it is generally assumed that cell wall-degrading enzymes produced by the bacteria are responsible, even though antibiotic may be produced at the same time. For example biocontrol of *Botrytis elliptica* Botrytis leaf blight of lily *Bacillus cereus* 28- exhibited on Botrytis leaf blight of lily was obtained using ChiCW isolate of *Bacillus cereus* 28-9 (54)

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2.4.4 Induced Systemic Resistance (ISR)

Certain bacteria trigger a phenomenon known as ISR phenotypically similar to systemic acquired resistance (SAR). SAR develops when plants successfully activate their defense mechanism in response to primary infection by a pathogen, notably when the latter induces a hypersensitive reaction through which it becomes limited in a local necrotic lesion of brown, desiccated tissue (23). As SAR, ISR is effective against different types of pathogens but differs from SAR in that the inducing PGPB does not cause visible symptoms on the host plant (23).

The ability to act as bioprotectants via ISR has been demonstrated for both rhizobacteria and bacterial endophytes, and considerable progress has been made in elucidating the mechanisms of plant-pathogen interaction. ISR is associated with an increase in sensitivity to these hormones rather than an increase in their production, which might lead to the activation of a partially different set of defense genes (56).

Bacteria triggered ISR fortifies plant cell wall strength and alters host physiology and metabolic responses, leading to an enhanced synthesis of plant defense chemicals upon challenge by pathogens and/or abiotic stress factors (57, 58). The type of bacterized plant response induced after challenge with a pathogen resulted in the formation of structural barriers, such as thickened cell wall papillae due to the deposition of callose and the accumulation of phenolic compounds at the site of pathogen attack (59, 60). Biochemical or physiological changes in plants (58) include induced accumulation of pathogenesis-related proteins (PR proteins) such as PR-1, PR-2, chitinases, and some peroxidases (58, 60, 61, 62).

2.5 Chitin

2.5.1 Difinition of chitin

Chitin is the second most abundant biopolymer (polysaccharide) in nature, after cellulose, which is a strong indication of its importance in nature. Chitin is an insoluble linear β-1,4 homopolymer of *N*- acetyl-*D*-glucosamine (GlcNAc). It is

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widely distributed as a structural component of crustaceans, insects, and other arthropods, as well as a component of the cell walls of most fungi and some algae. About 1,011 tons of chitin is produced annually in the aquatic biosphere alone. (63, 64, 65).

2.5.2 Chemical structure, molecular formula and properties of chitin

Chitin is non-electrolytic polymer, so it is unsoluble in general solvent, there is structure formula $(C_8H_{13}O_5)_n$. Chemically, it is classified as a polysaccharide, which are large molecules consisting of smaller sugar molecule strung together like pearls on a strand (14). Chitin is a linear polysaccharide polymer, β-(1,4)-2-acetamino-2-deoxy-D-glucose (N-acetyl-D-glucosamine) (66). It is chemically similar to cellulose, a polymer of β-1,4- linked glucose and its derivatives. The only difference is that chitin consists of NH-CO-CH_3 groups instead of hydroxyl groups (-OH) at the second carbon atom (14). Chitosan is a copolymer of β-1,4- linked glucosamine and *N*- acetylglucosamine (63, 64, 65).

Chitin is composed of repeating units of the monomer *N*-acetylglucosamine (GlcNAc) and contains carbon and nitrogen in a ratio of 8:1. Chitin degradation is a key step in the cycling of nutrients in the environment (67, 68).

Chitin does not exist alone, but is found in close association with calcium carbonate and/or protein and other organic substances, making the compound stable to most reagents (69). Chemical structures of them comparing with cellulose are shown in Figure 2-1.

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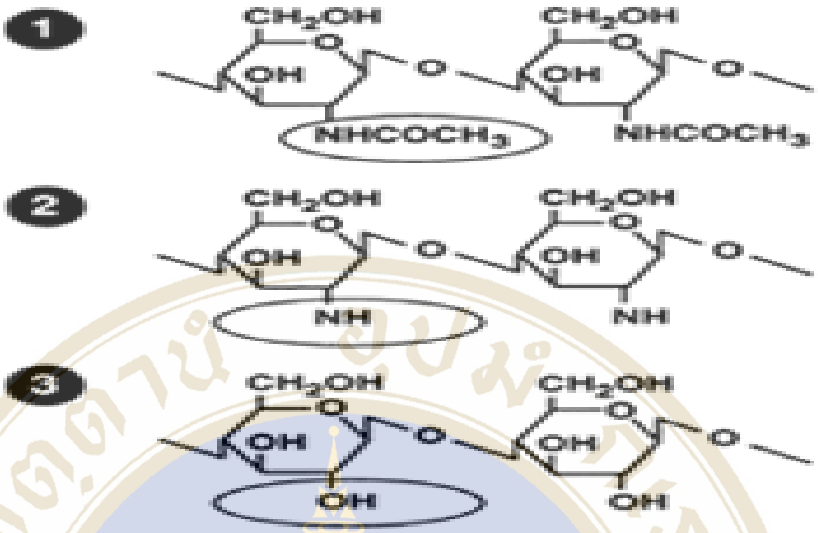

Figure 2-1 Chemical structures of 1. chitin, 2. chitosan and 3. cellulose (70)
This page was last modified May 23, 2002

2.5.3 Structures of chitin

Chitin is a crystalline polysaccharide widely spreading in nature with three structures: (63, 71)

1. α-chitin is the most abundant isomorphous form and is not soluble and does not swell in common solvents. It is tightly compacted due to its crystalline structure where the chains are in antiparallel fashion favoring strong hydrogen bounding. α-chitin are found in from crustaceans, insects, and fungi.
2. β-chitin has an arrangement in parallel with weaker intermolecular forces that leads to a less stable molecule than α-chitin and can be swollen in water as well as dissolved in formic acid. β-chitin are found in the spines of polychaete *Aphrodite,* pen of the squid *Loligo*, tubes of *Pogonophor*s and spines of certain marine diatoms.
3. γ-chitin is a mixture of both α-chitin and β-chitin. γ-chitin can be transform to α-chitin by reacting with Lithiumtriocyanate. γ-chitin has been reported from the stomach lining *Loligo*.

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2.5.4 Source of chitin

Chitin is a polysaccharide that perform be molecule structure by is the components of shrimp shell an insect and crab carapace and still the components of cell wall in fungus, yeast and invertebrates show in table 1 .

Table 2-1 Source of chitin and chitosan (63)

Organisms	Examples	Structure	Minearals	Chitin % org	Other organic constituents
FUNGI					
Ascomycota	*Aspergillus flavus, A. fumigatus, A. niger, Penicillum notatum, Neurospora crassa.*	cell walls			
Basidiomycota	*Blastcocladiella emersonii, Mucor rourii, Mortierella vinacen, Phycomycetes Blakesleanus, Rhizopus rhizopodiformis, Apodachlya.*	and structural membranes of mycelia stalds and spores		traces to 45	glucans, mannan or other polysaccharides
Imperficti	*Candida albicans, Alternaria kikuchiana.*				
ALGAE					
Chlorophyceae	*Ulva lactuca, Valonia ventricosa*			+	cellulose
PROTOZOA					
Rhizopzda	*Pelomyxa*	cyst wall		+	-
	Plagiopyridae	shell	silica	+	-
	Allogromia	shell	iron	+	proteins, lipid
	Vasicola ciliata.	cyst wall		+	proteins
CNIDARIA					
Hydrazoa					
Hydroidea		perisarc		3-30 α	proteins (tanned)
Milleporina	*Millepora alcicomis.*	coenosteum	$CaCO_3$	+ α	-
Siphonophora		pneumatophore		+ α	-
Anthozoa	*Metridium, Pocillopora*	"skeleton"	$CaCO_3$	+	proteins
Scyphozoa	*Aulai*	podocyst		+	proteins

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Table 2-1 Source of chitin and chitosan (63) (continued)

Organisms	Examples	Structure	Minearals	Chitin % org	Other organic constituents
ASCHELHNTES					
Rotifera		egg inner membr.		14	proteins
Nematoda	*Ascaris lombricoides.*	egg middle		16	proteins
Acanthocephala		egg capsule		+	proteins
Priapulida	*Priapulus caudatus.*	cuticle		+	proteins (tanned)
ENDOPROCTA	*pedicellina cemura*	cuticle		+	tanned proteins
BRYOZOA	*Cristatella mucedo*	ectocyst	$CaCO_3$	2-6	proteins
PHORONIDA		tubes		13	proteins
BRACHIOPODA					
Articulata		stalk cuticle		4	-
		stalk cuticle		+ γ	collagen
		shell		29 β	-
ECHIURIDA		hooked chaetae		+	-
ANNELIDA					
Polycheatea	*Aphroodite, Amphinome*	chaetae		20-38 β	quinine-tanned prot.
Eunicidae		jaws		0.3	proteins
Oligocheta	*Lombricus terrestris*	chaetae		+ β	
all		peritrophic member.		+	proteins
MOLLUSCA					
Polyplacophora	*Chiton*	shell plates	$CaCO_3$	12	proteins
		radula	iron	+	proteins
Castropoda	*Helix pomatia*	mother of pearl	$CaCO_3$	3-7	conchiolin
		radula	iron sil.	20 α	tanned proteins
		jaws		+	tanned proteins
Cephalopoda	*Sepia officinalis, Nautilus*	calcifield shell	$CaCO_3$	3-26 β	conchiolin
	prompilus, Loligo paclei,	pen		18 β	conchagen
	Octopus vulgaris,	jaw and redula		20 β	tanned proteins
	Omnztostrephes sagittalus	stomach cuticle		γ	
Lamellibranchia	*Nucula nitida, Ostrrea virginica*	shells	$CaCO_3$	up to 8	conchiolin
	Venus Mercenaria mercenaria	gastric shield		17	-

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Table 2-1 Source of chitin and chitosan (63) (continued)

Organisms	Examples	Structure	Minearals	Chitin % org	Other organic constituents
ARTHOPODA					
Crustacea	*Palinurus valgaris, Homarus vulgaris, Cancer magister, pagurus, Carcinus maenas, Astacus fluviatilis, Euphausia superba*	calcifield cuticle	$CaCO_3$	58-85 α	arthopodin+ sclerotin
		intersegmental m.	$CaCO_3$	48-80 α	arthopodin
Insecta	*Droaophila melanogaster, Locusta migratoria, Periplaneta Americana, Bombyx mori, Calliphora sp., Sarcophaga bullata, Tenebrio molitor, Schistocerca gregaria.*	hardened cuticle	$CaCO_3$	20-60 α	arthopodin+ sclerotin
		unhardened cuticle	$CaCO_3$	20-60 α	arthopodin+ sclerotin
Arachnida and Chilopoda	*Galeodes, Scolopendra*				
all	*Musca domestica*	peritrophic member		4-22	proteins+mucin
POCONOPHORA	*Oligobrachia ivanovi*	tubes		33 β	proteins

2.5.5 Properties of chitin

2.5.5.1 Biological properties

One of the most beneficial properties of chitin is its biological properties. Due to its biological properties namely biocompatibility, non-toxicity and biodegradability, it could provide the potential applications such as medical, food processing and agricultural applications (71).

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2.5.5.2 Soluble properties

Chitin is insoluble in water, alkali and organic solvent, but it is soluble in most solutions of organic acid when the pH of solution is less than 6 (72).

2.5.6 Applications of chitin

Nowadays, chitin and chitosan is used in a variety of products. Some major applications are summarized in Table 2-2.

Table 2-2 Applications of chitosan

Field	Applications
Biomedical uses	- Potential anti-cholesterol drugs - Wound healing care - Coating for eye bandages - Use in porous grindable contact lens - Dental bioadhesive and biodegradable structures
Personal care products	- Skin and hair care products to build coating - Cosmetics to build viscosity, coating, moisture retention
Biotechnological applications	- Matrix for immobilizing enzymes and cells - Filtration membrane for recovery of protein from products - Chromatography support for enzymes and cells

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Table 2-2 Applications of chitosan (continued)

Field	Applications
Water treatments	- Industrial waste stream treatments - Membrane filtration - Clarifying agent for fruit juices, food processing streams, and fermented beverages - Removal of metal and bacteria from swimming pools and spa water
Textile and paper applications	- Coating for textiles and non woven fabrics - Binder for paper and a retention aid to bind clays and cellulose
Agriculture uses	- Antimicrobial substances for some fungi and bacteria - Nematocide - Seed treatments and growth regulators - Animal feed additives at ≤ 0.1 %

Source: Weiner ML (73) and Kungsuwan A (74)

2.6 Chitinase

Chitinase be the enzyme in the hydrolase group which will digest chitin is free N-acetylglucosamine

Schomburg and Salzman (75) the systematization of the enzyme has the enzyme digests chitin 2 types

1. endo-chitinase or chitinase be known chemical name that poly- β-(1,4)-2-acetamide-2-deoxy-D-glucoside glycanohydrolase: EC 3.2.1.14 be known call other chitodextrinase, poly- β-glucosaminidase which digest chitin to random at the Glycosidic bound of chitin get oligomer and GlacNAc for example chitooligosaccha-

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ride, GlcNAc3 and chitotraose (GluNAc4) and GlcNAc if ripen keep long ago.

2. exo-chitinase or N-acetylglucosaminidase: EC 3.2.1.30 which be known call other chitobiase, β-N-acetylglucosaminidase this enzyme will digest the end non-reducing chain of N-acetyl-D-glucosamine the remainder from digesting with chitinase be chitobiose and oligomer, the other get the produce is N-acetylglucosamine and *p*-nitrophenol.

Flach et al. (76) divided the enzyme digests chitin 4 type

1. Endochitinase be that make enzyme digest polymer of chitin ruptures.
2. Separate exochitinase be the enzyme has that to digest chitin already chitobiose.
3. Chitobiase be the enzyme that digests chitobiose get GlcNAc.
4. N-acetylglucosaminidase be the enzyme has that to digest chain within chitin get GlcNAc.

2.6.1 Source of chitinase

2.6.1.1 Animal

Jeuniaux (1996) (77) meet the enzyme chitinase in the invertebrate for the first time in a snail, later meet in protozoa and gastric mucosa of coelenterates, nematodes, polychaets, oligochates, mollusks and arthopods for in animal chitin that eat enter digested be GlcNAc before then absorb go to use. Chitinase in the system digests the food is from 3 places the enzyme that the body makes to come to by oneself, the enzyme from the microorganism in the intestines and the alimentary canal and from the food that eat to enter (78). Besides the living things in the group nematodes chitinase pour from epidermis, while there is something egg sheath in the arthopods chitinase pour from epidermis, while there is sloughing in the fish reptiles amphibious and a bird that eat an insect is the food chitinase pour from the pancreas and gastric mucosa for digest the food. The parts in the mammal chitinase pour from gastric mucosa (77) chitinase in the blood of the fish turbod (*Scophthalmus Maxmas)* there is a role in disease prevention from a fungus and protect the parasite (79).

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2.6.1.2 Plant

In the plant has a substance surppression of a fungus which for example chitinase and β-1,3-glucanase in the plant was encouraged by the hormone Etherine or activate from pathogens an insect (80, 81). Chitinase and β-1,3-glucanase from pea pods there is the property in digesting wall the cell of a fungus by chitinase against separate get can suppression fungus *Trichoderma viride* (82), besides study chitinase in the plant and a seed such as barley grain (83), tomato (*Lycopersicon esculentum*) (84) bean leaves (85).

2.6.1.3 Microorganisms

In the environment marine microorganisms have polymer differ be powder from the seaweed the cellulose from the seaweed vacates the plant chitin from crustacean and cell wall of the plant polymer these will have digesting crumbles slowly and have the accumulation in a little sea except shore border (neritic sea) by digesting majority is born from location microorganism in the sea (86) the microorganism then is source of chitinase that important and the microorganism that can produce this enzyme can meet both of in the bacteria, fungus, and yeast. Chitinase at produce from many bacteria meet that have though intracellular chitinase and produce extracellular chitinase, often mainly meet extracellular chitinase more than.

2.6.1.3.1 Chitinase in fungus

Fungus that produce chitinase have various strains such as *Mucor mucedo* (87), *Tricoderma hazianum* (88) and *Penicillium oxalicum* (89) etc., which meets that fungus produce chitinase can apply in fungus plant disease supervision that meet in soil.

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Table 2-3 Fungus that builds chitinase control phytopathogenic fungi (90)

Chitinolytic fungi	Pathogenic fungi
Tricoderma hazianum.	*Sclerotium rolfsii* and *Rhizoctonia solani*.
T. hazianum	Soil bron plant pathogenic fungi
T. hazianum. strain P1	Fusarium, Botrytis, Ustilago, Uncinula and *T. hazianum*

Vyas and Despande (1989) (91)studied chitinase in *Myrothecium verrucaria* found that this strain can produce chitinase get rich in the medium that have chitin be carbon source and add 0.03% urea, found that there is the enzyme increases 4 times when compare with the group controls and if add 0.1% oxgallin the growth medium, ripen that room temperature for 7 day found that appraise the work of topmost enzyme, biochemistry property of chitinase found that there is temperature wide period be during 25-55 ^{o}C and pH during 4.0-6.5 intermittently that best compare with the enzyme that have commercial (Novozym 234 and Onozuka R-10) with the nutrient of *M. verrucaria* found that there is rich work 5-6 more times and the fiber of fungus has the production N-acetyl-D-glucosamine rather tall.

2.6.1.3.2 Chitinase in actinomycetes

Actinomycets no have chitin be an element of cell wall like fungus, but found that can produce chitinase particularly genus Streptomyces which have person study keep a lot of.

Gupta et al. (1995) (92) checked the *Streptomyces* 9 species that can choose found that *Streptomyces viridificans* produce chitinase well most which fungus. *Streptomyces viridificans* builds the enzyme is constitutive enzyme by found that there is building enzyme in low level although will use simplex and complex carbon source the sugar is arabinose make building enzyme increase 2 times while glucose suppression building last produce this enzyme has can durable the temperature wide 30-55^{o}C and pH 3.0-7.5 Mn^{2+} and Hg^{2+} enzyme

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suppression, the nutrient and the enzyme are pure can bring to antagonist pathogenic fungi by digesting crumbles cell wall of pathogenic fungi.

2.6.1.3.3 Chitinase in bacteria

There is report bacteria can produce chitinase widely throws such as *Serratia marcescens* (92), *Bacillus circulans* (93) and *Vibrio* sp. (94) etc., found that the bacteria that produce the enzyme for uses in digesting crumbles can give last result is N-acetylglucosamine which bacteria will apply next.

Osawa and Koga (1995) (95) study the bacteria that can use chitin 48 species which separate from a river and the Tokushima sea these bacteria has done checking already found that be *Vibrio fluvialis, V. parahamolyticus, V. alginolyticus, V. mimicus, Listonella anguillarum* and *Aeromonas hydrophila* every the species found that can build chitinase and chitobiase and have the ability in using NAG be carbon and nitrogen source.

2.6.2 The advantage of chitinase

2.6.2.1 Biocontrol agent

Rojas-Avelizapa et al., (1999) (96) studied bacteria strain *Bacillus thuringiensis* give precedence in 2 points firstly choose the strain can produce chitinase there is the property is bioinsecticide give a large amount, the points is second choose the strain that have the activity of topmost enzyme bring digest the remnant from the industry and found that strain Bt-112 *(B. thuringienisis* serovar *tolworthi*) produce chitinase have the poisonous builds in the family *Manduca sexta, Aedes aegypti* and *Leptinotarsa texana* and be poisonous young of *Manduca sexta*, in the phase Instar I. Lorito et al., (1998) (97) clone gene of fungus *Trichoderma harzianum* add in a tobacco leaves and the potato, for both plants have the property in antagonist from a fungus, because fungus can produce endochitinase and have the property is a suppression fungus.

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2.6.2.2 Chitooligosaccharide preparation

Chitooligosaccharide apply to medical advantage has many the way such as use decrease concentration of plasma when have hypercholesteral, help hurry give a wound is lost fast, encourage immune system there is the antagonist cancer (chitohexaose, chitohepaose) and suppression the progression of the bacteria. Chitooligosaccharide prepare from digesting chitin with acid or alkaline is which give a result low produce, later found that digesting with effective enzyme better because can fix the size of molecule, get the products that have the size particularly different follow a type of the enzyme, a substance that have the size differently has will physical property, biological differently and reaction do not be severe, then can induce apply in food industry, supplementary health food, medical profession and the agriculture (98).

2.6.2.3 Chitinase in the preparation protoplast of fungus

Separation protoplast from fungi be significant increase because protoplast can apply biological technology advantage has variously such as study building cell wall, driving extracellular enzyme, transformation of steroids and study about carcinogen because chitin be pillar components in the structure of cell wall of fungi, enzyme that digest chitin be pillar components in the structure, because of chitin be majority structure of cell wall of a fungus, the enzyme digests chitin then have a role in the separation protoplast from fungi. Chitinase in high-level help separate protoplast from the mycerium of efficiently fungi, mucolytic enzyme cooperate chitinase can make protoplast homogenous gene greatly in fast time (91, 99).

2.6.2.4 Preparation single-cell protein (SCP)

Production SCP is waste matter leads that have chitin be an element such as shrimp shell, crab carapace and squid carapace (100). The first stage the pulling takes the part that is the protein and other substance that do not want

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to go out for get especial chitin, then lead the microorganism that can digest chitin to digesting have been monomer of, N-acetylglucosamine will bring the media with yeast strain can produce SCP (101) and apply the food supplementary in domesticating animals and aquatic animals (102).

2.7 Phytopathogenic Fungi

2.7.1 *Colletotrichum gloeosporioides*

2.7.1.1 Biology

Colletotrichum gloeosporioides Penz. a facultative parasite belongs to the order *Melanconiales.* The fungus produces hyaline, one- celled, ovoid to oblong, slightly curved or dumbbell shaped conidia, 10-15 μm in length and 5-7 μm in width. Masses of conidia appear pink or salmon colored. The waxy acervuli that are produced in infected tissue are subepidermal, typically with setae, and simple, short, erect conidiophores. The pathogen initially infects intact, non-wounded immature green fruit in the field. Spores germinate and form appressoria on the fruit surface. The fungus, using its appressorium, enzymatically penetrates the cuticle and then remains as sub-cuticular hyphae until the post climacteric stage of fruit growth is attained. At this point, for reasons that are not understood, the fungus resumes growth and causes the characteristic symptoms. Environmental conditions favoring the pathogen are high temperatures, 28^{o}C being optimal, and high humidity. Spores must have free water to germinate; germination is negligible below 97% relative humidity. Spores are only released from acervuli when there is an abundance of moisture. Splashing from rain is a common means of spread. Severity of disease is related to weather and the fungus is relatively inactive in dry weather. Sunlight, low humidity and temperature extremes (below 18^{o}C or greater than 25^{o}C) rapidly inactivate spores.

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Table 2-4 Scientific classification of *Colletotrichum gloeosporioides* (141)

Kingdom:	Fungi
Phylum:	Deuteromycota
Class:	Coelomycetes
Order:	Melanconiales
Family:	Melanconiaceae
Genus:	*Collectrotrichum*
Species:	*C. gloeosporioides*
Binomial name	*Colletotrichum gloeosporioides*

2.7.2.2 Anthracnose disease (Leaf Blight disease)

Colletotrichum gloeosporioides causes of *Dendrobium* sp. Signs and symptoms as follow (103, 104):

2.7.2.2.1 Hosts

Colletotrichum gloeosporioides is known to infect a wide variety of hosts including orchid, papaya, mango etc.

2.7.2.2.2 Epidemiology

Primary inoculums can be disseminated by wind or rain.

2.7.2.2.3 Symptoms

Also may appear as irregular to circular spots 1 to 10 mm in diameter, sharply defined, occasionally slightly depressed and reddish-brown in color. These lesions are referred to as "chocolate spots." As the fruit ripens, these spots rapidly enlarge (up to 20 mm in diameter), to form the characteristic

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circular sunken lesions. Seedlings that emerge from infected seed can develop lesions on the root, hypocotyl or cotyledons. Lesions are generally oval shaped, pink to beige and up to 2 cm long. These cause the stem to bend and may progress to infect the pods and seeds.

2.7.2.2.4 Management

2.7.2.2.4.1 Non-chemical control

Hot water dips at 48°C for 20 min is an effective treatment for reducing anthracnose incidence. Although hot water dips do not completely eliminate anthracnose the reduction in disease is economically significant.

2.7.2.2.4.2 Chemical control

Orchard sprays applied at 14 - 28 day intervals, depending on rainfall, with an appropriate protective fungicide is commonly recommended. Post harvest fungicides; applied as a spray or dip, with a food-grade wax have also shown to be effective in reducing anthracnose.

2.7.2 *Sclerotium rolfsii*

A sclerotium is a compact mass of hardened mycelium (as an ergot) stored with reserve food material that in some higher fungi becomes detached and remains dormant until a favorable opportunity for growth occurs (104).

2.7.2.1 Biology

Synonyms of *Sclerotia rolfsii* include: *Athelia rolfsii* (Curzi) Tu and Kimbrough (Sexual stage) and *S. delphinii* (Synonyms for the sexual stage: *Corticium rolfsii, Pellicularia rolfsii*). *S. rolfsii* grows, survives, and attacks plants at or near the soil line. Before the pathogen penetrates host tissue it produces a

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considerable mass of mycelium on the plant surface, a process which can take 2 to 10 days. Penetration of host tissue occurs when the pathogen produces an enzyme which deteriorates the hosts' outer cell layer. This results in tissue decay, further production of mycelium and the formation of *sclerotia*. The latter two rely upon favorable environmental conditions. *Sclerotia* undergo either hyphal or eruptive germination. Hyphal germination is characterized by the growth of individual strands of hyphae from the sclerotial surface while eruptive germination is characterized by plugs or aggregates of mycelium bursting through the sclerotial surface. The quantity of mycelial growth and the energy needed for infection is dictated by the type of *sclerotial* germination that takes place. A food base of nonliving organic matter must be present for hyphally germinating sclerotia to infect host tissue because mycelial growth is sparse. However, mycelium from eruptively germinating *sclerotia* can infect host tissue without requiring an exogenous food base. *S. rolfsii* is able to survive (and thrive) within a wide range of environmental conditions. Growth is possible within a broad pH range, though best on acidic soils. The optimum pH range for mycelial growth is 3.0 to 5.0, and *sclerotial* germination occurs between 2.0 and 5.0. Germination is inhibited at a pH above 7.0. Maxiumum mycelial growth occurs between 25 and 35^{o}C with little or none at 10 or 40^{o}C. *Sclerotial* formation is also greatest at or near the optimum temperature for mycelial growth. Mycelium is killed at 0^{o}C, but *sclerotia* can survive at temperatures as low as -10^{o}C. High moisture is required for optimal growth of the fungus. *Sclerotia* fail to germinate when the relative humidity is much below saturation. However, there are some studies which assert that sclerotia germinate best at relative humidities of 25-35 %. One review summed it up by stating that soil moisture studies are difficult to interpret. Mycelial growth and sclerotial germination occur rapidly in continuous light, though they will occur in darkness if other conditions are favorable. Occasionally *S. rolfsii* has a sexual fruiting stage which develops on the margins of lesions and in locations that are shaded from the sun. Two or four thin-walled colorless spores are borne on short spines at the ends of slightly enlarged short threads. To what extent this stage aids in the reproduction and spread of the organism under field conditions is unknown. The spores are so light that if produced in large quantities they could be carried long distances in the air. This

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stage is not frequently seen in the field and is not believed to be of primary importance in disease transmission.

Table 2-5 Scientific classification of *Sclerotium rolfsii* (142)

Kingdom:	Fungi
Phylum:	Basidiomycota
Class:	Basidiomycetes
Order:	Agaricales
Family:	Typhulaceae
Genus:	*Sclerotium*
Species:	*S. rolfsii*
Binomial name	*Sclerotium rolfsii*

Figure 2-3 *S. rolfsii* caused orchid dry rot disease (105)
A : symptoms of orchid dry rot causing by *S. rolfsii* Sacc.
B : Colony of *S. rolfsii* Sacc. on PDA for 3-5 days
C : Sclerotia of *S. rolfsii* Sacc. on PDA

2.7.2.2 Stem rot disease

Sclerotium rolfsii causes of *Dendrobium* sp. Signs and symptoms as follow (104, 106):

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2.7.2.2.1 Hosts

S. rolfsii has an extensive host range; at least 500 species in 100 families are susceptible. The most common hosts are the legumes, crucifers, and cucurbits. Other reported hosts (worldwide) include: alfalfa, amaryllis, artichoke, banana, bean, beet, Brussels sprouts, cabbage, canteloupe, carrot, cauliflower, celery, chrysanthemum, coffee, cotton, cucumber, delphinium, endive, escarole, garlic, ginger, gourd, iris, lettuce, mango, muskmelon, mustard, narcissus, onion, parsley, southern pea, peanuts, pineapple, potato, pumpkin, radish, rhubarb, soybean, squash, tobacco, tulip, turf (i.e., golf greens, bermudagrass and crabgrass), turnip, and yam. The fungus persists in many weed hosts as well.

2.7.2.2.2 Epidemiology

S. rolfsii can overwinter as mycelium in infected tissues or plant debris. It usually persists as *sclerotia. Sclerotia* are disseminated by cultural practices (infested soil and contaminated tools), infested transplant seedlings, water (especially through irrigation), wind, and possibly on seeds. In addition, a small percentage of sclerotia may survive passage through sheep and cattle, and thus, could be spread through fertilizers.

2.7.2.2.3 Symptoms

S. rolfsii primarily attacks host stems, although it may infect any part of a plant under favorable environmental conditions including roots, fruits, petioles, leaves, and flowers. The first signs of infection, though usually undetectable, are dark-brown lesions on the stem at or just beneath the soil level; the first visible symptoms are progressive yellowing and wilting of the leaves. Following this, the fungus produces abundant white, fluffy mycelium on infected tissues and the soil. *Sclerotia* of relative uniform size are produced on the mycelium: roundish and white when immature then becoming dark brown to black. Mature sclerotia resemble mustard seed. The fungus occasionally produces basidiospores (the sexual stage of

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reproduction) at the margins of lesions and under humid conditions, though this form is not common. Seedlings are very susceptible and die quickly once they become infected. Older plants that have formed woody tissue are gradually girdled by lesions and eventually die. Invaded tissues are pale brown and soft, but not watery.

2.7.2.2.4 Management

2.7.2.2.4.1 Non-Chemical control

Control of *Sclerotium* diseases is difficult and depends on a combination of cultural, biological and/or chemical methods. Good cultural practices include roguing, eliminating weed hosts, and avoiding crop injury during cultivation. A dense canopy increases disease incidence, thus increasing plant spacings can help keep infection down. A delayed planting date may also help reduce disease incidence if planting is timed so that the dense canopy forms after temperatures fall so that infection is not as likely. Also, keeping plant bases free of dead leaves (and weeds) will deny the pathogen a food source, helping to keep disease incidence down.

Crop Rotation: Because *S. rolfsii* has such a broad host range, crop rotation has less of a chance of being successful as there are few resistant crops. There are some grasses and grains that are not as susceptible to the fungus that help in reducing soil inoculum levels. Onion is susceptible to *S. rolfsii,* however, some cultivars have been shown to reduce the viability of *sclerotia* when cultivars are planted in winter when temperatures are too low for disease development. A significant increase in yield and reduction in disease incidence was reported for summer peanut crops when appropriate onion cultivars were planted the previous winter. It has been postulated that onion exudates cause the pathogen to become susceptible to antagonistic microflora in the soil.

Plowing: Deep plowing (at least 20 cm) with a moldboard extention inverts soil so that organic matter, sclerotia, and plant debris are buried at least 10 cm beneath the surface. This helps to eliminate inoculum

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when plowing occurs just prior to planting. Buried soil must not be re-surfaced during the growing season.

Amendments: Compost, oat, or straw added to the soil has been shown to limit disease incidence. The addition of an amendment may increase populations of antagonistic soil microorganisms (see biocontrol section). This method may be reasonable for small-scale farms and greenhouses, but is probably not practical for large farms unless it is combined with crop rotation.

Soil Solarization: Soil solarization or solar heating is a relatively recent method for controlling *S. rolfsii* inoculum. *Sclerotia* grown in vitro are still viable after 12 hours at 45^{o}C, but are killed in 4-6 hours at 50 ^{o}C and in 3 hours at 55^{o}C. Covering soil with transparent polyethylene sheets during the hot season increases soil temperatures and kills *sclerotia* when the temperature under the sheets get hot enough for an appropriate length of time. Most field trials have achieved *sclerotia* degradation at 1 cm, but eradication at greater depths usually did not occur. In addition, this method requires immediate planting, which excludes crops that are planted in spring because temperatures are not high enough to affect *sclerotia*. Soil solarization combined with the addition of *Trichoderma harzianum* (a mycoparasite, see biocontrol section) has been shown to decrease disease incidence more than either treatment alone. However, the practicality of soil solarization is questionable. First, the length of time of solarization may be limited; a trial in Arizona reported that the tarps disentigrated after 6 weeks. Second, it is not known what affect solarization has on the existing soil microflora and what affect any microflora change would have on the crop. Third, it is not known what affect solarization has on non-target and/or temperature-tolerant pathogens.

Black Plastic Mulch: Mulching with black plastic has been shown to reduce disease incidence and perhaps provide greater crop yields. Black plastic mulch (BPM) prevents or reduces the "bridge" of dead tissue between the soil and plant and may increase temperatures, conserve soil moisture, and help control weeds for a higher crop yield. BPM alone and BPM with floating row covers both provide better control than no treatment. Disease incidence can still be

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high, but significantly lower than no treatment. BPM alone and BPM with floating row covers combined with a chemical treatment (PCNB) provide even better control.

Biological Control: A number of antangonistic fungi have been shown to provide control against *S. rolfsii* in controlled experiments, though field trial results vary. Some of the commonly used organisms are: *Trichoderma harzianum, T. viride, Bacillus subtilis, Penicillium* spp., and *Gliocladium virens. Trichoderma* spp. are known mycoparasites of a number of plant pathogens. *T. harzianum* colonizes *S. rolfsii* hyphae, disrupts mycelial growth, and kills the organism. Field studies where effective control was obtained, involved application rates in the range of 140 to 600 to 1500 kg/ha. However, the populations of the pathogen and the antagonist were not monitored over time. *T. viride* has been shown to provide good control, especially when used in combination with an herbicide or pesticide. When combined with EPTC (an herbicide) in autoclaved soil, *S. rolfsii* activity was diminished, eventhough EPTC alone stimulated growth of the pathogen. In natural soil, the effectiveness of *T. viride* was reduced in the presence of EPTC, indicating the involvement of other soil microorganisms. *T. viride* in combination with PCNB has been shown to provide good disease control and better yield in artificially inoculated field plots (tomato) than in non-inoculated, untreated field plots. *T. viride* without PCNB provided statistically similar disease control but a lower yield. PCNB alone was less effective than PCNB with *T. viride* or *T. viride* alone. *Gliocladium virens* have been shown to rapidly degrade *S. rolfsii* strain SR-1 in soil. *G. virens* will colonize *S. rolfsii* strain SR-3 but *sclerotia* can germinate under good conditions. The different reactions between *S. rolfsii* strains may be due to the size of the *sclerotia* (SR-3 sclerotia are up to 15-20 times larger than SR-1 sclerotia) and the amount of melanin in the sclerotial rind (SR-3 sclerotia has more melanin). In vitro studies show that varying concentrations of *G. virens* provide a corresponding variation in the germination of *S. rolfsii* (SR-1) sclerotia, but all concentrations result in a low percentage of *S. rolfsii* infection. In most cases there were always sclerotia that germinated but the pathogen did not infect plant tissues. *G. virens* had little effect on the germinability and infectivity of *S. rolfsii* (SR-3). Data such as these suggest considerable specificity in biocontrol due to differences in susceptibility of strains of The same pathogen to a single biocontrol strain, in addition to specific due to various

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strains of a biocontrol agent.

2.7.2.2.4.2 Chemical control

Control measures include chemical disinfection of vegetative propagation material, adjustment of soil pH by liming, adjustment of fertilizer regime, and use of herbicides for weed control. Formalin, chlorobromopropene and methyl bromide are among the most promising fumigants for treatment of seed beds or fields for valuable crops. Pre-plant chemicals and application techniques: fumigants such as metamsodium (Vapam), Vorlex, methyl bromide, and chloropicrin, when applied to soil, reduce southern blight incidence.

2.8 Plant and Pathogenic Fungi Interaction

2.8.1 Fungal disease development

The development of a fungal disease in a host plant may be arranged into distinct steps in sequence as follows (107): (a) Infection, denoting the processes from the entry of the fungus and its initial invasion till it has been able to establish itself inside the host tissues, (b) Incubation, the time period between the establishment of infection and the expression of symptoms, and (c) Disease development, the process occuring after the establishment of infection till the disease is finally and completely expressed.

The initial step in the infection process of a host plant by a fungal pathogen is its entry into the host plant, which is known as penetration. Fungi enter inside the host plant as hyphal form and the fungal spores must germinate to initiate the infection process (108). It is dependent on the presence of spore germination capable and suitable environmental conditions for the spore germination. Regarding the environmental factors, moisture is the most important and needed for a sufficiently long period. Most spores require free water for germination till the entry of the fungus inside the host tissue. Besides, relative humidity in the surrounding of the spore should be very high and as near saturation as possible during this period. Temperature is

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another determining factor because germination can only take place within the limit range of temperature. It may be from 0-34 °C, however for each fungus, the value of minimum, optimum and maximum are different (107).

The germ tube or hyphae of the fungus may penetrate the host tissues through uninjured intact plant surfaces, *i.e.*, cuticle and epidermis, or through special unthickened organs of the host, *e.g.*, stigma and root hair. They may enter through natural openings like stomata, lenticels and hydathodes, whereas certain fungi which are weak parasites can enter only through wounds (107). After the fungus has entered the host tissues, it must be capable of growing inside the host tissues and deriving nutrition from the host cells to establish it and cause infection. Besides, some fungi can kill the cells and disorganize the host tissues in advance of penetration with the help of enzymes and/or toxins secreted by their hyphae. These lead to biochemical changes within the plant that result in more or less conspicuous disease sypmtoms like yellowing of leaves, wilting, necrosis or distortion of plant form which reduce the vitality of the plant and, with time, may finally end in the death of plant parts or even the whole plant (109).

2.8.2 Inducible defense responses in plant

In spite of the fact that a fungus gains its entry inside the host tissues and has the necessary enzymes or toxins to kill the tissues, the host plant may offer the resistances to the progress of pathogen, thereby eliminating or delimiting the attack (108).

In order to protect themselves from pathogens, plants have evolved a number of defense responses that are elicited during their life cycle in response to developmental signals and to pathogen attack. One of the inducible defense responses in plant is the expression of pathogenesis-related (PR) proteins (110). PR proteins, a class of polypeptide, are found in the intercellular fluids of plants infected with phytopathogens, or after treatment of plants with biotic or abiotic elicitors. Their apparent function is to restrict the growth of the invading microorganism. These PR proteins include the enzymes involved in the synthesis of low molecular weight antimicrobial substances such as phytoalexins, and certain hydrolytic enzymes such as

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chitinase and glucanase (111). Among PR proteins that have been studied, the lytic enzyme chitinase and β-(1,3)-glucanase have long been speculated to play a crucial role in plant defense against fungal pathogens. This is based on the following indirect evidences: Firstly, high activities of chitinase and β-(1,3)-glucanase are frequently found in higher plants. Although chitinase has no known substrate in plants itself, while the substrate for β-(1,3)-glucanase, callose, usually presents only in small amount (112). However, chitin and β-(1,3)-glucan which are important structural elements of the cell walls of many fungi, may represent the natural substrates for the two plant hydrolases. Secondly, purified plant chitinase and β-(1,3)-glucanase can partially degrade isolated fungal cell walls (113). Thirdly, chitinase and β-(1,3)-glucanase can be coordinately induced by ethylene and by pathogenic infection or derived pathogenic elicitors in various plants (114).

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CHAPTER III
METHODOLOGY

3.1 Materials and Equipment

3.1.1 Phytopathogenic fungal strains

- *Collectotrichum gloeosporioides* DOAC 1690
- *Sclerlotium rolfsii* DOAC 1521

Fungal strains were obtained from Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand

3.1.2 Culture media and chemicals

- Colloidal Chitin agar (CCA)
- Potato Dextrose Agar (PDA)
- Potato Dextrose Broth (PDB)
- Tryptic Soy Agar (TSA)
- Tryptic Soy Broth (TSB)
- R2A medium
- Chitin powder
- Hydrochloric acid
- Sodium acetate
- Sodium carbonate
- Sodium hydroxide
- Sodium hypochlorite
- Tween-20

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- Phenol crystal
- Glycerol
- Glycol chitin
- Cotton blue dye
- Bradford's reagent
- N-acetyl-D-glucosamine (GlcNAc)
- Bovine Serum Albumin (BSA)
- Proteinase K
- Ethanol
- Potassium ferricyanide

All chemicals were purchase from Sigma (USA), Merck (Germany), BDH chemicals (Australia), Ajak (Australia), Fluka (Switzerland) and Lab-Scan (Ireland).

3.1.3 Materials and equipments

- Adjustable volume pipette (20, 200 and 1,000 µl) (Rainin, USA)
- Needle
- Loop
- Cork borer
- Buchner funnel
- Suction flask
- Filter holder
- Syringe
- 0.45 µm membrane filter paper
- Desiccator
- Magnetic stirrer
- Vacuum pump (Gast model DAA-V174-ED)
- Refrigrated centrifuge (HERMLE model 2323K)
- Autoclave (Hirayama model HA-300MII)
- Hot-air oven (Contherm model CAT 260M)
- Incubator (Termaks model KB8400 and Termaks model 6000)

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- Incubator shaker (Thermo electron model 481)
- Microbiological safety cabinet (Flufrance model Atlas 2000)
- Analytical balance (Gec Avery model WA250)
- Vortex mixer (Vortex-Genie2 model G-560E)
- UV/VIS spectrophotometer (Thermo model 4001/4)
- pH meter (Orion model EA940)
- Light microscope (Olympus model CH-2)
- Hot plate (Labinco model L-82)
- Glassware

3.2 Isolation of Bacteria from Fermented Liquid Bioextract (FBE) and Shrimp Shells

3.2.1 Sample collection

Some fermented liquid bioextract (FBE) samples were purchased from companies and others were obtained from local agriculturist. Each sample contained the different main ingredients used as raw materials for fermentation. All samples have no chemical addition that might effect on microbial growth. Samples of shrimp shell were collected in from shrimp farms at Chonburi and Chachoengsao provinces. Shrimp shell samples were aseptically collected sterile plastic tubes and kept in 4^{o}C until uses for isolation.

3.2.2 Isolation and screening of chitinolytic and non-chitinolytic bacteria

All samples of FBE and shrimp shell were diluted in TSB (Appendex A No. 1). Appropriated dilutions of each samples were spreaded on R2A (Appendex A No. 2) and TSA (Appendex A No. 3) plates incubated at 28^{o}C for 2-3 days. Appeared colonies on agar plates were selected according to their morphological characteristics and patch on Colloidal chitin agar (CCA) (Appendex A No. 4) to observe chitinolytic activity (70). After 5 days of incubation period, that the diameters of clear zone and colony were measured and calculated. The isolates that exhibited the ratio of diameters

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of clear zone and colony more than 5 mm were selected (71) as chitinolytic bacteria. Furthermore, all appeared colonies were pick up for antagonistic activity testing.

3.2.3 Pure culture preparation

Isolated bacterial from No. 3.2.2 were streaked on TSA plates. Single colony of each isolate was picked up and restreaked on TSA plate to ensure the pure culture. Each isolate of pure culture was subcultured in TSA slant and kept at 4^{o}C.

3.3 Screening for Antifungal Activity of Isolated Bacteria Against Phytopathogenic Fungi

The potential of isolated bacteria on the growth suppression of 2 strains of phytopathogenic fungi, including *C. gloeosporioides* and *S. rolfsii* was evaluated.

3.3.1 Preparation of bacterial inoculum

Isolated bacteria were maintained on TSA plates. Bacterial cells were cultivated into TSB at 28^{o}C, 120 rpm for overnight (15-18 hr) used as cell inoculum.

3.3.2 Fungal inoculum preparation

Each phytopathogenic fungi namely, *C. gloeosporioides* and *S. rolfsii* was cultured on PDA (Appendix A No. 5) plates at 28°C. Mycelium tip of 5 to 7-day-old culture was used for this experiment.

3.3.3 Determination of antifungal activity of chitinolytic bacteria by dual culture technique

All isolates were screened for their *in vitro* antagonism against two strains of fungal pathogens namely, *C. gloeosporioides* and *S. rolfsii* according to the

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modified method of Nautiya (1997) (138) and De Boer et al. (1998) (123) Bacterial inoculum in No. 3.3.1 was measured all turbidity by spectrophotometer and adjust cell density to give OD_{600} ~ 0.1. The 10-µl of bacterial inoculum was point inoculated onto one side of PDA plate. The mycelial tip of 7-day-old of phytopathogenic fungus that maintained on PDA plate was cut in the diameter of 0.6 mm. The mycelial plug of fungus was aseptically placed onto another side of PDA plate at positio 4-cm away from bacteria and incubated at 28°C for 3-5 days. Fungal plug was placed on uninoculated PDA plates separately as control treatment. Each experiment was done in five replications. After incubation, the radial of fungus was determined compared to control. Inhibition was indicated when *C. gloeosporioides* and *S. rolfsii* mycelial growth in the direction of the bacterial colonies was retarded or inhibited. Growth inhibition of *C. gloeosporioides* and *S. rolfsii* defined as hyphal growth less abundant and growth retarded on the area of the plate to the side where the bacteria had been grown.

The level of inhibition was recorded and calculated as shown in Equation 3-1 that mentioned by Yuan and Crawford (1995) (115)

$$\Delta\gamma = \gamma_0 - \gamma \qquad \text{.....3-1}$$

Where; $\Delta\gamma$: the level of inhibition (mm)

γ_0 : the fungal growth radius of a control culture (mm)

γ : the distance of the growth in the direction of antagonists (mm)

The rating used was modified from those of Yuan and Crawford (1995) (115).

$\Delta\gamma <$ 5mm = 0

$\Delta\gamma \geq$ 5 - 9 mm = +

$\Delta\gamma \geq$ 10 - 19 mm = ++

$\Delta\gamma \geq$ 20 mm = +++

The high potential antagonistic bacteria that exhibited a strong inhibition of fungal growth were selected for further study.

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3.4 Growth Curve Determination and Cell Preservation of Selected Antagonistic Bacteria

Isolates of selected bacteria were maintained on TSA plates. Each isolate was cultured into TSB and overnight incubated at 28°C, 120 rpm. Overnight cell culture was transferred into 20-ml of fresh TSB in 125 ml-Erlenmeyer flask to give the OD_{600} ~ 0.1 and then incubated at 28°C, 120 rpm. The bacterial culture was collected at 0, 4, 8, 12, 24, 36, 48 and 72 hr, respectively. Samples were measured cell turbidity by spectrophotometer at wavelength 600 nm. Growth curve was expressed by plotting graph between time and cell turbidity at OD_{600}. For long-term storage of bacteria isolates, fresh colonies were inoculated into TSB with agitation until exponential growth phase. Subsequence, 800 µl of the cultures were mixed with 400 µl of 45% sterile glycerol to give a final concentration of 15% glycerol and kept at -70 °C deep freezer.

3.5 Morphological Study and Identification of Antagonistic Bacteria

The potent bacterial antagonists were identified. Selected bacterial isolates were strained by Gram's stain (77). Cell morphology was observed by scanning electron microscope (SEM). Identification of bacterial genus and species was determined by 16S rDNA sequencing. The 755-bp fragment of 16S rDNA was amplified in a thermocycler (Perkin Elmer Cetus Model 480) by using universal primers of 27F (5' –AGA GTT TGA TCC TGG CTC AG -3') and 782R (5' ACC AGG GTA TCT AAT CCT GT -3'). The amplify DNA fragment was sequenced by ABIPRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit with sequencing enzyme AmpliTag DNA polymerase from Applied Biosystem (ABI, USA) (143). This experiment was conducted at Chulabhorn Research Institute (CRI). The 16S rDNA sequence of selected bacterial isolate was analyzed by using BLASTn program (116) from Genbank database (http: www.ncbi.nlm.nih.gov).

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3.6 Quantitative Determination of Extracellular Chitinase Enzyme Produced by Antagonistic Bacteria

3.6.1 Preparation of bacterial cell-free supernatant

Overnight cell inoculum of each selected bacterium from No. 3.3.3 was performed as previously described in No. 3.3.1. Overnight cell inoculum was cultured into 20 ml of TSB in 125 ml-Erlenmeyer flask with continuous shaking at 28^{o}C for 4, 8, 12, 24, 36, 48 and 72 hr, respectively. Each sampled was centrifuged at 10,000 rpm for 15 min at 4^{o}C. Cell-free supernatant was collected to analyze the chitinase activity.

3.6.2. Chitinase activity assay

The chitinase activity was determined by measuring reducing end group (N-acetyl-D-glucosamine, GlcNAc) degrade from glycol chitin (Appendix B No 2). as substrate by the modified method described by Schales procedure (117). Reaction mixtures containing 0.1 ml of enzymes solution, 0.1 ml of 0.1% glycol chitin and 0.2 ml of 10 mM potassium phosphate buffer (pH 7.0) were incubated at 37^{o}C for 20 min. The glycol chitin was removed from the mixture by centrifugation at 13,000 rpm for 10 min. The supernatant was transferred to new tube, then 2.0 ml of potassium ferricyanide solution (Appendex B No. 4) and 0.7 ml of distilled water were added. The mixture was boiling water for 15 min. The release of reducing sugar was measured by spectrophotometer at the wavelength of 420 nm (A_{420}). A standard curve was established with solutions of GlcNAc ranging from 0.005 to 0.1 mg/ml versus their corresponding A_{420}.

One unit (U) of chitinase activity is defining as the amount of enzyme liberating 1 μM of GlcNAc equivalent from the substrate per ml per min under the assay conditions. Specific activity of chitinase is expressed as units per mg of total protein.

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3.6.3 Total protein determination

The total protein of bacterial supernatant was determined by Coomassie Blue Protein Assay according to the sensitive method of Bradford (144). The 0.1 ml of bacterial supernatant was added with 3 ml of Bradford's reagent. After incubation at room temperature for 5 min, total protein was measured by spectrophotometer at the wavelength of 595 nm (A_{595}) in glass or polystyrene cuvettes. A standard curve of 0.02 to 0.1 mg/ml of Bovine Serum Albumin (BSA) is used for calibration. Total protein in sample could be calculated as show in equation 3-2.

$$\text{Total protein (mg/ml)} = \frac{A_{595} \text{ of sample} \times \text{Standard concentration (mg/ml)}}{A_{595} \text{ of standard}} \quad \text{.....3-2}$$

3.7 Efficiency of Extracellular Antifungal Metabolites Produced by Selected Bacterial Antagonists on the Growth Suppression of Phytopathogenic Fungi

3.7.1 Preparation of cell-free culture filtrate as extracellular antifungal metabolites

Each bacteria antagonist was cultured in 20-ml of TSB at the same condition as described in No. 3.6.1 Fermentation broth was collected at exponential (4-hr) and stationary (24-hr) phase of growth. Cell were removed by centrifugation at 10,000 rpm for 20 min at 4^{o}C. Then cell-free supernatant was aseptically filtered through sterile 0.45 µm-pore size membrane and stored at 4^{o}C until use for antifungal assay.

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3.7.2 Effect of extracellular antifungal metabolites on the radial growth suppression of phytopathogenic fungi

To evaluate the effect of cell-free culture filtrates on the radial growth of each phytopathogenic fungi the experiment was conducted according to the method of Prapagdee et al. (2007) (118) as follows.

Briefly, exponential and stationary culture filtrates prepared according to method in No.3.7.1 were separately added into warm-molten PDA (45^{o}C) to give a final concentration at 10. 20 and 30% (v/v) and placed until solidify. The control plate was added an equal volume of sterile distilled water instead of culture filtrate. Each plate was seeded with 6-mm-diameter mycelial plugs taken from the margin of a 5-day-old culture of *C. gloeosporioides* or *S. rolfsii* Inoculated plates were incubated at 28°C and fungal growth was recorded at 1-day intervals until those of the control plates reaching the edge of the plate.

Fungal growth inhibition was expressed as the percentage of inhibition of radial growth relative to the control as shown in equation 3-3.

$$\text{Inhibition of radial growth (\%)} = \frac{\text{Colony Ø of control} - \text{Colony Ø of sample}}{\text{Colony Ø of control}} \times 100 \quad \text{.....3-3}$$

3.7.3 Effect of extracellular antifungal metabolites on the submerged growth suppression of phytopathogenic fungi

Inhibitory effect of extracellular antifungal metabolites in cell-free culture filtrates on submerged growth of *C. gloeosporioides* and *S. rolfsii* were evaluated in Potato Dextrose Broth (PDB) (Appendix A No.3). The 10% of cell-free cultured filtrates collected exponential (4-hr) and stationary (24-hr) phase in No. 3.7.1 were aseptically added into a fresh 20-ml PDB medium in 125 ml-Erlenmeyer flask. The 4 fungal discs of each tested funngus were inoculated into PDB medium. Control experiment was also performed by adding an equal volume of sterile distilled water in place of the cell-free culture filtrates. All flasks were incubated on incubator shaker at 120 rpm, 28^{o}C for 12, 24 hr, 2, 3, 5, 7 and 9 day, respectively. The fungal growth

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monitored by dry weight determination. Fungal cultures were filtrated by using the membrane filter apparatus. After filtration, the membrane filter paper with cell mass was dried in oven at 80°C for 3 hr and then held in desiccators until cool down, in order to calculate the constant dry weight. Before filtration, the membrane filter paper is dry and calculates for the constant weight the same. From the constant weight of membrane filter paper and the constant dry weight of membrane filter paper with cell mass, cell dry weight could be calculated.

3.7.4 Effect of extracellular antifungal metabolites on the fungal mycelial morphology

Cell-free culture filtrates prepared according to method in No. 3.7.1 were added into 20-ml of warm-molten PDA to give a final concentration of 10% (v/v) and placed until solidify. Prepared PDA was cut in the size 1 cm^2 and 5-mm-thick and placed on the steriled slide that put on Petri-dish. Mycelial tip of each phytopathogenic fungus was inoculated on the surface of piece of PDA and covered with cover slip. Inoculated slides were incubated at 28^oC in dark condition for 3 days. After incubation, fungal hypha on slides were stained with lactophenol cotton blue dye (Appendix B No. 2). Fungal mycelial morphology was observing under 40X light microscope.

3.7.5 Effects on heat and proteinase K on antifungal activity of extracellular antifungal metabolites

To investigate some chemical properties of antifungal metabolites, cell-free culture filtrates collected from exponential (4-hr) and stationary (24-hr) phase were either treated with boiling (100^oC) for 45 min or 1.0 mg/ml of proteinase K (Sigma, USA) to give a final concentration at 10 µg/ml for 1 hr. Inhibitory effect of treated culture filtrates on the radial growth of *C. gloeosporioides* and *S. rolfsii* were using method determined as describe in No. 3.7.2. The percentage of radial growth inhibition by treated and untreated culture filtrates were calculated

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3.8 Statistical Analysis

The means and standard deviation of inhibition levels, radial growth, submerged growth and chitinase activity were calculated. All experiments were independently repeated at least three times. Data were analyzed by one-way-analysis of varience (ANOVA). Significant differences ($p \leq 0.05$) between means were determined by t-test Dependent.

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CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation and Screening of Chitinolytic Bacteria

Seven samples of FBE and 10 samples of shrimp shell were used to isolation of bacteria. Forty six strains of bacteria were isolated from FBE and 97 strains were isolated from shrimps shell waste, totally 143 strains. Several investigations reported that chitinase are considered hydrolytic enzyme that degrade cell walls of pathogenic fungi (54, 119, 122, 133). Thus, all isolated bacteria were tested for chitinolytic activity on CCA plates. Clearing zone of colloidal chitin formed by chitinase produced by chitinolytic bacteria was shown in Appendix D (Figure No. D-1). Among 143 isolates, only 12 strains or 8.4% of total isolated bacteria showed chitinolytic activity on CCA plates (Figure 4-1) the chitinolytic activity of each bacteria strain isolated from FBE and shrimp shells was shown in Table D-2 and D-3 (Appendix D)

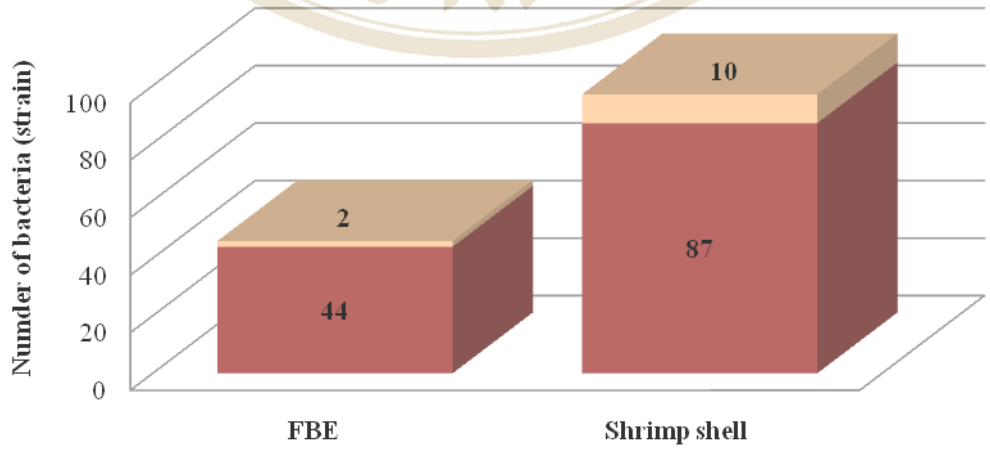

Figure 4-1 Number of chitinolytic (□) and non-chitinolytic (■) bacteria isolated from FBE and shrimp shells.

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Wang et al. (2002) (119) founded that *Bacillus amyloliquefaciens* V656 produced antifungal enzymes and the antifungal enzymes displayed chitinase activities. *Bacillus thuringiensis* chitinase may contribute to the biocontrol of *S. rolfsii* and other phytopathogenic fungi Antifungal chitinase activity on phytopathogenic fungi was investigated in growing cultures and on soybean seeds infected with *Sclerotium rolfsii*. Fungal inhibition was found to be 100% for *S. rolfsii*; 55% to 82% for *Aspergillus terreus*, *Aspergillus flavus*, *Nigrospora* sp, *Rhizopus* sp, *Aspergillus niger*, *Fusarium* sp, *Aspergillus candidus*, *Absidia* sp, and *Helminthosporium* sp; 45% for *Curvularia* sp; and 10% for *A. fumigates* (120). *Bacillus cereus* 28-9 is a chitinolytic bacterium. This bacterium exhibited biocontrol potential on Botrytis leaf blight of lily as demonstrated by a detached leaf assay and dual culture assay and an *in vitro* assay showed that the purified chitinase (ChiCW) had inhibitory activity on conidial germination of *Botrytis elliptica*, a major fungal pathogen of lily leaf blight (54, 121).

4.2 Antifungal Activity of Bacteria Isolated from FBE and Shrimp Shell Waste

A collection of 143 bacteria isolated from FBE and shrimp shell waste were screened for potential antagonists toward *C. gloeosporioides* and *S. rolfsii* by dual culture technique. The levels of growth inhibition were determination by the difference between the distress of the radial growth of a control culture (γ_0) and the radial growth in the direction of bacteria of paired-culture (γ) as indicated in the equation 3-1 (No. 3.3.3) (115).

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Table 4-1 Antifungal activity of isolated bacteria against *C. gloeosporioides* and *S. rolfsii*

Activity fungal activity[a]	Numbers of strains (%) of bacteria against tested fungi		
	C. gloeosporioides	*S. rolfsii*	*C. gloeosporioides* and *S. rolfsii*
-	133 (93.01)	140 (97.90%)	140 (97.90%)
+	2 (1.42%)	0	0
++	2 (1.42%)	0	0
+++	6 (4.20%)	3 (2.10%)	3 (2.10%)

[a] - = $\Delta\gamma < 5$ mm (No antifungal activity)

\+ = $\Delta\gamma \geq 5$ - 9 mm

\+ + = $\Delta\gamma \geq 10$ - 19 mm

\+ + + = $\Delta\gamma \geq 20$ mm

The results of antifungal activity from Table 4-1 strains exhibited a weak or non-antagonistic activity 133 and 140 towards *C. gloeosporioides* and *S. rolfsii* respectively. Only, 6 and 3 bacteria isolates had high levels of antagonistic activity (+++) to *C. gloeosporioides* and *S. rolfsii*, respectively. The percentages of isolated bacteria with antifungal activity to either *C. gloeosporioides* or *S. rolfsii* were 4.2% and 2.1% respectively. Among 143 bacterial isolates, the high potential bacterial antagonists towards both tested fungal phytophatogen were strain SSCHC4, EMC4 and SSE4. The colony morphology of 3 potent antagonists was shown in figure 4-3. Strain SSCHC4 showed chitinolytic activity that isolated from shrimp shell waste while non-chitinolytic bacteria were SSE4 and EMC4 isolated from shrimp shells waste and FBE, respectively.

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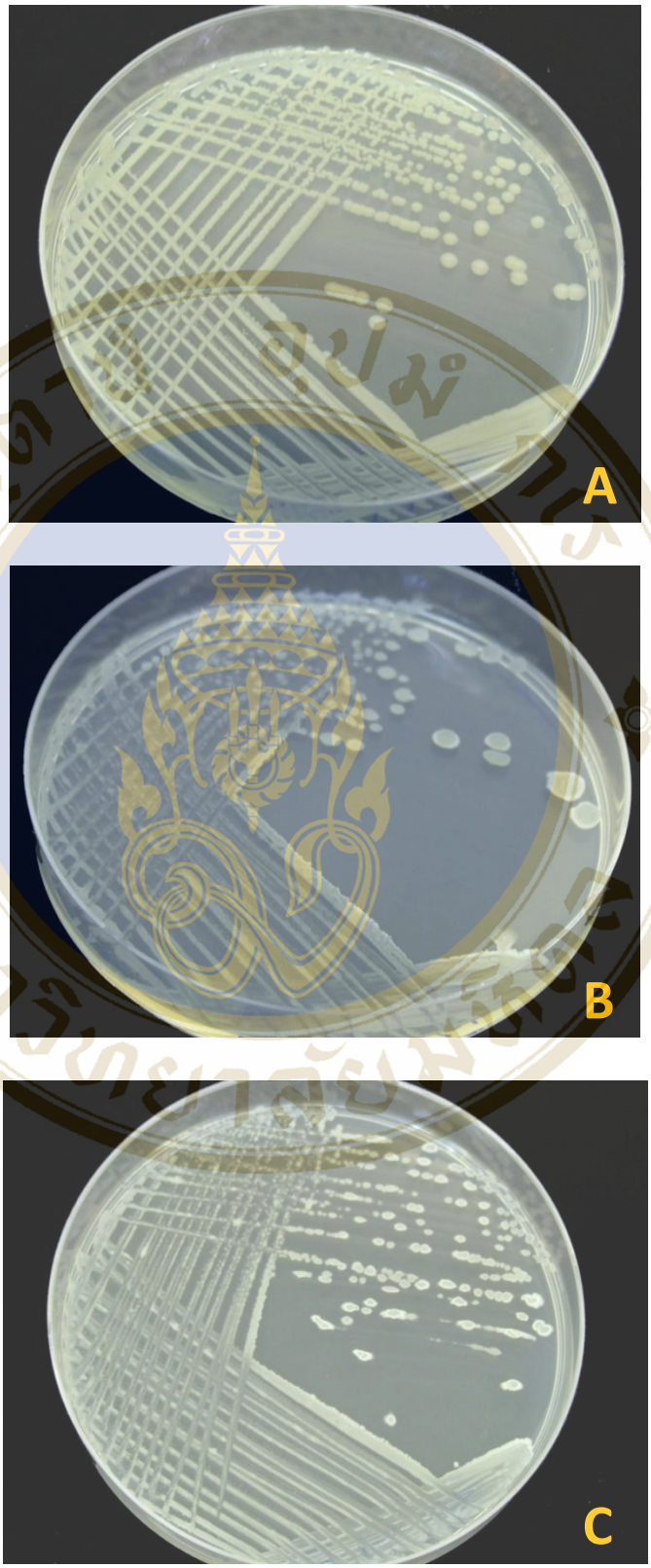

Figure 4-2 Colony morphology of (A) SSCHC4 (B) EMC4 (C) SSE4 cultured on TSA plates after incubation at 28^{o}C for 24 hr

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These results indicated that the strain SSCHC4, SSE4 and EMC4 grown on PDA plates excreted an extracellular metabolite(s) that inhibited hyphal growth of both *C. gloeosporioides* and *S. rolfsii*. Antagonistic potential of SSCHC4 may involve the production of extracellular chitinase enzymes. *Bacillus* spp. strains antagonistic to phytopathogenic fungi, 19 were found to possess chitinolytic activity although the enzymatic preparations of most of these strains inhibited the growth of the phytopathogenic fungus, *Helminthosporium sativum*. (122). De Bore et al. (1998) (123) reported that *Pseudomonas* spp. were the most abundant culturable, chitin-degrading bacteria at the lime-poor sites, whereas *Xanthomonas* spp. and *Cytophaga* spp. were important at the lime-rich site. Chitinolytic actinomycetes were relatively abundant at all three sites.

Besides the production of chitinase enzyme, secondary antifungal compounds as antibiotics have been involved in antifungal activity of many antagonistic bacteria such as *Bacillus* spp. *Pseudomonas* spp. The pervious study reported that antagonistic of chitinolytic bacteria involved the production of antibiosis (123). Woo et al. (2002) (124) reported that *Pseudomonas* spp. produce membrane-disrupting lipodepsipeptides (LDPs), syringotoxins (SP) and syringomycins (SR). SR are considered responsible for the antimicrobial activity, and SP for the phytotoxicity. Cell wall degrading enzymes (CWDEs) of *Trichoderma* spp. synergistically increased the toxicity of P25−A or Syringomycin E (SRE) purified from *Pseudomonas. syringae* against fungal pathogens. The positive interaction between LDPs and CWDEs may be part of the biocontrol mechanism in some *Pseudomonas* strains (124). Moreover, Besson (1978) found that *Bacillus subtilis* were produced the antifungal antibiotics of the iturin group. The characterization of these antibiotics was made on the basis of antifungal activity against *Penicilluim chrysogenum* (125). The *Bacillus subtilis* strain KS03 was inhibited the anthracnose disease fungus, *Gloeosporium gloeosporioides* (126). Thus, the strain SSE4 and EMC4 may produce secondary antifungal compound(s).

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4.3 Growth Curve of Selected Antagonistic Bacteria

The growth assays of selected antagonistic bacteria, strain SSCHC4, SSE4 and EMC4, were determined by measuring the cell density with spectrophotometer at wavelength 600 nm (OD_{600}). Overnight cell culture of each selected bacteria was transferred into 20-ml of fresh TSB in 125 ml-Erlenmeyer flask to give the OD_{600} ~ 0.1 and then incubated at 28°C, 120 rpm. The bacterial culture was aseptically collected at 0, 4, 8, 12, 24, 36, 48 and 72 hr, respectively. Samples were measured. Growth curve was expressed by plotting graph between time and cell turbidity. The growths of each bacteria strain results were illustrated in Figure 4-4.

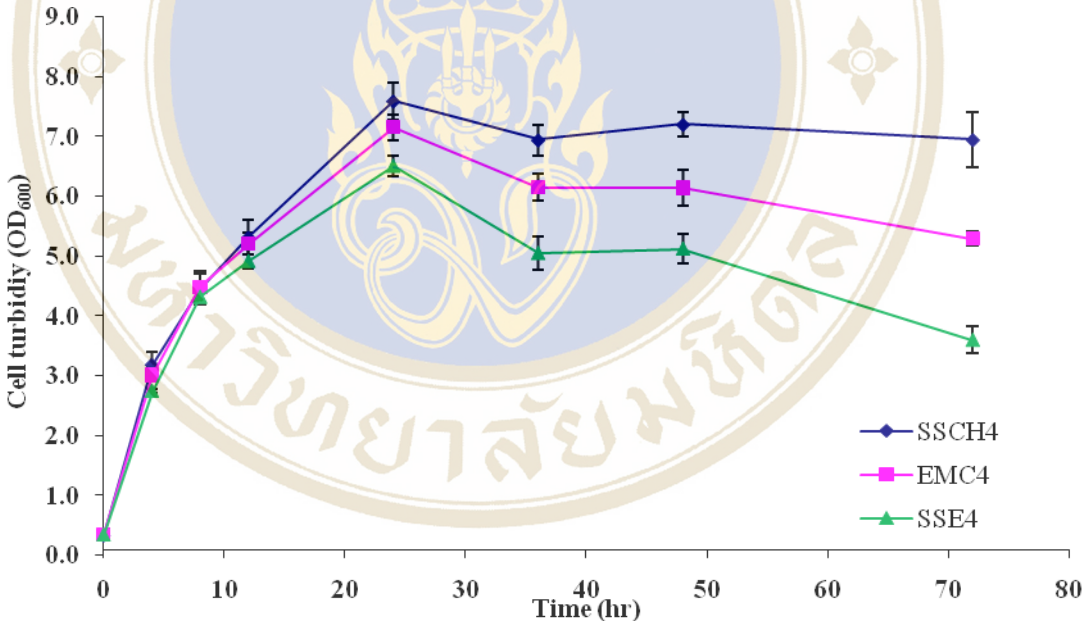

Figure 4-3 Growth curves antagonistic bacteria, strain SSCHC4, EMC4 and SSE4 cultivated in TSB at 28°C

No different in the growth curves of 3 bacterial strains was observed. The growth was sharply increase into exponential phase within 4 hr (OD_{600} ~ 2.75-3.19). After that cell proliferation was slightly increase until 12 hr of incubation period. Growth of each strain entered the mid-stationary phase at 24 hr. The cell growth was

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slightly constant until 72 hr of growth period. Data of growth curve of each bacteria strain were shown in Appendix D No.3.

4.4 Morphology and Identification of Selected Antagonistic Bacteria

The potent antagonistic bacteria, SSCHC4, EMC4 and SSE4 were determined the Gram by Gram's stain and observed morphology by scanning electron microscope (SEM) (Table 4-2). All strains were Gram positive and rod shape. In addition, endospore formation was observed in both the strain EMC4 and SSE4.

Table 4-2 Morphological characteristics and Gram of selected antagonistic bacteria

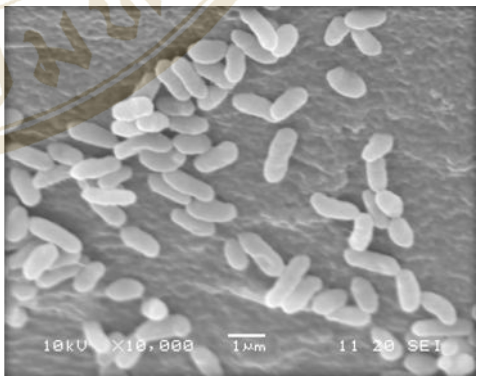
Bacteria No.	Gram' stain	Cell shape	Cell morphology order SEM (10,000X)
SSCHC4	Positive	Short rod	

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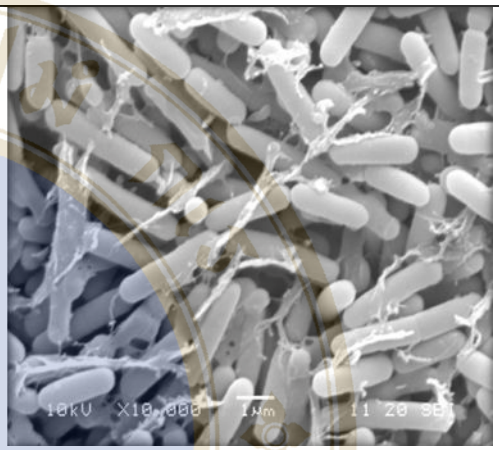
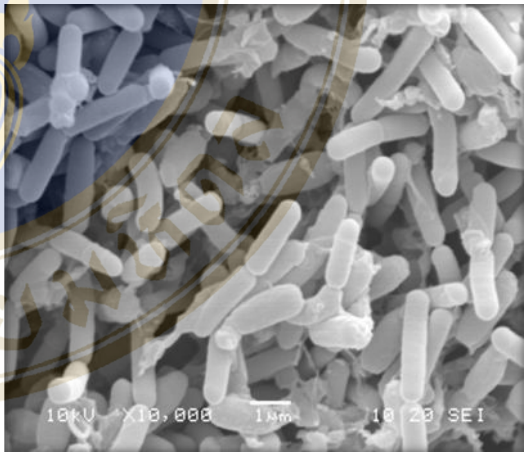
Table 4-2 Morphological characteristics and Gram of selected antagonistic bacteria (Continued)

Bacteria No.	Gram' stain	Cell shape	Cell morphology under SEM (10,000X)
EMC4	Positive	Long rod	
SSE4	Positive	Long rod	

The genus and species of 3 bacterial antagonists were identified by partial 16S rDNA sequencing. Bacterial strains were cultured in TSB medium for 24 hours at 28^{o}C. Cell were harvested and extracted for genomic DNA. PCR of 16S rDNA was conducted using primers 27F and 782R. PCR products were purified and sequenced. The results of 16S rDNA partial sequence of SSCHC4, EMC4 and SSE4 exhibited in Figure 4-5, 4-6 and 4-7, respectively. The results found that SSCHC4 (Table4-3) showed a highly similarity to *Aeromonas salmonicida* (97% similarity) from Genbank

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database accession number AM931169. (Appendix D, Figure D-2). Analysis of 16S rDNA sequence of SSE4 and EMC4 revealed that SSE4 and EMC4 showed a highly similarity to *Bacillus subtilis* EMC4 (98% similarity) from Genbank accession number EF617316 and *Bacillus subtilis* SSE4 (98% similarity) from Genbank accession number EF472261.1 (Appendix D, Figure D-3 and Figure D-4).

CTGCAGCGCACGGAAGACTTGTCTTTTGCGGCGAGCGCGGACGGGTGAGTAATGCTGGGG
ATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTAC
GGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCCAGGTGGGATTAGCT
AGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGGGAAACCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGNCTTCGGGTTGTA
AAGCACTTTCAGCGAGGAGGAAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCG
CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGT
TAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGC
CCCGGGCTCAACCTGGGAATGCATTTAAAACTGTCCAGCTAGNNTCTTGTAGAGGGGGGTA
GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGAATACCGGGGAAAGCGCCNC
CTGACAAAGAAGAG

Figure 4-4 Partial sequence of 16S rDNA of a potent antagonist SSCHC4

CNTGCGCGTGCTATCATGCAGTCGAGCGGCAGATGGGAGCTTGCTCCCTGATGTTAGCGGC
GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCG
GGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTA
CCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
GACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAC
GCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGACAAGTGCC
GTTCAAATAGGGCGGCACCTTGACGGTACCTAACAGAAAGCCACGGCTAACTACGTGCCA
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCG
CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAA
ACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGCGAAGNGACTCTCTGTCTGAACNGCGTAGAGGAA

Figure 4-5 Partial sequence of 16S rDNA of a potent antagonist EMC4

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NCTGCGCGTGCTATCATGCAAGTCGAGCGGCAGATGGGAGCTTGCTCCCTGATGTTAGCGG
CGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC
GGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCT
ACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG
CGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA
CGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTG
CCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAGCCACGGCTAACTACGTGC
CAGCACCGCGGTAATCGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTC
GCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGA
AACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG
TAGAGATGTGGAGGAACACCAGTGGCGAAGGGACTCTCTGTCTGAACTGCGCTAGAGNAA
NGGG

Figure 4-6 Partial sequence of 16S rDNA of a potent antagonist SSE4

Table 4-3 Identification of bacterial antagonists by partial 16S rDNA sequence analysis

Bacterial antagonists	Closest strains	Sequence analysis (Length of nucleotides)	Accession number
SSCHC4	- *Aeromonas salmonicida* strain 242	97% (644/661)	AM931169
	- *Aeromonas salmonicida* subsp. *flounderacida* strain HQ010320-5	97% (644/661)	AY786178
	- *Aeromonas salmonicida* subsp. *flounderacida* strain HQ010320-1	97% (644/661)	AY786177
EMC4	- *Bacillus subtilis* strain SB2	98% (713/727)	EF617316
	- *Bacillus subtilis* strain QD517	97% (715/731)	EF472261
	- *Bacillus subtilis* strain LQ20	97% (714/729)	EU346662
SSE4	- *Bacillus subtilis* strain QD517	98% (715/725)	EF472261
	- *Bacillus subtilis* strain LQ20	98% (714/724)	EF472266
	- *Bacillus subtilis* strain Pab02	98% (713/724)	EU346662

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There were several studies about the antagonistic activity of *Bacillus subtilis* toward phytopathogenic fungi. Nutrient-limited *B. subtilis* cells are able to sporulate, an elaborate process that results in the release of an endospore from the terminally differentiated, apoptotic mother cell (127). Strikingly et al. (2001) (128) documented that sporulation is tightly intertwined with the development of highly ordered and surface-associated cell clots, 'fruiting-bodies', that are characterized by spore-specific gene expression. The formation of similar aerial hyphae in multicellular organism like fungi need the generation of surface-active molecules (129, 130). Unfortunately *A. salmonicida* cause disease in fish, particularly order Salmoniformes (131, 132). Thus, antifungal activity of *A. salmonicida* SSCHC4 did not performed in further study.

4.5 Production of Chitinase Enzyme by Bacterial Antagonists

The chitinase activity was determined by the modified method described by Schales procedure (117). One unit (U) of chitinase activity is defining as the amount of enzyme liberating 1 μM of GlcNAc equivalent from the substrate per ml per min under the assay conditions. Specific activity of chitinase is expressed as units per mg of total protein. The results were illustrated in Figure 4-7.

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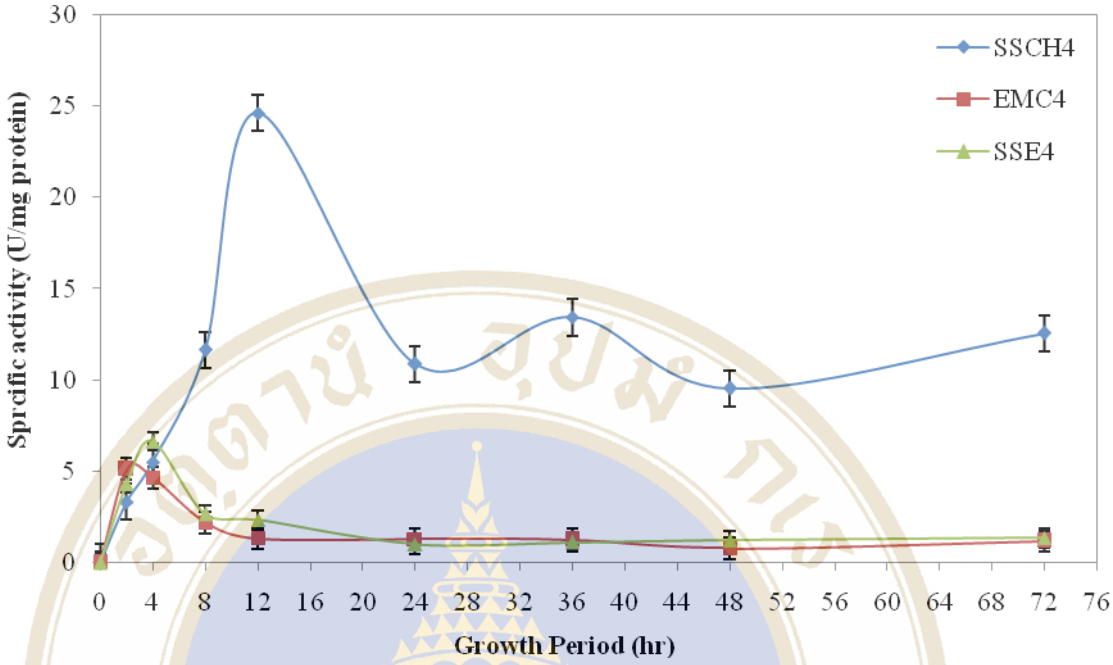

Figure 4-7 The levels of chitinase enzymes produce by selected antagonist at different growth phases

Then, analysis result of chitinase assay founded that *A. salmonicida* SSCHC4 produced higher level of chitinase than *B. subtilis* EMC4 and *B. subtilis* SSE4. The highest chitinase activity in *A. salmonicida* SSCHC4 was observed at 12 hr of growth period (24.62 U/mg protein). The level of chitinase enzyme produced by *A. salmonicida* SSCHC4 was rapidly decreased when cells entered the stationary phase (24 hr). In addition, *B. subtilis* EMC4 produced the highest chitinase enzyme at 2-hr of growth period (5.13 U/mg protein) and slowly decrease until stable. *B. subtilis* SSE4 produced the highest chitinase enzyme at 4-hr of growth period (6.62 U/mg protein) after that it instantly decrease and stable.

B. subtilis EMC4 and *B. subtilis* SSE4 were non-chitinolytic bacteria but the inhibitory effects of *B. subtilis* EMC4 and *B. subtilis* SSE4 to phytopathogenic fungi were involved in the production of antifungal metabolites. As stated in many investigators, *B. subtilis* producers an antifungal compounds like antibiotics, including iturin, bacillomycin D and fengycin A and B (134, 135, 136, 137).

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4.6 Efficiency of Antifungal Metabolites Produced by Bacteria Antagonists on the Growth of Phytopathogenic Fungi

4.6.1. Radial growth suppression by extracellular antifungal metabolites

To evaluate the inhibitory effect of extracellular antifungal metabolites in cell-free culture filtrate on the radial growth, *C. gloeosporioide* and *S. rolfsii* were cultivated on PDA plates amended with 10%, 20% and 30% (v/v) of cultured filtrates of selected antagonists. Antifungal potential of culture filtrate collected from exponential (4hr) and stationary (24 hr) phases of each antagonist was compared. The fungal growth on agar plates was daily measured at 4 and 6 days for *S. rolfsii* and *C. gloeosporioide*, respectively. The antifungal activity of culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 toward *C. gloeosporioide* was shown in Figure 4-8 and 4-11, respectively. The apparent radial growths of *C. gloeosporioide* on PDA plates supplemented with exponential and stationary culture filtrates of *B. subtilis* EMC4 were shown in Figure 4-9 and 4-10, respectively.

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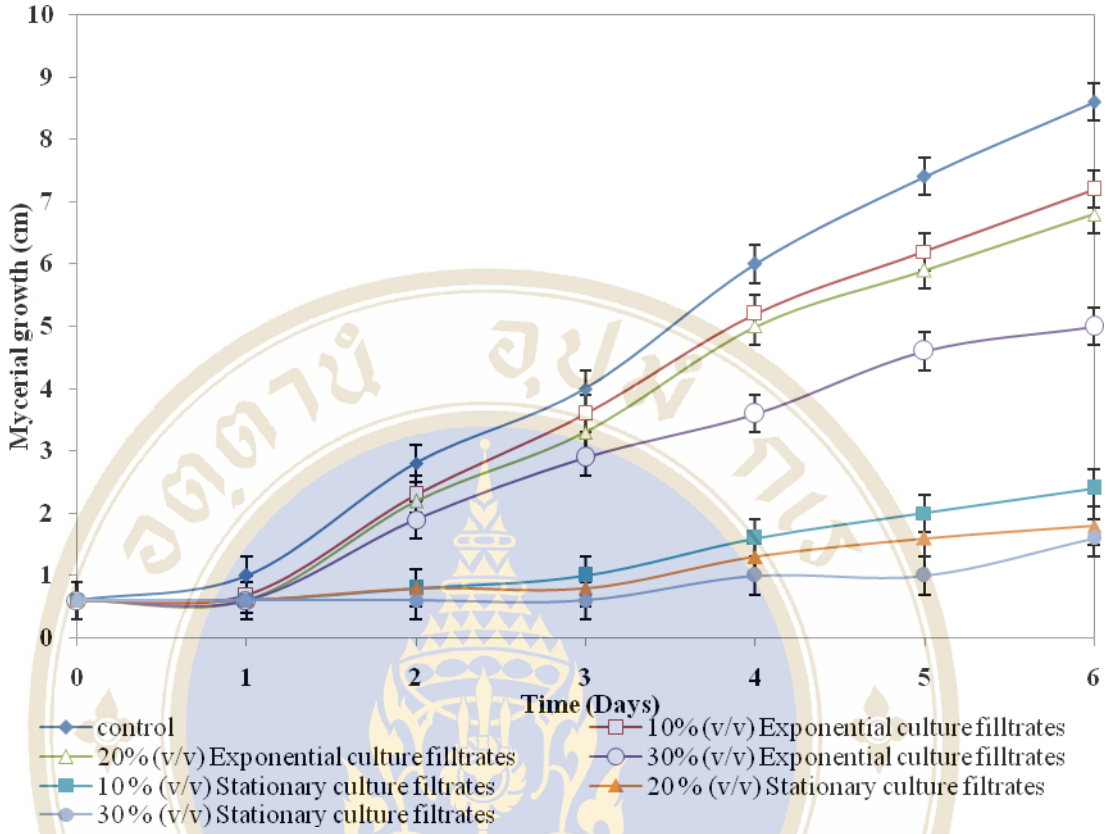

Figure 4-8 Antifungal effects of extracellular antifungal metabolites of *B. Subtilis* EMC4 on the radial growth of *C. gloeosporioides*

From Figure 4-8, radial growth of *C. gloeosporioides* on PDA plate was inhibited by the exponential and stationary culture filtrates at concentration up to 10% (v/v) as compared to the control culture. The percentages of radial growth inhibition of *C. gloeosporioides* by 10% (v/v) of exponential and stationary culture filtrates of *B. subtilis* EMC4 were 17.85 and 71.42 at 2-day of incubation period, respectively. The potential of antifungal activity was increased when the concentration of cell-free culture filtrate was increased from 10% to 30% (v/v). Moreover, the potential of fungal growth inhibition by stationary culture filtrates was higher than that of exponential culture filtrate.

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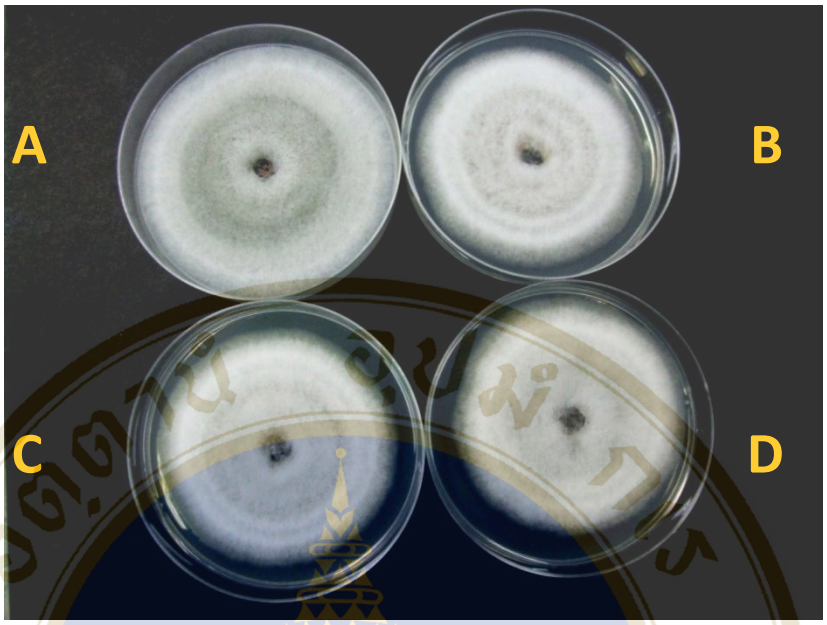

Figure 4-9 The radial growth of *C. gloeosporioides* on PDA plates amended with exponential culture filtrate of *B. subtilis* EMC4 in various concentrations (A) Control, (B) 10%, (C) 20% and (D) 30% (v/v) of culture filtrate

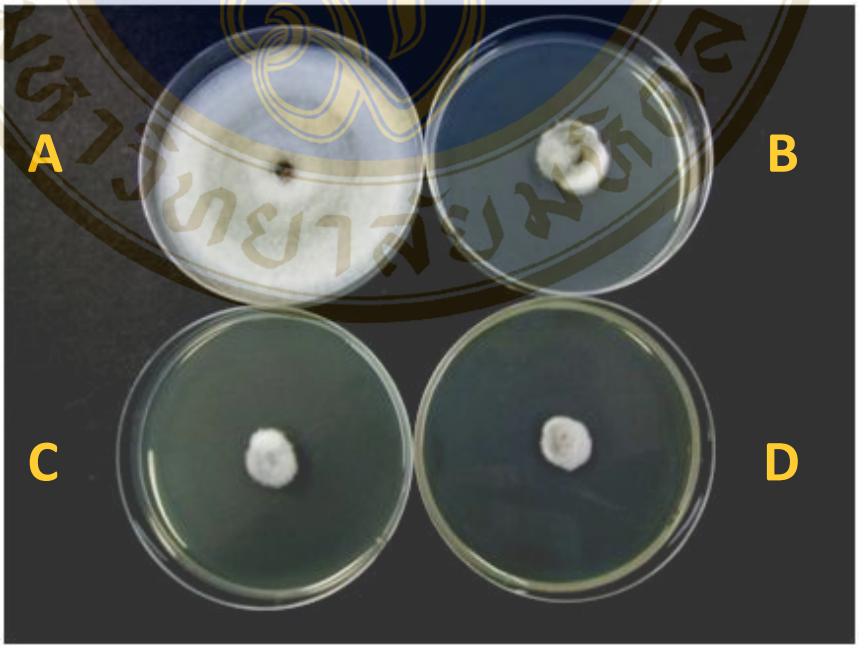

Figure 4-10 The radial growth of *C. gloeosporioides* on PDA plates amended with stationary culture filtrate of *B. subtilis* EMC4 in various concentrations (A) Control, (B) 10%, (C)20% and (D) 30% (v/v) of culture filtrate

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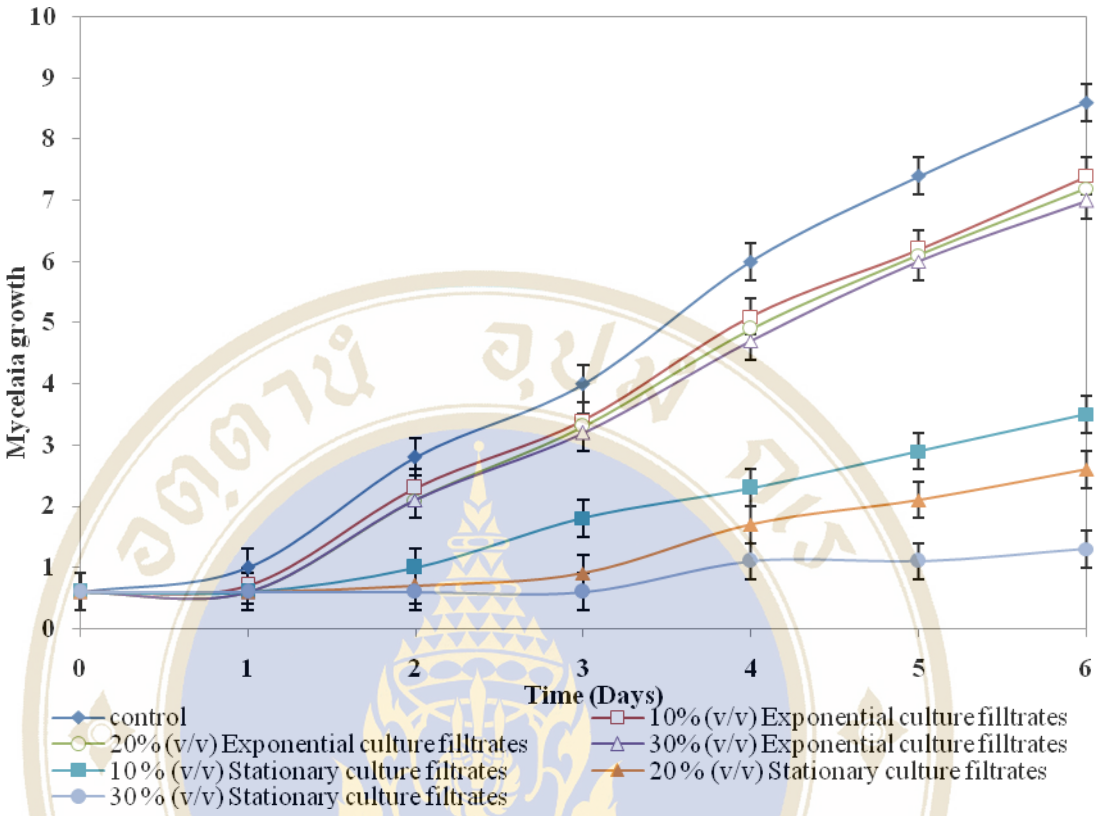

Figure 4-11 Antifungal effects of extracellular antifungal metabolite of *B. subtilis* SSE4 on the radial growth of *C. gloeosporioides*

From Figure 4-11, radial growth of *C. gloeosporioides* on PDA plate was inhibited by exponential and stationary culture filtrates at concentration up to 10% (v/v) as compared to the control culture. The percentages of radial growth inhibition of *C. gloeosporioides* by 10% (v/v) of exponential and stationary cultured filtrates of *B. subtilis* SSE4 were 17.85 and 64.28 at 2-day of incubation period respectively. The potential of antifungal effects activity was increased when the concentration of cell-free culture filtrate was increased from 10% to 30% (v/v). Similar to *B. subtilis* EMC4, the antifungal activity of stationary culture filtrate of SSE4 was higher than that of exponential cultured filtrate. The apparent radial growths of *C. gloeosporioides* on PDA plates amended with various concentration of exponential and stationary culture filtrates of SSE4 were shown in Figure 4-12 and 4-13, respectively.

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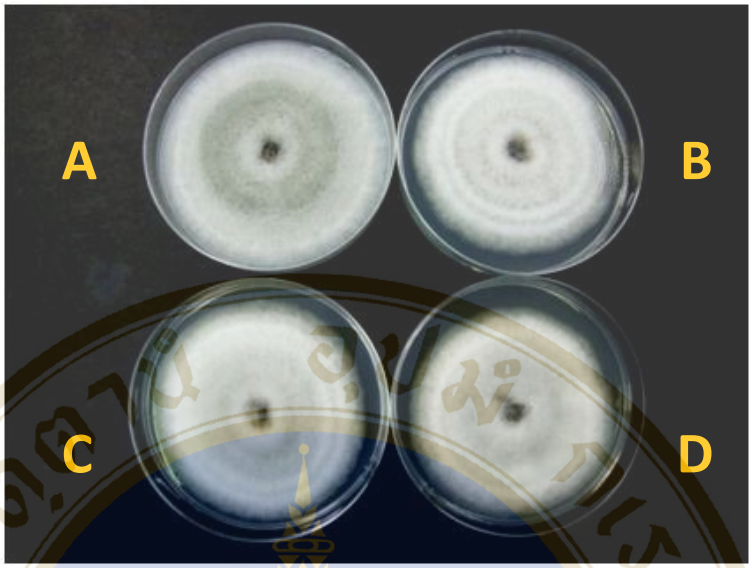

Figure 4-12 The growth of *C. gloeosporioides* on PDA plates amended with exponential culture filtrate of *B. subtilis* SSE4 in various concentrations. (A) Control, (B) 10%, (C) 20% and (D) 30% (v/v) of culture filtrate

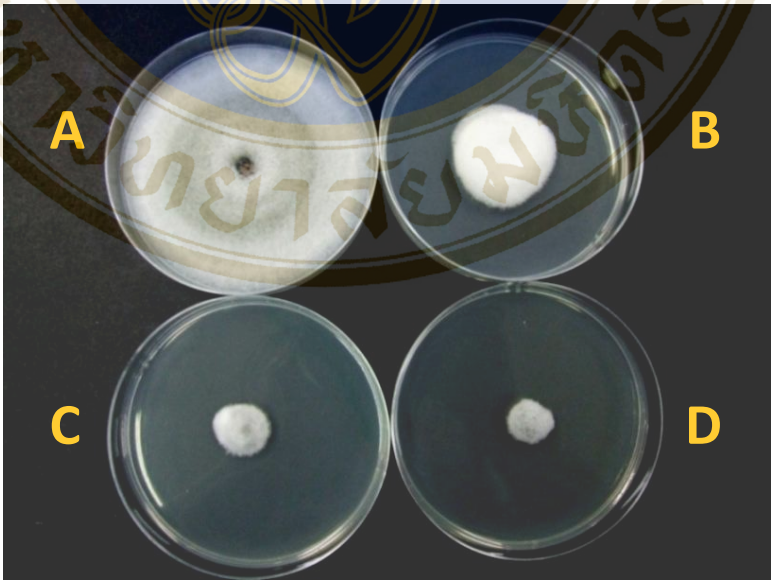

Figure 4-13 The radial growth of *C. gloeosporioides* on PDA plates amended with stationary culture filtrate of *B. subtilis* SSE4 in various concentrations. (A) Control, (B) 10%, (C) 20% and (D) 30% (v/v) culture filtrate

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Inhibitory effects of culture filtrates produced by *B. subtilis* EMC4 and *B. subtilis* SSE4 on the radial growth inhibition of *S. rolfsii* were performed. The radial growth inhibition of *S. rolfsii* by culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 was shown in Figure 4-14 and 4-17, respectively. The apparent radial growths of *S. rolfsii* on PDA plates supplemented with exponential and stationary culture filtrates of *B. subtilis* EMC4 were shown in Figure 4-15 and 4-16, respectively.

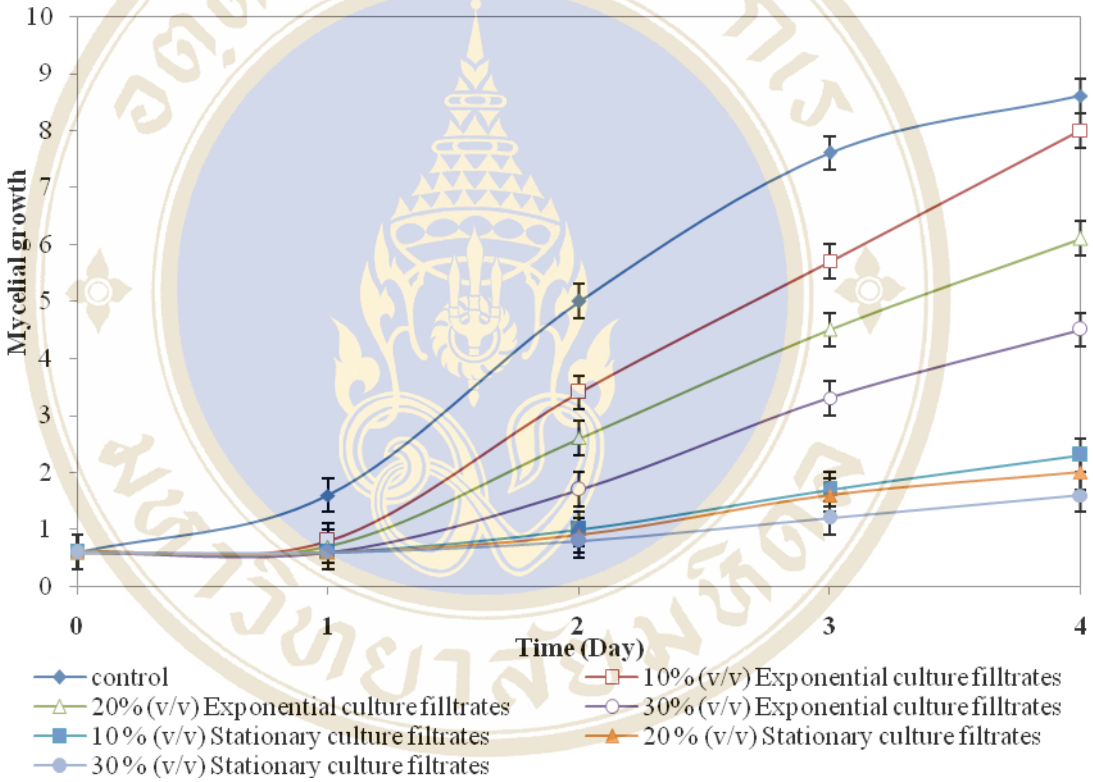

Figure 4-14 Antifungal effects of extracellular antifungal metabolite of *B. subtilis* EMC4 on the radial growth of *S. rolfsii*

From figure 4-14, radial growth of *S. rolfsii* on PDA plate was inhibited by exponential and stationary culture filtrates at concentration up to 10% (v/v) as compared to the control culture. The percentage of radial growth inhibition of *S. rolfsii*

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by 10% (v/v) of exponential and stationary culture filtrates of *B. subtilis* EMC4 were 18.6 and 80.0 at 2-day of incubation period. The potential of antifungal activity was increased when concentration of cell-free culture filtrate was increased from 10% to 30% (v/v). Similar to *B. subtilis* EMC4, the antifungal activity of stationary culture filtrate of EMC4 was higher than that of exponential cultured filtrate.

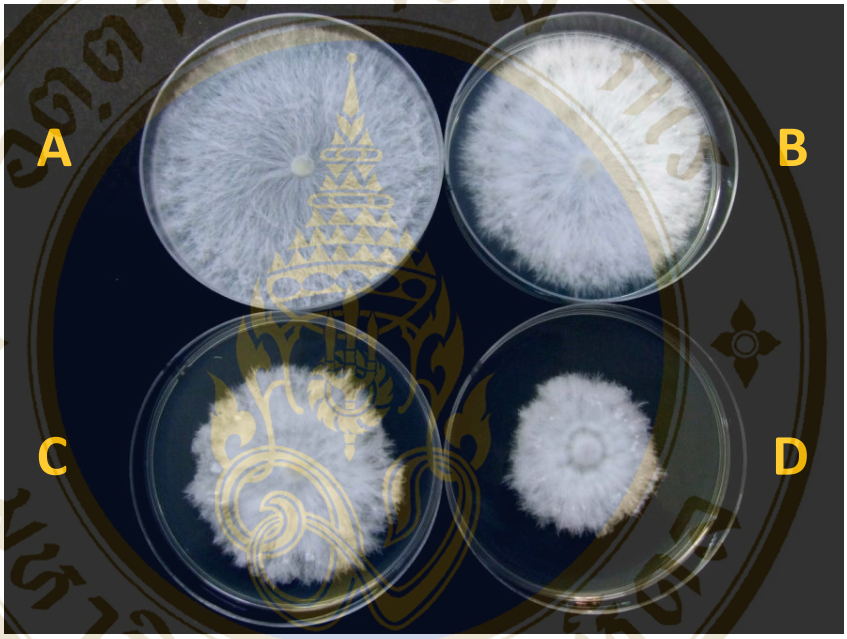

Figure 4-15 The radial growth of *S. rolfsii* on PDA plates amended with exponential culture filtrate of *B. subtilis* EMC4 in various concentrations. (A) Control, (B) 10%, (C) 20% and (D) 30% (v/v) of culture filtrate

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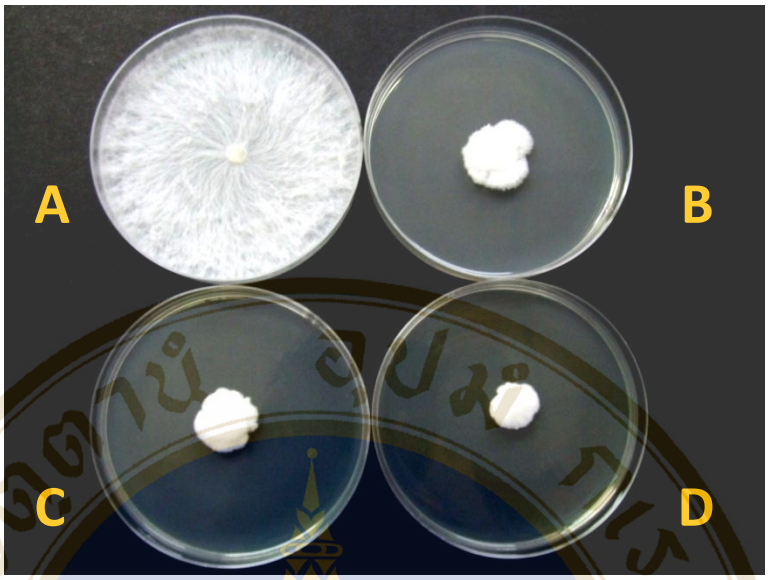

Figure 4-16 The radial growth of *C. gloeosporioides* on PDA plates amended with stationary culture filtrate of *B. subtilis* EMC4 in various concentrations. (A) Control, (B) 10%, (C) 20% and (D) 30% (v/v) of culture filtrate

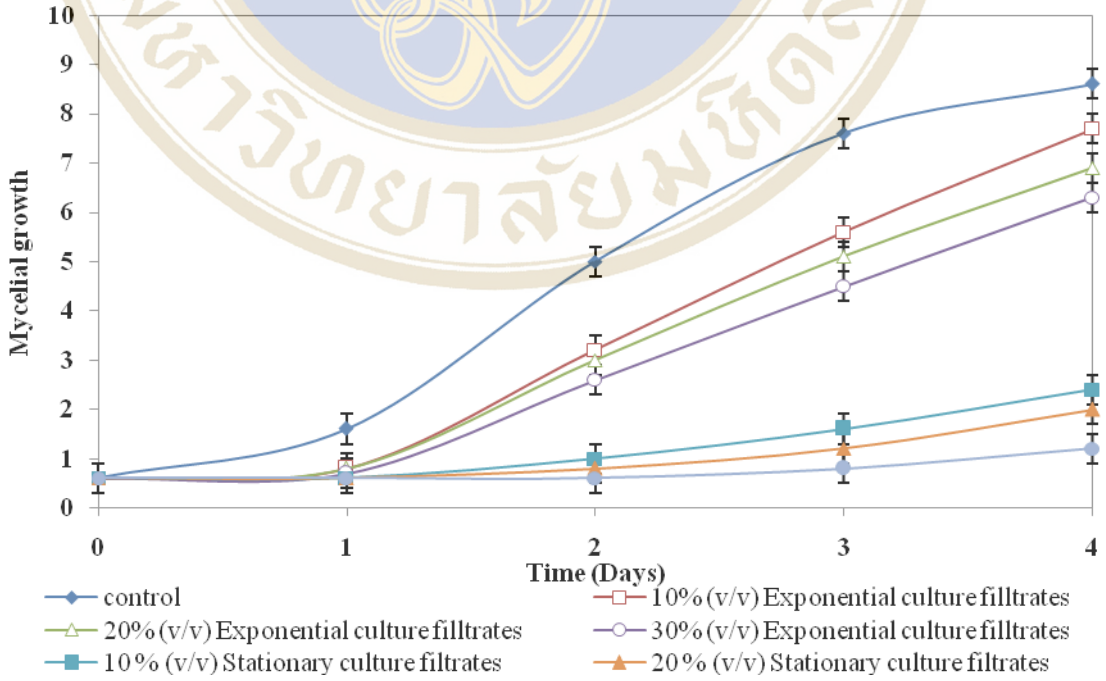

Figure 4-17 Antifungal effects of extracellular antifungal metabolite of *B. subtilis* SSE4 on the radial growth of *S. rolfsii*

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From figure 4-17 radial growth of *S. rolfsii* on PDA plate was inhibited by exponential and stationary culture filtrates at concentration up to 10% (v/v) as compared to the control culture. The percentage of radial growth inhibition of *S. rolfsii* by 10% (v/v) of exponential and stationary culture filtrates of *B. subtilis* SSE4 were 36.0 and 80.0 at 2-day of incubation period. The potential of antifungal activity was increased when concentration of cell-free culture filtrate was increased from 10% to 30% (v/v). Similar to *B. subtilis* EMC4, the antifungal activity of stationary culture filtrate of *B. subtilis* SSE4 was higher than that of exponential cultured filtrate. The apparent radial growths of *S. rolfsii* on PDA plates amended with various concentrations of exponential and stationary culture filtrates of *B. subtilis* SSE4 were shown in Figure 4-18 and Figure 4-19, respectively.

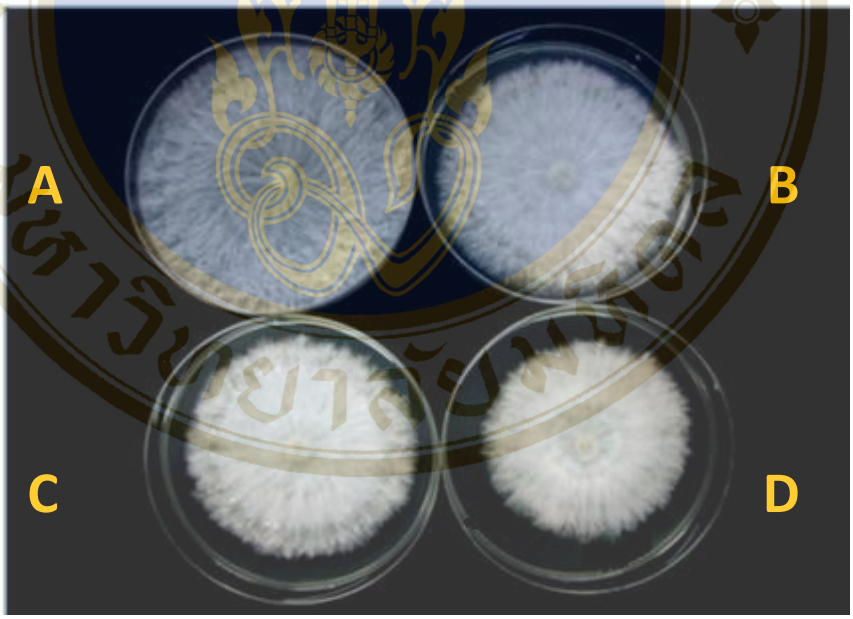

Figure 4-18 The radial growth of *S. rolfsii* on PDA plates amended with exponential culture filtrate of *B. subtilis* SSE4 in various concentrations. (A) Control, (B) 10%, (C) 20% and (D) 30% (v/v) cultured filtrate

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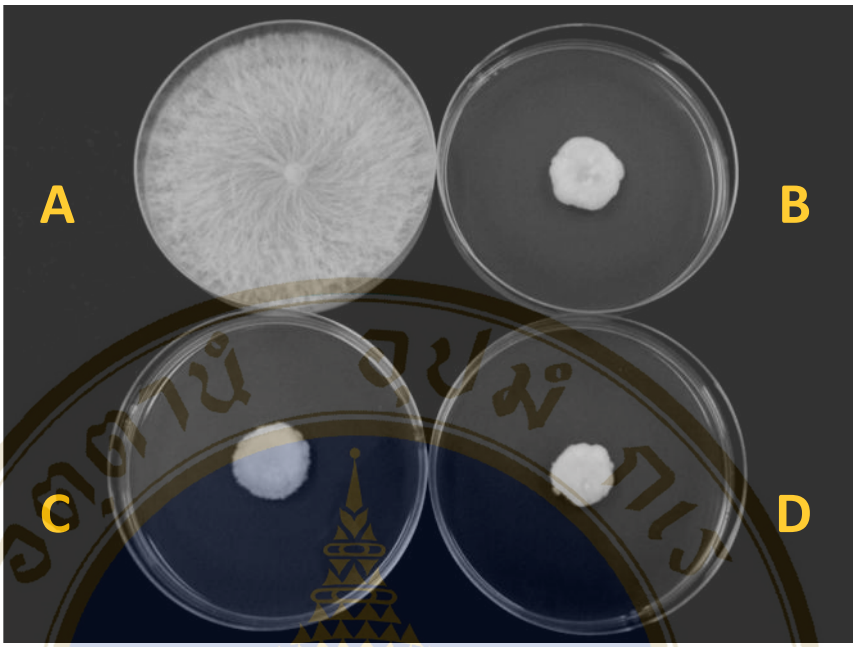

Figure 4-19 The radial growth of *S. rolfsii* on PDA plates amended with stationary culture filtrate of *B. subtilis* SSE4 in various concentrations. (A) Control, (B) 10%, (C) 20% and (D) 30% (v/v) culture filtrate

Table 4-4 The percentage of radial growth inhibition of *C. gloeosporioides* and *S. rolfsii* by exponential and stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 after 3-day of incubation

Concentration of culture filtrate (v/v)	% Radial growth inhibition			
	C. gloeosporioides		*S.rolfsii*	
	Exponential culture filtrate	Stationary culture filtrate	Exponential culture filtrate	Stationary culture filtrate
B. subtilis EMC4				
- 10%	11.76	88.23	25.71	82.85
- 20%	20.59	94.11	44.29	85.71
- 30%	35.29	100	57.14	91.42

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Table 4-4 The percentage of radial growth inhibition of *C. gloeosporioides* and *S. rolfsii* by exponential and stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 after 3-day of incubation (Continued)

Concentration of culture filtrate (v/v)	% Radial growth inhibition			
	C. gloeosporioides		*S.rolfsii*	
	Exponential culture filtrate	Stationary culture filtrate	Exponential culture filtrate	Stationary culture filtrate
B. subtilis SSE4				
- 10%	17.65	70.58	28.57	85.71
- 20%	20.59	91.17	35.71	91.42
- 30%	23.53	100	42.86	97.14

Table 4-4 showed the comparison of the percentage of radial growth inhibition of *C. gloeosporioides* and *S. rolfsii* by culture filtrates of both bacterial antagonists. The results revealed that the percentage of radial growth inhibition of *C. gloeosporioides* by stationary culture filtrate of *B. subtilis* EMC4 was significantly higher than that of *B. subtilis* SSE4. However, there was no significant difference between the percentages of growth inhibition of *S. rolfsii* by exponential and stationary culture filtrate of *B. subtilis* EMC4 and *B. subtilis* SSE4.

4.6.2 Submerged growth suppression by extracellular antifungal Metabolites

The result of radial growth inhibition by culture filtrates from No. 4.6.1 indicated that antifungal potential of culture filtrate was obviously observed at concentration 10% (v/v) (Appendix D No. 7, Tabled-28 – D 29). Thus, the inhibitory effects of culture filtrate on submerge growth by using 10% (v/v) exponential and stationary culture filtrates. *C. gloeosporioides* and *S. rolfsii* were grown in PDB medium amended with 10% (v/v) of culture filtrates. The fungal growth was daily monitored by dry weight determination for 9 days. The result of the antifungal

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potential of culture filtrates produced by *B. subtilis* EMC4 and *B. subtilis* SSE4 toward *C. gloeosporioides* and *S. rolfsii* were shown in Figure 4-20 and 4-21, respectively.

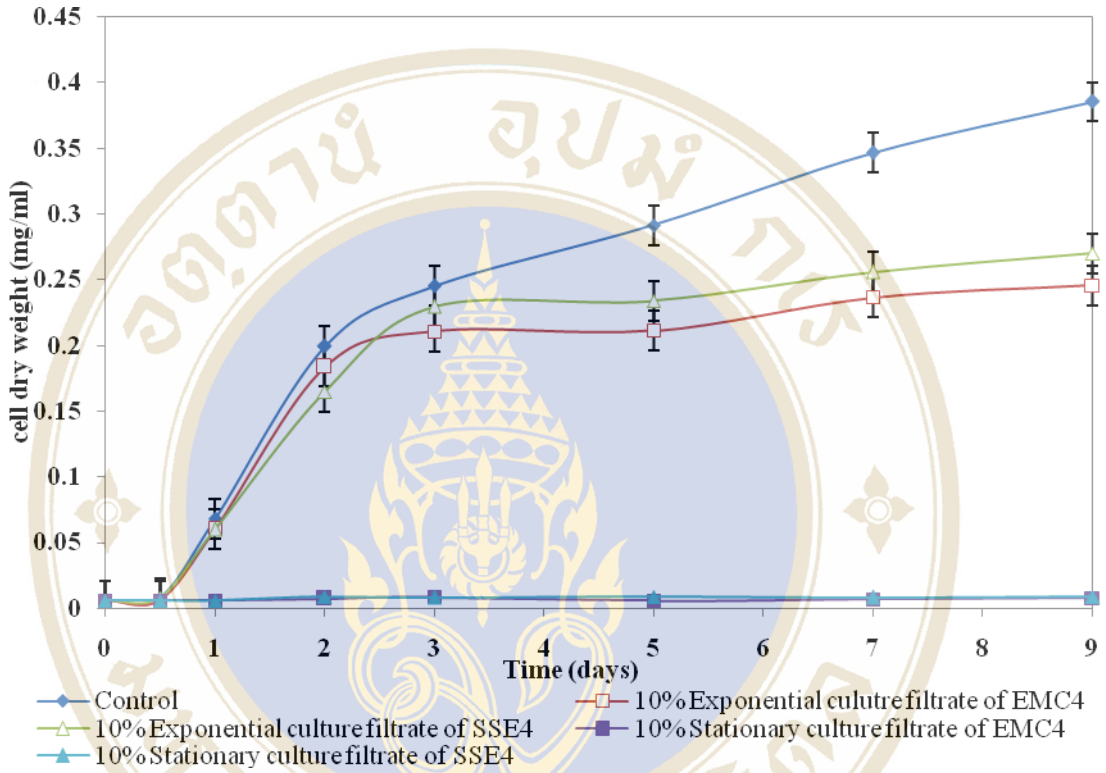

Figure 4-20 Antifungal effects of 10% (v/v) extracellular antifungal metabolites of *B. subtilis* EMC4 and *B. subtilis* SSE4 on the submerged growth of *C. gloeosporioides*

From Figure 4-20, submerged growth of *C. gloeosporioides* was almost absolutely inhibited by stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4. At 2-day of incubation, exponential culture filtrate of *B. subtilis* SSE4 (17.40%) more exhibited antagonistic activity to *C. gloeosporioides* higher than exponential culture filtrate of *B. subtilis* EMC4 (7.87%). Nevertheless, the exponential culture filtrate of *B. subtilis* SSE4 more exhibited antagonistic activity to *C. gloeosporioides* than the exponential culture filtrate of *B. subtilis* EMC4 after 5-day of incubation. The

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percentages of submerge growth inhibition of *C. gloeosporioides* by *B. subtilis* EMC4 and *B. subtilis* SSE4 at 5-days were 27.48 and 19.73, respectively.

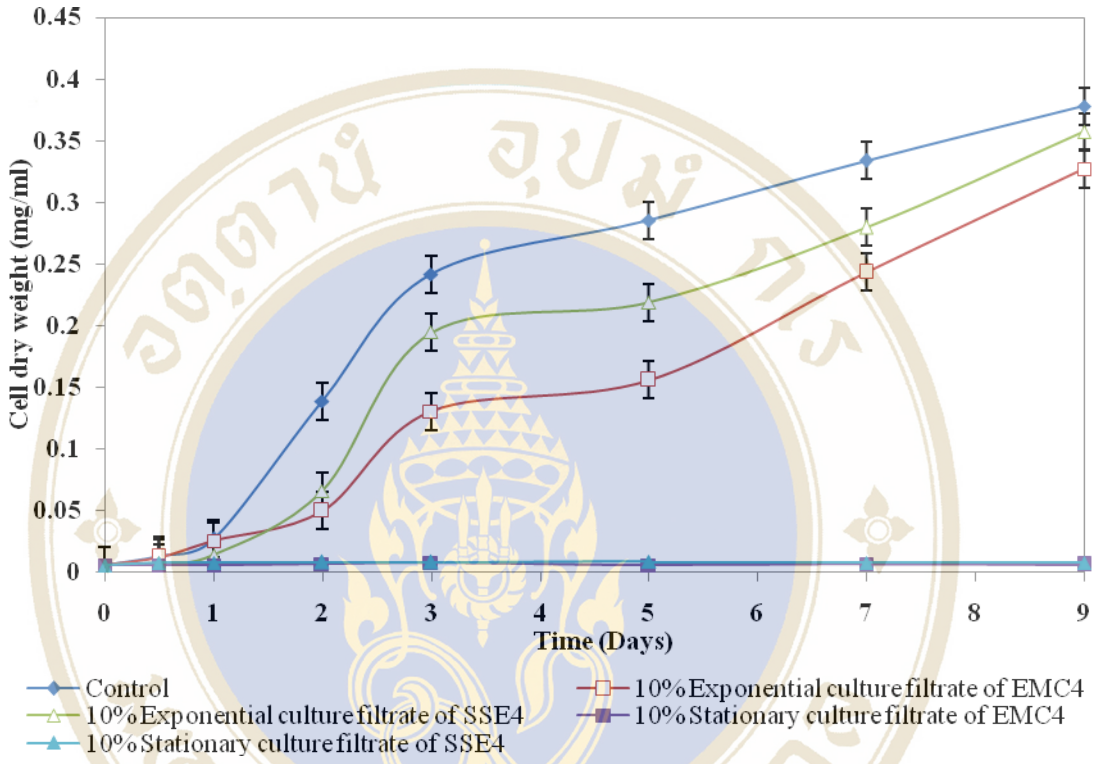

Figure 4-21 Antifungal effects of 10% (v/v) extracellular antifungal metabolites of *B. subtilis* EMC4 and *B. subtilis* SSE4 on the submerge growth of *S. rolfsii*

From Figure 4-21, submerged growth of *S. rolfsii* was almost absolutely inhibited by the stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4. The exponential culture filtrate of *B. subtilis* EMC4 more exhibited higher antagonistic activity to *S. rolfsii* than that of *B. subtilis* SSE4. The percentages of submerged growth inhibition of *S. rolfsii* by *B. subtilis* EMC4 and *B. subtilis* SSE4 at 5-day of incubation period were 45.30 and 23.28, respectively.

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4.6.3 Effects of heat and protinase K on the antifungal activity of extracellular antifungal metabolites

Based on the results from the *in vitro* studies, extracellular chitinase enzyme or secondary antifungal metabolite(s) play a major role for inhibition of fungal growth. To clarify this assumption, exponential and stationary culture filtrates of both *B. subtilis* EMC4 and *B. subtilis* SSE4 were treated with heat at 100^{o}C for 45 min or proteinase K. *In vitro* antifungal bioassay was performed to determine the antifungal activity against *C. gloeosporioides* and *S. rolfsii*. Antifungal potential to *C. gloeosporioides* by 10% (v/v) exponential and stationary culture filtrate of *B. subtilis* EMC4 and *B. subtilis* SSE4 were significant unchanged after treatment with boiling or proteinase K by and respectively, compared to the activity of 10% (v/v) untreated culture filtrate (Figure 4-22 and Figure 4-23).

For *S. rolfsii*, the potential of growth inhibition by 10% (v/v) untreated and heat- and proteinase K–treated exponential and stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 were significantly unchanged both of treatment when a comparison to untreated culture filtrate (Figure 4-24 and Figure 4-25). The results revealed that antifungal activity of heat- and proteinase K-treated exponential and stationary culture filtrates were significantly reduced when compare to untreated exponential culture filtrates. The result indicated that exponential and stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 were not an enzyme but were probably a thermostable secondary antifungal compound(s) for fungal growth inhibition. A variety of antibiotics have been identified that were produced by *Bacillus*. spp. for antifungal metabolites were antibiotics including iturin, bacillomycin D and fengycin A and B (134, 135, 136, 137).

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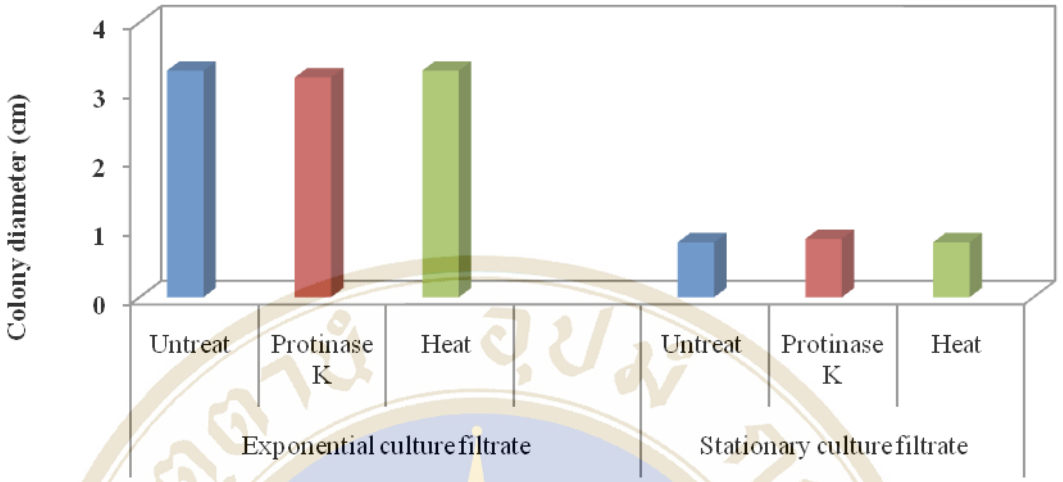

Figure 4-22 Effects of heat and proteinase K on the antifungal activity of 20% (v/v) culture filtrates of *B. subtilis* EMC4 to *C. gloeosporioides* at 3-day of incubation

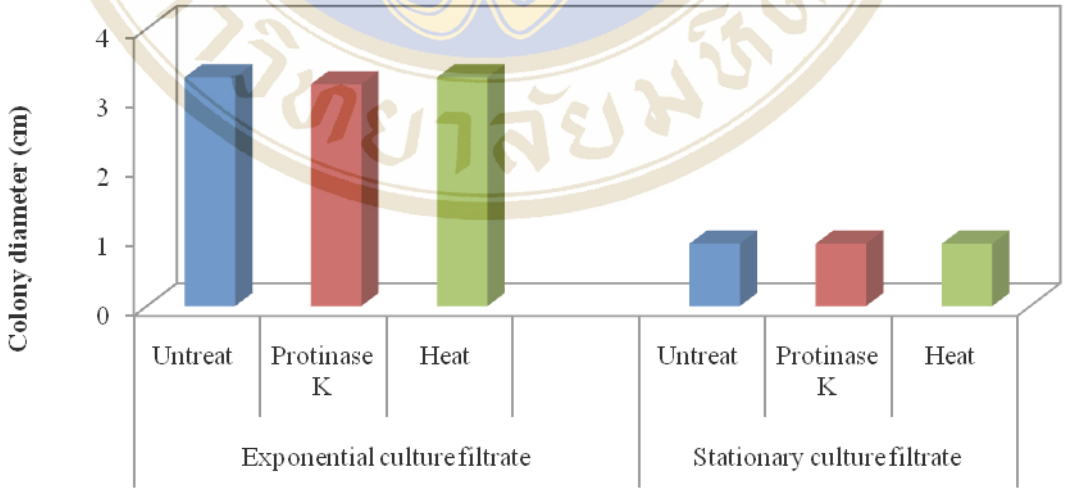

Figure 4-23 Effects of heat and proteinase K on the antifungal activity of 20% (v/v) culture filtrates of *B. subtilis* SSE4 to *C. gloeosporioides* at 3-day of incubation

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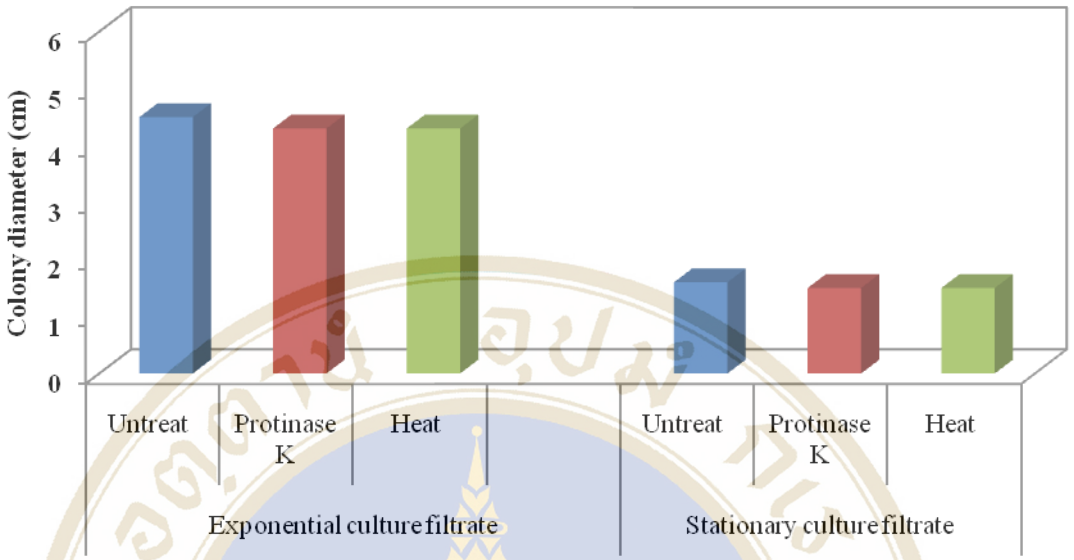

Figure 4-24 Effects of heat and proteinase K on the antifungal activity of 20% (v/v) culture filtrates of *B. subtilis* EMC4 to *S. rolfsii* at 3-day of incubation

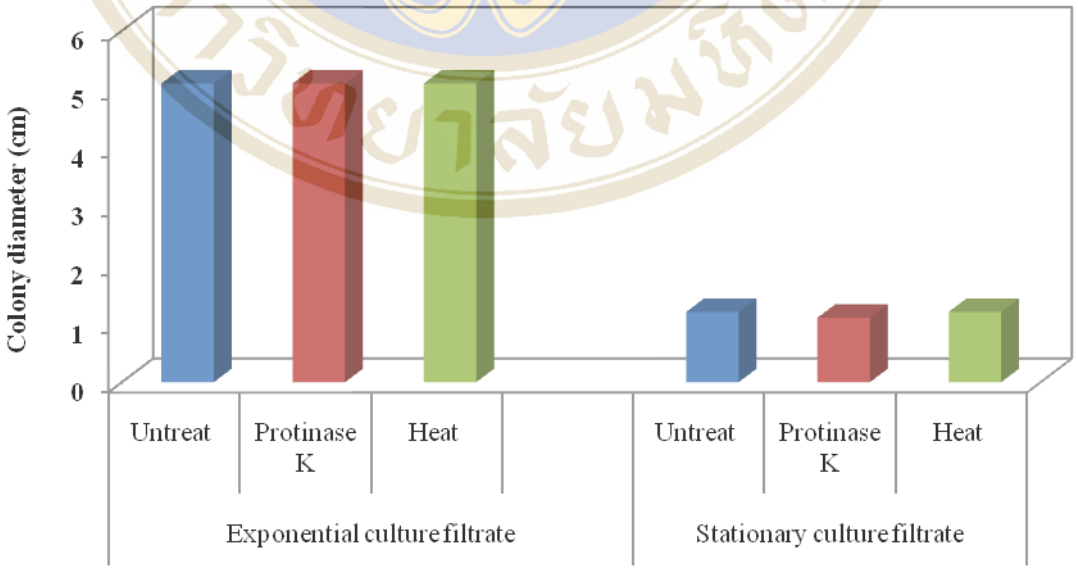

Figure 4-25 Effects of heat and proteinase K on the antifungal activity of 20% (v/v) culture filtrates of *B. subtilis* SSE4 to *S. rolfsii* at 3-day of incubation

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4.7 Effects of Extracellular Antifungal Metabolites on the Hyphal Morphology of Phytopathogenic Fungi

The hyphal morphology of *C. gloeosporioides* and *S. rolfsii* grown on PDA plates amended with 10% exponential and stationary culture filtrates was observed under light microscope at magnification 400X for 3 and 5 days of growth period, respectively. The microscopic results of *C. gloeosporioides* and *S. rolfsii* were illustrated in Figure 4-25 and Figure 4-26, respectively.

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Figure 4-26 Effects of 10% (v/v) culture filtrates on the morphology of 5-day-old *C. gloeosporioides*, (A) Control (B) Exponential culture filtrate of *B. subtilis* SSE4 (C) Stationary culture filtrate of *B. subtilis* SSE4 (D) Exponential culture filtrate of *B. subtilis* EMC4 (E) Stationary culture filtrate *B. subtilis* EMC4

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Figure 4-27 Effects of 10% (v/v) culture filtrates on the morphology of 3-day-old *S. rolfsii*, (A) Control (B) Exponential culture filtrate of *B. subtilis* SSE4 (C) Stationary culture filtrate of *B. subtilis* SSE4 (D) Exponential culture filtrate of *B. subtilis* EMC4 (E) Stationary culture filtrate *B. subtilis* EMC4

Exponential and stationary culture filtrates from *B. subtilis* EMC4 and *B. subtilis* SSE4 caused the swelling and lysis of hyphal structure of both tested fungi. Morphological changes in hyphal swelling began to be noticeable (as marked in Figure 4-26 and 4-27). Swelling was initially observed at diverging angles of branched

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mycelium as compared to the control. Hyphae of *C. gloeosporioides* and *S. rolfsii* grew in abnormal sharps such as marked swelling, cytoplasm aggregation. Light microscope investigation revealed that growth inhibition of *C. gloeosporioides* and *S. rolfsii* as a response to extracellular antifungal metabolites in culture filtrates were accompanied by marked cellular changes including hyphal swelling, distortion and cytoplasm aggregation. According to the report of Bottone (139) described a compound produced by *Bacillus. pumilus* (MSH) that has fungicidal activity against Mucoraceae and *Aspergillus* spp. The compound is of low molecular mass and inhibits spore germination and hyphal elongation, rendering it versatile in potential prophylaxis against, and treatment of, opportunistic infections caused by the above angio invasive fungal pathogens. The effect of the crude fungicide on the hyphal growth was examined using a light microscope from *B. subtilis* W113 crude fungicide on the morphology of *F. oxysporum* were swelling, degradation, and lysis (140).

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CHAPTER V
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this study, the selected antagonistic bacteria strain SSCH4 was chitinolytic bacteria, isolated from shrimp shell waste and 2 strains that EMC4 and SSE4 were non-chitinolytic bacteria, isolated from FBE and shrimp shell waste respectively, which showed an inhibition activity against *C. gloeosporioides* and *S. rolfsii* that were phytopathogenic fungi caused anthracnose and stem rot disease respectively. Culture filtrates of selected antagonistic bacteria were inhibited phytopathogenic fungi in laboratory scale. The conclusion can be drawn from this study are as follows:

5.1.1 Isolation of bacteria from Fermented bioextract (FBE) and shrimp shell waste

Totally, 143 strains of bacteria 46 strains were isolated from FBE and 97 strains were isolated from shrimp shell waste. Only 12 strains or 8.4% of total isolated bacteria showed chitinolytic activity.

5.1.2 Antifungal activity of isolated bacteria

The antifungal activity of selected bacteria, 133 and 140 strains exhibited a weak or non-antagonistic activity towards *C. gloeosporioides* and *S. rolfsii*, respectively. Only 6 and 3 bacterial strains had a high antagonistic activity toward *C. gloeosporioides* and *S. rolfsii*, respectively. The percentage of isolated bacteria with antifungal activity to both *C. gloeosporioides* and *S. rolfsii* was 2.10%. Three bacterial strains, designated as SSCHC4, SSE4 and EMC4, showed the highly antifungal activity toward *C. gloeosporioides* and *S. rolfsii* were selected for further study.

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5.1.3 Growth curve of selected antagonistic bacteria

The growth curves of 3 bacterial strains were not significantly different. The growth was sharply increased into exponential phase within 4 hr (OD_{600} ~ 2.75-3.19). After that cell proliferation was still increased until 12 hr of incubation period. Growth of each strain entered the mid-stationary phase at 24 hr. The cell growth was slightly constant until 72 hr of growth period

5.1.4 Morphology and identification of selected antagonistic bacteria

From the Gram staining, all strains were Gram positive and rod shape. Morphology of bacterial cells was also observed by SEM at 10,000X. In addition, endospore formation was observed in the strain SSE4 and EMC4. The 3 bacterial antagonists were identified by partial 16S rDNA sequence analysis. The results found that strain SSCHC4 showed a high similarity to *Aeromonas salmonicida* (97% similarity) from Genbank while SSE4 and EMC4 showed a high similarity to *Bacillus subtilis* (98% similarity) from Genbank database. Due to its pathogenicity to fish of *A. salmonicida*, there was no determination of antifungal activity in strain SSCHC4.

5.1.5 Efficiency of extra cellular antifungal metabolites of antagonistic bacteria on the growth suppression of phytopathogenic fungi

The efficiency of extra cellular metabolites of antagonistic bacteria on the growth suppression of phytopathogenic fungi on the radial growth of *C. gloeosporioides* by *B. subtilis* EMC4 were 17.85 and 71.42 at 2-days of 10% cell-free culture filtrate of exponential and stationary phase respectively. When the potential of antifungal effects were increasingly while increase concentration of cell-free culture filtrate.

The percentage of radial growth inhibition of *C. gloeosporioides* by *B. subtilis* EMC4 were 17.85 and 64.28 at 2-days of 10% cell-free culture filtrate of exponential and stationary phase respectively. When the potential of antifungal effects were increasingly while increase concentration of cell-free culture filtrate.

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The efficiency of extra cellular metabolites of antagonistic bacteria on the growth suppression of phytopathogenic fungi on the radial growth of *S. rolfsii* by *B. subtilis* EMC4 were 18.60 and 80.00 at 2-days of 10% cell-free culture filtrate of exponential and stationary phase respectively. When the potential of antifungal effects were increasingly while increase concentration of cell-free culture filtrate.

The percentage of radial growth inhibition of *S. rolfsii* by *B. subtilis* SSE4 were 36.00 and 80.00 at 2-days of 10% cell-free culture filtrate of exponential and stationary phase respectively. When the potential of antifungal effects were increasingly while increase concentration of cell-free culture filtrate.

The study of effect of exponential and stationary culture filtrates on the submerged growth of *C. gloeosporioides* by *B. subtilis* EMC4 was almost absolutely inhibited by stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4**.** The exponential culture filtrates of *B. subtilis* EMC4 more exhibited antagonist to *S. rolfsii* than *B. subtilis* SSE4. The percentages of submerge growth inhibition of *S. rolfsii* by *B. subtilis* EMC4 and *B. subtilis* SSE4 at 5-days were 45.30 and 23.28 respectively.

The study of effect of exponential and stationary culture filtrates on the submerged growth of *S. rolfsii* was almost absolutely inhibited by stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4**.** The exponential culture filtrates of *B. subtilis* EMC4 more exhibited antagonist to *S. rolfsii* than *B. subtilis* SSE4. The percentages of submerge growth inhibition of *S. rolfsii* by *B. subtilis* EMC4 and *B. subtilis* SSE4 at 5-days were 45.30 and 23.28 respectively.

To clarify this assumption, exponential and stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 were treated with heat at 100^{o}C for 45 min or protinase K. Antifungal potential to *C. gloeosporioides* and *S. rolfsii* by 10% (v/v) exponential culture filtrate was no significant unchanged after treatment with boiling or protinase K by and respectively, compared to the activity of 10% (v/v) untreated culture filtrate

The effect of exponential and stationary culture filtrates from *B. subtilis* EMC4 and *B. subtilis* SSE4 on the morphology of *C. gloeosporioides* and *S. rolfsii* have been studied and found that both of culture filtrates concentration at 10% (v/v) could induce morphological changes to *C. gloeosporioides* and *S. rolfsii*. Culture filtrates from *B. subtilis* EMC4 and *B. subtilis* SSE4 caused the swelling and lysis of

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hyphal structure of both tested fungi. Morphological changes in hyphal swelling began to be noticeable. Swelling was initially observed at diverging angles of branched mycelium as compared to the control

5.2 Recommendation

The recommendation can be drawn from this study are as follows:

5.2.1 The screening and isolation of antagonistic bacteria should be performed in other sources such as FBE, soil, plant or plant debris in the disease-occurrence area.

5.2.2 Evaluation of the effect of culture filtrates from selected antagonistic bacteria on the growth of other phytopathogenic fungi or other bacterial plant pathogens should be performed for further studies.

5.2.3 To obtain the highest yields of extracellular antifungal metabolites in cell-free culture filtrates of bacterial antagonists, the optimal condition such as pH, temperature types and composition of cultural medium should be investigated.

5.2.4 Chemical structure and properties of extracellular antifungal metabolites in cell-free culture filtrates produce by from the antagonistic bacteria should be characterized.

5.2.5 The effects of extracellular antifungal metabolites in cell-free culture filtrates on the plants and other indigenous soil microorganisms should be studied.

5.2.6 For further application, extracellular antifungal metabolites in cell-free culture filtrates or bacterial cells for disease control should be performed in the greenhouse and field study under the control of parameters.

5.2.3 To obtain the highest efficiency of culture filtrates produce from antagonistic bacteria, some condition factors such as degree of deacetylation of secondary metabolites, optimum pH, kind of cultural medium should be determined.

5.2.4 Investigation on the effective condition of culture filtrates from on the control of disease should be performed in the greenhouse and field study under the control of parameters.

5.2.5 Investigation on the effect of culture filtrates on the bacteria – fungal pathogen interaction.

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5.2.6 Investigation on the secondary metabolites produce from the antagonistic bacteria.

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REFERENCES

1. Office of Agricultural Economics. Agricultural Statistics of Thailand Crop Year 1997/1998. Ministry of Agriculture and Cooperatives, Thailand. 1999.
2. Department of Agriculture. Sustainable agriculture: The future of Thai farmers. Pages 118-129 in: Annual Technical Bulletin for 1993. Ministry of Agriculture and Cooperatives, Thailand. 1993.
3. มูฮำหมัดซาหารี สะแลแม . โรคต้นและใบแห้งในต้นพริก. [Online] Available from http://www.yalor.yru.ac.th/~dolah/notes4902-1-48G12/SEMREP/Sm_404652024.doc [Access on 2007 Mar 15]
4. Chau, KF, Alvarez AM. A histological study of anthracnose on Carica papaya. Phytopathology. 1983; 73:1113–1116.
5. Donkin, DJ, Oosthuyse SA. Quality evaluation of sea-exported South African mangoes in Europe during the 1995–1996 seasons. S.A. Mango Growers' Assoc. Yearbook. 1996; 16:1–5.3.
6. Okereke VC, Wokocha RC. *In vitro* growth of four isolates of *Sclerotium rolfsii* Sacc in the humid tropics. African Journal of Biotechnology.2007; 6(16): 1879-1881.
7. Okabe I, Morikawa C, Matsumoto N. Variation in southern blight fungus in Japan detected by ITS-RFLP analysis. 2000; 34:93-97.
8. Howarth FG. Environmental impacts of classical biological control. Annu. Rev. Entomol. 1991; 36: 85-509.
9. Hajek Ann E. Natural Enemies: an introduction to biological control. United Kingdom: Cambridge University Press; 2004.
10. Eilen berg J, Hajek A, Lomer C. Suggestions for unifying the terminology in biological control. BioControl. 2001; 46:387-400.
11. Hajek Ann E. Natural Enemies: an introduction to biological control. United Kingdom: Cambridge university press; 2004.

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12. Van Driesche RG. Biological control. New York: Chapman & Hall; 1996

13. Gilbert GS, Parke JL, Clayton MK. and Handelsman J. Effect of an introduced bacterium on bacterial communities on roots. Ecology. 1993; 74: 840-509.

14. Osburn RM, Milner JL, Oplinger ES, Smith RS, Handelsman J. Effect of *Bacillus cereus* UW85 on the yield of soybean at two field sites in Wisconsin. Plant Dis. 1995; 79:551-556.

15. Hou X, Boyetchko SM, Brkic M, Olson D, Ross A, Hegedus D. Characterization of the anti fungal activity of a *Bacillus* spp. associated with sclerotia from *Sclerotinia sclerotiorum*. Appl. Microbiol. Biotechnol. 2006; 72:644–653.

16. Douville Y, Boland GJ. A note on the antibiotic properties of *Bacillus subtilis* against *Collectotrichum trifolii*. Phytoprotection. 1992;73: 31-36

17. Kelemu S, Badel JL. In vitro inhibition of *Collectotrichum gloeosporioides* and other phytophatogenic fungi by an Amazonian isolate of *Bacillus subtilis* and its cell free culture filtrate. Aust. Plant pathol. 1994; 23:41-45.

18. Touré Y, Ongena M, Jacques P, Guiro A, Thonart P. Role of lipopeptides produced by Bacillus subtilis GA1 in the reduction of grey mould disease caused by Botrytis cinerea on apple. J. Appl. Microbiol. 2004; 96:1151–1160.

19. Chan YK, McCormick WA, Seifert KA. Characterization of an antifungal soil bacterium and its antagonistic activities against Fusarium species. Can. J. Microbiol. 2003; 49:253–262.

20. Souto GI, Correa OS, Montecchia MS, Kerber NL, Pucheu NL, Bachur M, García AF Genetic and functional characterization of a *Bacillus* sp. strain excreting surfactin and antifungal metabolites partially identified as iturin-like compounds. J. Appl. Microbiol. 2004; 97:1247–1256.

21. Lin D, Qu LJ, Gu H, Chen Z A 3.1-kb genomic fragment of *Bacillus subtilis* encodes the protein inhibiting growth of *Xanthomonas oryzae* pv. oryzae. J. Appl. Microbiol. 2001; 91:1044–1050.

22. Shoda M, Ano T. Basic analysis of *Bacillus subtilis* NB22 and its application to biological control. Bioprocess Technol. 1994; 19:641–664.

23. Van Loon LC, Bakker PAHM, Pieterse CMJ. Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol.1998; 36: 453–483.

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24. Whipps JM. Microbial interaction and biocontrol in the rhizosphere. J. Exp. Bot. 2001; 52: 487-511.

25. DeBach P, Rosen D. Biological Control by Natural Enemies. 2nd ed. Cambridge: Cambridge University Press. 1991.

26. Greathead DJ. Historical biological control. Antenna. 1994; 18: 187-199.

27. Goden RD, Andrés LA. Biological control of weed in terrestrial and aquatic environments.Ann. Rev. Entomol. 1975; 20: 31-46.

28. Whipps JM, Davies KG. Success in biological control of plant pathogens and nematods by microorganisms. In Gurr G, Wratten SD, Waage J. Biological Control: Measures of Success. Boston : Kluwer Academic Publishers. 2000.

29. Food and Agriculture Organisation of the United Nations (FAO), Code of conduct for the import and release of exotic biological control agents. International Standards for Phytosanitary Measures. 1996; 3: 19.

30. Coombs J, Hall KE. Dictionary of Biological Control and Integrated Pest Management. CPL Press, Newbury, UK. 1998.

31. Nordlund DA. Biological control, integrated pest management and conceptual models. Biocontrol News and Information. 1999; 17: 35N–44N.

32. Van Driesche RG, Bellows TS. *Biological Control*. Publ. Chapman Hall. 1996.

33. Hunter-Fujita FR, Entwistle PF, Evans HF, Crook NE. Insect Viruses and Pest Management. Wiley, Chichester, UK. 1998.

34. Shiomi Y, Nishiyama M, Onizuka T, Marumoto T. Comparison of Bacterial Community Structures in the Rhizoplane of Tomato Plants Grown in Soils Suppressive and Conducive towards Bacterial Wilt. Appl. Environ. Microbiol. 1999; 65: 3996-4001.

35. Postma J, Willemsen-De Klein M, van Elsas JD. Effect of the Indigenous Microflora on the Development of Root and Crown Rot Caused by *Pythium aphanidermatum* in Cucumber Grown on Rockwool. Phytopathology.2000; 90(2): 125-133.

36. Cody RM. Distribution of Chitinase and Chitobiase in *Bacillus*. Curr. Microbiol. 1989; 19: 201-205.

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37. Frankowski J, Lorito M, Scala F, Schmidt R, Berg G, Bahl H.Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. Arch. Microbiol. 2001; 176:421–426.

38. Singh PP, Shin YC, Park CS, Chung YR. Biological control of *Fusarium* wilt of cucumber by chitinolytic bacteria. Phytopathology. 1999; 89: 92–99.

39. Handelsman J, Stabb EV. Biocontrol of soilborne plant pathogens. The Plant Cell. 1996; 8: 1855-1869.

40. DeSouza JT, DeBoer M, DeWaard P, VanBeek TA, Raaijmakers JM. Biochemical, genetic, and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. Appl. Environ. Microbiol. 2003.69**:**7161–7172.

41. Nielsen TH, Sørensen D, Tobiasen C, Andersen JB, Christeophersen C, Givskov M, et al. Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar beet rhizosphere. Appl. Environ. Microbiol. 2002; 68: 3416–3423.

42. Nielsen TH, Sørensen J. Production of cyclic lipopeptides by *Pseudomonas fluorescens* strains in bulk soil and in the sugar beet rhizosphere. Appl. Environ. Microbiol. 2003; 69: 861–868.

43. Raaijmakers JM, Vlami M, de Souza JT. Antibiotic production by bacterial biocontrol agents. Antonie Leeuwenhoek 2002; 81:537–547.

44. HashidokoY, Nakayama T, Homma Y, Tahara S. Structure. elucidation of xanthobaccin A, a new antibiotic produced from *Stenotrophomonas* sp. strain SB-K88. Tetrahedron Lett. 1999; 40: 2957–2960.

45. Kim BS, Moon SS, Hwang BK.. Isolation, identification and antifungal activity of a macrolide antibiotic, oligomycin A, produced by *Streptomyces libani*. Can. J. Bot. 1999; 77: 850–858.

46. Nakayama T, Homma Y, Hashidoko Y, Mizutani J, Tahara S. Possible role of xanthobaccins produced by *Stenotrophomonas* sp. Strain SB-K88 in suppression of sugar beet damping-off disease. Appl. Environ. Microbiol. 1999; 65: 4334–4339.

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47. Compant S, Duffy B, Nowak J, Cle´ment C, Barka EA. Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects. Appl. Environ. Microbiol. 2005; 71(9): 4951–4959.

48. Duffy B K, De´fago G. Controlling instability in *gacS-gacA* regulatory genes during inoculum production of *Pseudomonas fluorescens* bicontrol strains. Appl. Environ. Microbiol. 2000; 66: 3142–3150.

49. Duffy B K, De´fago G. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol. 1999; 65: 2429–2438.

50. Duffy B K, De´fago G.Potential role of pathogen signaling in multitrophic plant-microbe interactions involved in disease protection. Appl. Environ. Microbiol. 2004; 70:1 836–1842.

51. Haas D, Keel C. Regulation of antibiotic production in rootcolonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annu. Rev. Phytopathol. 2003; 41:117–153.

52. Notz R, Maurhofer M, Schnider-Keel U, Duffy B, Haas D, De´fago G.. Biotic factors affecting expression of the 2,4-diacetylphloroglucinol biosynthesis gene *phlA* in *Pseudomonas fluorescens* biocontrol strain CHA0 in the rhizosphere. Phytopathology 2001; 91:873–881.

53. Pettersson M, Bååth E. Effects of the properties of the bacterial community on pH adaptation during recolonization of a humus soil. Soil Biol. Biochem. 2004; 36: 1383–1388.

54. Huang CJ, Wang TK, Chung SC, Chen CY. Identification of an Antifungal Chitinase from a Potential Biocontrol Agent, *Bacillus cereus* 28-9. J. Biochem. Mol. Biol. 2005; 38(1): 82-88.

55. Nelson EB, Chao WL, Norton JM, Nash GT, Harman GE. Attachment of *Entorobactor cloacae* to hyphae of *Pythium ultimum*: possible role in biological control of *Pythium* pre-emergence damping-off. Phytopathology. 1986; 75: 327-335. In Whipps JM. Microbial interaction and biocontrol in the rhizosphere. J. Exp. Bot. 2001; 52: 487-511.

Copyright by Mahidol University

56. Hase S, van Pelt JA, van Loon LC, Pieterse CMJ. Colonization of *Arabidopsis* roots by *Pseudomonas fluorescens* primes the plant to produce higher levels of ethylene upon pathogen infection. Physiol. Mol. Plant P. 2003; 62: 219–226.

57. Nowak J, Shulaev V. Priming for transplant stress resistance in in vitro propagation. In Vitro Cell. Dev. Biol. Plant. 2003; 39: 107–124.

58. Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V, Smaiyappan R. Induction of systemic resistance by plant growth-promoting rhizobacteria in crop plants against pests and diseases. Crop Prot. 2001 20:1–11.

59. Benhamou N, Kloepper JW, Tuzun S. Induction of resistance against Fusarium wilt of tomato by combination of chitosan with an endophytic bacterial strain: ultrastructure and cytochemistry of the host response. Planta. 1998; 204: 153–168.

60. M'Piga P, Belanger RR, Paulitz TC,. Benhamou N. Increased resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63-28. Physiol. Mol. Plant Pathol. 1997; 50: 301–320.

61. Jeun YC, Park KS, Kim CH, Fowler WD, Kloepper JW. Cytological observations of cucumber plants during induced resistance elicited by rhizobacteria. Biol. Control. 2004; 29: 34–42.

62. Park KS, Kloepper JW. Activation of PR-1a promoter by rhizobacteria which induce systemic resistance in tobacco against *Pseudomonas syringae* pv. *tabaci*. Biol. Control. 2000; 18: 2–9.

63. Muzzarelli RAA. Chitosan-based dietary foods. Carbohyd. Polym. 1996; 29: 309–316.

64. Patil RS, Ghormade V, Deshpande MV. Chitinolytic enzymes: an exploration. Enzyme Microbial Technol. 2000; 26: 473–83.

65. Tsigo I, Bouriotis V. Purification and characterization of chitin deacetylase from *Colletotrichum lindemuthianum*. J. Biol. Chem. 1995; 270: 26280–91.

66. Expert JM, Digat B. Biocontrol of *Sclerotinia* wilt of sunflower by *Pseudomonas fluorescens* and *Pseudomonas putida* strains. Can. J. Microbiol. 1995; 41: 685–691.

Copyright by Mahidol University

67. Gooday GW. The ecology of chitin degradation. Adv. Microb. Ecol. 1990; 11: 387–430.

68. Poulicek M, Gaill F, Goffinet G. Chitin biodegradation in marine environments. In: Stankiewicz BA, van Bergen PF (eds.) Nitrogen-containing macromolecules in the bio- and geosphere. American Chemical Society, Washington, DC. 1998: 163–210.

69. Gu BG. Production and uses of biopesticides in China. In: Tang WH, Cook RJ, Rovira A. (eds.), Advances in Biological Control of Plant Diseases, China Agricultural University Press, Beijing1996; 332–334.

70. Leffler M. Tresure from trash is there profit crab waste?. Available from http://www.mdsg.umd.edu/MarineNotes/Mar-Apr97/index.html [Access on 2006 Dec 10]

71. Yong-Woo Cho, Jang J, Park CR, Ko SW. Preparation and Solubility in Acid and Water of Partially Deacetylated Chitins. Biomacromolecules 2000; 1: 609-614.

72. Einbu A, Naess SN, Elgsaeter A, Varum KM. Solution Properties of Chitin in Alkali. Biomacromolecules. 2004; 5:2048-2054.

73. Weiner ML. An overview of regulatory status and of the safety of chitin and chitosan as food and pharmaceutical ingredient. In: Brine CJ, Sandford PA, Zikakis JP, editors. Advances in chitin and chitosan. London: Elsevier Applied Science; 1992: 663-72.

74 อัธยา กังสุวรรณ. ไคติน : ผลิตภัณฑ์ธรรมชาติสำหรับศตวรรษที่ 21. วารสารการประมง 2541; 1: 57-67.

75. Schomburg D, Salzman M. Enzyme Handbook vol.4. Springer Verlag, New York. 1991.

76. Flach J, Piet PE, Jolles P. What's new in chitinase research?. Experientia. 1992; 48: 701-706.

77. Jeuniaux C. Chitinase. Methods Enzymol. 1996; 8: 644-650.

Copyright by Mahidol University

78. Place RA. The biochemical basis and ecological significance of chitin digestion. 1996; 2:39-54. In Muzzarelli RAA, ed. Chitin Enzymology, Proceeding of the 2nd International Symposium on Chitin Enzymology. Senigallia, Italy. 1996; May 8-11.

79. Manson FDC, Fletcher TC, Gooday GW. Location of chitinolytic enzyme in blood pf turbot, *Scophthalmus maximus*, and their possible roles in defence. J. Fish Biol. 1992; 40: 919-927.

80. Schomburg D, Mauch M, Vogeli U, Boller. Plant chitinase are potent inhibitors of fungal growth. Nature. 1986; 324: 365-367.

81. Verburg JG, Huynh QK. Purification and characterization of an anitifubgal. Plant Physiol.1991; 95:450-455.

82. Mauch F, Hadwiger LA, Boller T. Antifungal hydrolyses in pea tissue: II purification and characterization of two chitinase and β-1,3-glucanase differentially regulated during development and in response to fungal infection. Plant Physiol. 1988; 87: 325-333.

83. Folman LB, De Klein MJEM, Postmab BJ, Veen JA. Production of antifungal compounds by Lysobacter enzymogenes isolate 3.1T8 under different conditions in relation to its efficacy as a biocontrol agent of *Pythium aphanidermatum* in cucumber. Biol. Control. 2004; 31: 145–154.

84. Pegg GF. Chitinase from *Verticillium albo-atrum*. In Wood WA, Kellogg ST. eds. Methods Enzymol. 1988; 161: 474-479. Biomas, Part B: Lignin, Pectin and Chitin. Academic Press, Inc., San Diego.

85. Match F, Staehelin LA. Functional implications of the subcellular localization of ethlence-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell. 1989; 87: 325-333.

86. Tsujibo H, Yashida Y, Imada C, Okami Y, Miyamoto K, Inamori Y. Isolation and characterization of a chitin degrading marine bacterium belonging to the genus *Altoromonas*. Nippon Suisan Gakk. 1991; 57: 2127-2131.

87. Humphreys AM, Gooday GW. Properties of chitinase activities from *Mucor mucedo*: Evidence for membrane-bound zymogenic Form. J.Gen. Microbiol. 1984; 130: 1359-1366.

Copyright by Mahidol University

88. Ulhoa CJ, Peberdy JF. Purification and characterization of an extracellular chitinase produced by *Trichoderma harianum*. Enzyme Microbial Technol. 1992; 14: 236-240.

89. Rodiguez J, Copa-Patino J, Perez-Leblic MI. Purification and properties of a chitinase from *Penicillum oxalicum* autolysates. Letters in Appl. Microbiol. 1995; 20: 26-49.

90. มยุรา ศุภลักษณากร. การแยกและคัดเลือกเชื้อราที่ผลิตไคติเนส. วิทยานิพนธ์. วิทยาศาสตรมหาบณฑิต ภาควิชาชีววิทยา คณะวิทยาศาสตร์. มหาวิทยาลัยเชียงใหม่. 2539.

91. Vyas P, Deshpande M. Chitinase production by *Myrothecium verrucaria* its significanc for fungal mycelia degradation. J. Gen. Appl. Microbiol. 1989; 35: 343-350.

92. Gupta R, Saxena RK, Chaturvedi P, Virdi JS. Chitinase production by *Streptomyces viridificans*: its potential in fungal cell wall lysis. J. Appl. Bacteriol. 1995; 78: 378-383.

93. Watanabe T, Yamada T, Oyanagi W, Suzuki K, Tanaka H. Pyrification and some properties of chitinase B1 from *Bacillus circulans* WL-12. Biosci. Biotechnol. Biochem. 1992; 56(4):682-683.

94. Takahashi M, Tsukiyama T, Suzuki T. Purification and some properties of chitinase produced by *Vibrio* sp. J. Ferment. Bioeng. 1993; 75: 457-459.

95. Osawa R, Koga T. Aninvestigation of aquatic bacteria capable of utirizing chitin as the sole source of nutrients. Letter in J. Appl. Microbiol. 1995; 21: 288-291.

96. Rojas-Avelizapa LI, Cruz-Camarillo R, Guerrero MI, Rodriguez-Vazquez R, Ibarra JE. Selection and characterization of a proteo-chitinolytic strain of Bacillus thuringiesis, able to grow in shrimp waste media. World J. Microbiol. Biotechnol. 1999; 15: 261-268.

97. Lorito M, Woo SL, Fernandez IG, Harman GE, Pintor-Toro JA, Filippone E et al. Gene from mycoparasitic fungi as a improving plant resistance to funga' phathodans. Proc. Natl. Acad. Sci. USA. 1998; 95:7860-7865.

Copyright by Mahidol University

98. รัฐ พิชยางกูร. 2542. เอนไซม์ไคติเนส: การคัดเลือกและการทำพันธุวิศวกรรมและวิศวกรรมโปรตีน, น. 68. ใน การสัมมนาวิชาการเรื่อง ควาทร่วมมือของภาครัฐ และเอกชนในการพัฒนาและเอกชนในการพัฒนาการผลิต และการใช้สารไคติน ไคโตซานแบบครบวงจร, 2-3 เมษายน 2542. โรงแรมไอเฟลม ระนอง.

99. Koga D, Hirata T, Sueshiges N, Tanaka S, Ide A. Induction patterns of chitinase in yam callus by inoculation with autoclaved Fusarium oxysporum, ethylene, and chitin and chitosan oligosaccharides. Biosci. Biotechnol. Biochem. 1992; 56:280-285.

100. Carroad PA, Tom RA. Bioconversion of shellfish chitin wastes: process conception and selection and selection of microorganism. J. Food Sci. 1978; 43:1158-1161.

101. Wilke CR, Yang RD, Stocker U. Preliminary cost analyses for enzymatic hydrolysis of newsprint. Biotech. Bioen. 1976; 6:155.

102. Revah-Moiseev S, Carroad PA. Conversation of the enzymatic of shellfish waste chitin to single-cell protein. Biotechnol. Bioeng. 1981; 23:1067-1078.

103. Dickman MB. *Colletrotrichum gloeosporioides*. [Online] Available from http://www.extento.hawaii.edu/Kbase/crop/type/c_gloeo.htm [Access 2007 Mar 18]

104. Duff J, Daly A. Orchid Diseases in the Northern Territory. Agnote. 2002; 568.

105. Vithoonjit C. Investigation on Orchid Dry Rot: Morphology of *S. rolfsii* Sacc., Disease Causing Agent and Attempt on Antagonistic Fungi. [Online] Available from: http://kucon.lib.ku.ac.th/FullText/KC4301082.pdf [Accessed 2007 Mar 18]

106. Ferreira SA, Boley RA. *Sclerotium rolfsii*. [Online] Available from http://www.extento.hawaii.edu/Kbase/crop/type/s_rolfs.htm [Access 2007 Mar 18]

107. Mundkur BB. Fungi and plant disease. Bombay: Macmillan; 1967.

108. ไพโรจน์ จ๋วงพานิช. หลักวิชาโรคพืช. พิมพ์ครั้งที่ 2. กรุงเทพฯ: สารมวลชน; 2525.

Copyright by Mahidol University

109. Prell HH, Day PR. Plant-fungal pathogen interaction: a classical and molecular view. Berlin: Springer; 2001.

110. Linthorst HJM. Pathogenesis-related proteins in plants. Crit. Rev. Plant Sci. 1991; 10:123-50.

111. Ferreira RB, Monteiro S, Freitas R, Santos C, Chen Z, Batista LM, et al. The role of plant defence proteins in fungal pathogenesis. Mol. Plant Pathol. 2007; 8(5):677–700.

112. Mauch F, Mauch-MaNi B, Boller T. Antifungal hydrolase in pea tissue II inhibition of fungal growth by combinations of chitinase and β-(1,3)-glucanase. Plant Physiol 1988; 88:936-42.

113. Keen NT, Yoshikawa M. β-(1,3)-glucanase from soybean leaves elicitor-active carbohydrates from fungal cell walls. Plant Physiol 1983; 71: 460-5.

114. Mauch F, Hadwiger LA, Boller T. Ethylene: symptom, not signal for the induction of chitinase and β-(1,3)-glucanase in pea pod by pathogens and elicitors. Plant Physiol 1984; 76:607-11.

115. Yuan WM, Crawford DL. Characterization of *Streptomyces lydicus* WYCE108 as a potential biocontrol agent against fungal root and seed rots. Appl. Environ. Microbiol. 1995; 61:311-3128.

116. Altschul F.Stephen. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic. Acids. Res. 1997; 25: 3389–3402

117. Imoto T, Yagishita K. A simple activity measurement of lysozyme. Agr. Biol. Chem. Tokyo. 1971; 35: 1154–1156.

118. Prapagdee B, Kotchadat K, Kumsopa A, Visarathanonth N. The role of chitosan in protection of soybean from sudden death syndrome caused by *Fusarium solani* f. sp. *glycines*. Bioresource Technol. 2007; 98: 1353-1358.

119. Wang SL, Shih IL, Liang TW, Wang CH. Purification and Characterization of Two Antifungal Chitinases Extracellularly Produced by *Bacillus amyloliquefaciens* V656 in a Shrimp and Crab Shell Powder Medium. J. Agr. Food Chem. 2002; 50: 2241-2248

120. Reyes-ramírez A, Escudero-Abarca BI, Aguilar-Uscanga G, Hayward- Jones PM, Barbozacorona JE. Antifungal Activity of *Bacillus thuringiensis* Chitinase

Copyright by Mahidol University

and Its Potential for the Biocontrol of Phytopathogenic Fungi in Soybean seeds. J. food Sci. 2004; 69: 311-314

121. Huang CJ, Chen CY. Gene cloning and biochemical characterization of chitinase CH from *Bacillus cereus* 28-9. Annu. Rev. Microbiol. 2004; 54: 289–297.
122. Aktuganov1 GE, Melent'ev AI, Yu Kuz'mina L, Galimzyanova NF, Shirokov AV. The Chitinolytic activity of *Bacillus* cohn aacteria antagonistic to phytopathogenic Fungi. Microbiology. 2003;72(3): 313–317.
123. DeBoer W, Gunnewiek Klein PJA, Lafeber P, Janse JD, Bendien ESpit , Woldendorp JW. Anti-fungal properties of chitinolytic dune soil bacteria Soil. Soil Biol. Biochem. 1998; 30(2): 193-203.
124. Woo S, Fogliano V, Scala F, Lorito M. Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. Antonie van Leeuwenhoek. 2002; 81: 353–356.
125. Besson F, Peypoux F, Michel G, Delcambe L. Identification of antibiotics of iturin group in various strains of *Bacillus subtilis*. J Antibiot. 1978; 31: 284-288.
126. Cho SJ, Lee SK, Cha BJ, Kim YH, Shin KS. Detection and characterization of the *Gloeosporium gloeosporioides* growth inhibitory compound iturin A from *Bacillus subtilis* strain KS03. FEMS Microbiol. Lett. 2003; 223: 47-51.
127. Errington, J. Regulation of endospore formation in *Bacillus subtilis*. Nat. Rev. Microbiol. 2003; 1:117–126.
128. Strikingly, et al, (2001) (128) documented that sporulation is tightly intertwined
129. Wösten HAB, van Wetter MA, Lugones LG, van der Mei HC, Busscher HJ, Wessels JG. How a fungus escapes the water to grow into the air. Curr. Biol. 1999; 9: 85–88.
130. Kodani S, Hudson ME, Durrant MC, Buttner MJ, Nodwell JR, Willey JM. The SapB morphogen is a lantibiotic-like peptide derived from the product of the developmental gene *ramS* in *Streptomyces coelicolor*. Proc. Natl. Acad. Sci. USA. 2004; 101**:** 11448–11453.
131. Jutfelt F, Sundh H, Glette J, Mellander L, Thrandur Bȷrnsson B, Sundell K. The involvement of *Aeromonas salmonicida* virulence factors in bacterial

Copyright by Mahidol University

translocation across the rainbow trout, *Oncorhynchus mykiss* (Walbaum), intestine. J. Fish Dis. 2008; 31: 141–151.

132. Masada CL, LaPatra SE, Morton AW, Strom MS. An *Aeromonas salmonicida* type IV pilin is required for virulence in rainbow trout *Oncorhynchus mykiss.* Dis Aquat Organ. 2002; 51: 13–25.
133. Chang WT, Chen YC, Jao CL. Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. Bioresource Technology. 2007; 98:1224–1230.
134. Stein T. *Bacillus subtillis* antibiotics: structures, syntheses and specific functions. Mol. Microbiol. 2005; 56(4):845-857
135. Cazorla FM, Romero D, Pe´rez-Garcı A,. Lugtenberg BJJ, DeVicente A, Bloemberg G. Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. J. Appl. Microbiol. 2007; 103:1950–1959.
136. Oleinnikova GK, Dmitrenok AS, Voinov VG, Chaikina EL, Shevchenko LS, Kuznetsova TA. Bacillomycin from the marine isolate of *Bacillus subtillis* KMM 1922. Chem. Nat. Compd. 2005; 42:461-464
137. Chitarra GS, Breeuwer P, Nout MJR, Van Aelst AC, Rombouts FM, Abee T. AN antifungal compound produced by *Bacillus subtilis* YM 10-20 inhibits germination of *Penicillium roqueforti* conidiospores. J. Appl. Microbiol. 2003; 94:159-166.
138. Nautiyal, C.S. Rhizosphere competence of *Pseudomonas* sp. NBRI9926 and *Rhizobium* sp. NBRI9513 involved in the suppression of chickpea (*Cicer arietinum* L.) pathogenic fungi. FEMS Microbiol. Ecol. 23: 145-158, 1997.
139. Bottone EJ, Peluso RW. Production by Bacillus pumilus (MSH) of an antifungal compound that is active against Mucoraceae and *Aspergillus* species: preliminary report. J. Medical Microbiol. 2003; 52: 69–74.
140. Wang SL, Shih IL, Wang CH, Tseng KC, Chang WT, Twu YK, et al. Production of antifungal compounds from chitin by *Bacillus subtilis*. Enzyme Microb. Tech. 2002; 31(3): 321-328.

Copyright by Mahidol University

141. Department of plant pathology of Kasetsart University. [Online] Available from http://ppath.agr.ku.ac.th/DATA/CLASSES/015281/LAB/THAI/Chapt11.pdf [Access on 2008 May 5]

142. Global diversity information facility. [Online] Available from http://data.gbif.org/species/14434250/ [Access on 2008 May 5]

143. Lane DJ. 16S/23S rRNA sequencing. In Stackebrant E, Goodfellow M, editors. Nucleic acid technique in bacterial systematic. Chichester: John Wiley&sons: 1991.

144. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

145. Hsu SC, Lockwood JL. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl. Microbiol. 1975; 29(3): 422–426.

146. Trudel J, Asselin A. Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal. Biochem. 1989. 178: 362.366. in Botha AM, Nagel1 MAC, Van der Westhuizen AJ, Botha FC. Chitinase isoenzymes in near-isogenic wheat lines challenged with Russian wheat aphid, exogenous ethylene, and mechanical wounding Bot. Bull. Acad. Sin. 1998; 39: 99-106.

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APPENDIX

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APPENDIX A
CULTURAL MEDIA

1. Tryptic Soy Broth (TSB)

This medium was prepared by mixing the following ingredients together:

Pancreatic digest of casein	200 g
Enzymatic digest of soybean meal	5.0 g
Dextrose	2.5 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Distilled water	1 L

The ingredients were boiled for 1 min and then sterile by autoclaved at 121 °C under pressure 15 psi for 15 min.

2. R2A Agar (R2A)

This medium was prepared by mixing the following ingredients together:

Yeast extract	0.5 g
Protest prptone No. 3	0.5 g
Casamino acids	0.5 g
Dextrose	2.5 g
Soluble starch	0.5 g
Sodium pyruvate	0.3 g
Dipotassium phosphate	0.3 g
Magenesium sulfate	0.05 g
Agar	15 g
Distilled water	1 L

The ingredients were boiled for 1 min and then sterile by autoclaved at 121 °C under pressure 15 psi for 15 min.

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3. Tryptic Soy Agar (TSA)

This medium was prepared by mixing the following ingredients togerther:

pancreatic digest of casein	200 g
Enzymatic digest of soybean meal	5.0 g
Sodium chloride	5.0 g
Agar	20 g
Distilled water	1 L

The ingredients were boiled for 1 min and then sterile by autoclaved at 121 °C under pressure 15 psi for 15 min.

4. Colloidal Chitin Agar (Modify from Singh PP, *et al*., 1999)

This medium was prepared by mixing the following ingredients togerther:

Colloidal chitin	10 g
$MgSO_4*7H_2O$	0.5 g
K_2HPO_4	0.7 g
KH_2PO_4	0.3 g
$FeSO_4*H_2O$	0.01 g
$ZnSO_4$	0.001 g
$MnCl_2$	0.001 g
$(NH_4)_2SO_4$	1 g
Agar	20 g
Ketoconazole	100 mg
Distill water	1 L

The medium was autoclaved at 121 °C under pressure 15 psi for 15 min

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5. Potato Dextrose Agar (PDA)

This medium was prepared by mixing the following ingredients togerther:

potato (scrabbed and diced)	200 g
dextrose	15 g
agar	20 g
Distill water	1 L

New potatoes should be avoided. Potatoes were boiled for 1 hr. Then the mixture was passed through a fine sieve, added with agar and boiled until dissolved. Finally, the mixture was added with dextrose and stirred. The medium was autoclaved at 121 °C under pressure 15 psi for 15 min.

6. Potato Dextrose Broth (PDB)

This medium was prepared by mixing the following ingredients togerther:

potato (scrabbed and diced)	200 g
dextrose	15 g
Distill water	1 L

New potatoes should be avoided. Potatoes were boiled for 1 hr. Then the mixture was passed through a fine sieve, added with dextrose and stirred. The medium was autoclaved at 121 °C under pressure 15 psi for 15 min.

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APPENDIX B
CHEMICAL AGENTS

1. Colloidal Chitin preparation (Modify from S. C. Hsu and J. L. Lockwood, 1975) (145)

The chitin powder 40 g was dissolved in concentrate HCl 600 ml
by stirring for 30 to 50 min.

↓

The chitin was precipitated as a colloidal suspension by adding
it slowly to 2 liters of water at 5 to 10 C.

↓

The suspension was collected by filtration with suction on
a coarse filter paper

↓

Then washed by suspending it in about 5 liters of distill water refiltering

↓

The washing was repeated at least three times or until
the pH of the suspension was about 3.5

↓

For use, sufficient water was added to resuspend the chitin

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2. Glycol chitin

Glycol chitin was obtained by acetylation of glycol chitosan using a modification of the method of Molano et al. (1979). Five grams of glycol chitosan was dissolved in 100 mL of 10% acetic acid by grinding in a mortar, and the viscous solution was allowed to stand overnight at 22°C. Methanol was added, and the solution was vacuum filtrated through a Whatman No. 4 filter paper. The filtrate was transferred into a beaker and 7.5 mL of acetic anhydride was added. The gel was covered with methanol and homogenized. The suspension was centrifuged at 27,000 g for 15 min at 4°C. The gelatinous pellet was resuspended in 1 volume of methanol, homogenized, and centrifuged as in the preceding step. The pellet was resuspended in 500 mL distilled water containing 0.02% (m/v) sodium azide (146).

3. Sodium acetate buffer

The 4.10 g of sodium acetate was dissolved in 1 L of distilled water and the pH was adjusted to 5.0 or 5.6 with HCl.

4. Potassium ferricyanide solution

The 0.5 g of potassium ferricyanide was dissolved in 1 L of 0.5 M sodium carbonate and stored in a brown bottle.

5. Bradford's reagent

The 10 mg of Coomassie Blue G was dissolved in 100 ml of mixture of 85 % phosphoric acid, 95 % ethanol and water at 10:50:40 by volume.

6. Lactophenol cotton blue dye

The 20 g of phenol crystal was dissolved in 20 ml of lactic acid, 40 ml of glycerene and 20 ml of distilled water, then 0.1 g of cotton blue dye was added. The solution was stirred and filtrated before use.

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APPENDIX C
STANDARD CURVES

1. Standard curve of N-acetyl-D-glucosamine (GlcNAc)

The standard curve of GlcNAc was constructed using standard solution of GlcNAc at concentration of 0.005, 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1 mg/ml. The 2 ml of potassium ferricyanide solution was added into 1.5 ml of standard GlcNAc at various concentrations. The mixture was mixed and incubated in boiling water for 15 min. The color development was measured for the absorbance at wavelength of 420 nm (A_{420}) using distilled water as a blank (see table). A regression line was drawn between GlcNAc concentration against their absorbance value at 420 nm (see graph).

Table B-1 The correlation between standard GlcNAc concentration (mg/ml) and their absorbance value at 420 nm

GlcNAc concentration (mg/ml)	Absorbance value at 420 nm
0	0
0.1	0.131
0.2	0.209
0.4	0.325
0.5	0.383
0.6	0.516
0.08	0.682
1	0.801
1.5	0.99

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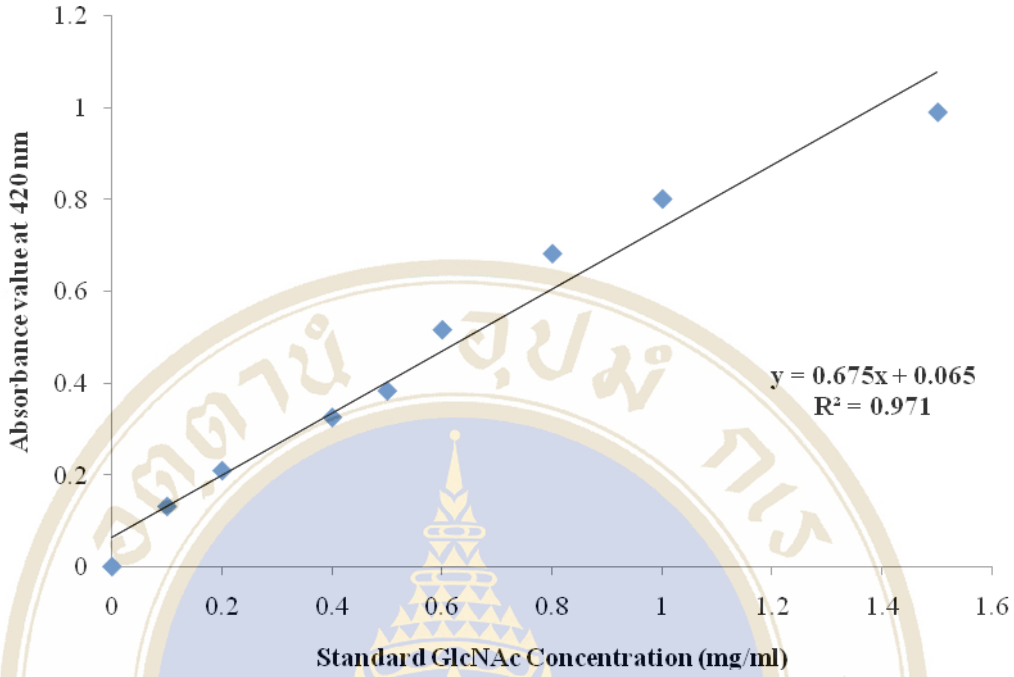

Figure B-1 The standard curve of GlcNAc (mg/ml) and their absorbance value at 420 nm

2. Standard curve of Bovine Serum Albumin (BSA) for chitinase activity assay

The standard curve of BSA for chitinase activity assay was constructed using standard solution of BSA at concentration of 0.02, 0.04, 0.06, 0.08 and 0.1 mg/ml. The 0.2 ml of each BSA concentration was added into 0.8 ml of Bradford's reagent. The mixture was incubated at room temperature for 5 min. The color development was measured for the absorbance at wavelength of 595 nm (A_{595}) using Bradford's reagent as a blank (see table). A regression line was drawn between BSA concentration against their absorbance value at 595 nm (see graph).

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Table B-2 The correlation between standard BSA concentration (mg/ml) and their absorbance value at 595 nm for chitinase activity assay

BSA concentration (µg/ml)	Absorbance value at 595 nm
0	0
2	0.101
4	0.201
6	0.321
8	0.376
10	0.465
12	0.531
14	0.641
16	0.712

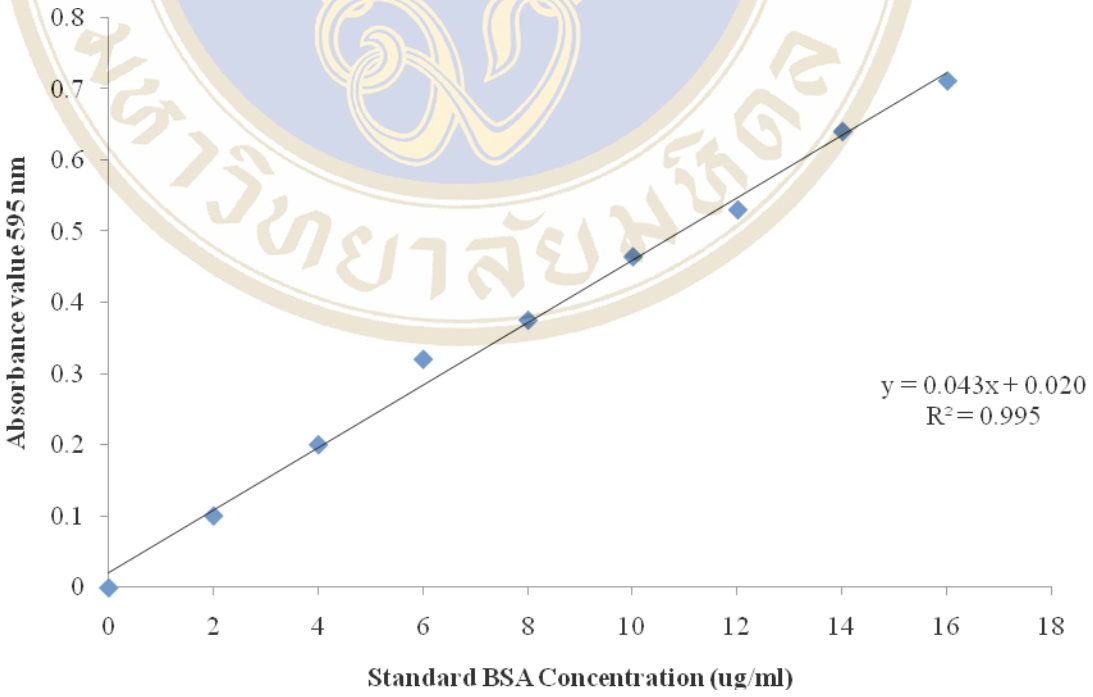

Figure B-2 The standard curve of BSA (µg/ml) and their absorbance value at 595 nm for chitinase activity assay

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APPENDIX D
RAW DATA

1. Number of chitinolytic and non-chitinolytic bacteria

Table D-1 Number of chitinolyitc and non-chitinolytic bacteria isolated from FBE and shrimp shells

Samples	chitinolytic activity	non-chitinolytic bacteria
FBE	44	2
Shrimp shell	87	10

Figure D-1 Clear zone of chitinolytic bacteria on CCA plates

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2. Level of antagonistic bacteria

Table D-2 Chitinolytic bacteria of isolated bacteria from FBE

Bacterial Isolated	chitinolytic activity
1. Company 1	
EMACH001	+
EMACH002	+
EMA003	-
EMA004	-
EMA005	-
EMA006	-
EMA007	-
EMA008	-
EMA009	-
EMA010	-
EMA011	-
EMA012	-
EMA013	-
2. Local 1	
EMB001	-
EMB002	-
EMB003	-
EMB004	-
EMB005	-
EMB006	-
EMB007	-
3. Local 2	
EMC001	-
EMC002	-
EMC003	-
EMC004	-

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Table D-2 Chitinolytic bacteria of isolated bacteria from FBE (Continued.)

Bacterial Isolated	chitinolytic activity
4. Company 2	
EMD001	-
EMD002	-
EMD003	-
EMD004	-
EMD005	-
EMD006	-
EMD007	-
5. Local 3	
EME001	-
EME002	-
6. Local 4	
EMF001	-
EMF002	-
EMF003	-
EMF004	-
EMF005	-
EMF006	-
EMF007	-
EMF008	-
7. Company 3	
EMG001	-
EMG002	-
EMG003	-
EMG004	-
EMG005	-

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Table D-3 Chitinolytic bacteria of isolated bacteria from shrimp shell waste

Bacterial Isolated	chitinolytic activity
1. Shrimp farm, Chachoengsao province 1	
SSCHA001	+
SSCHA002	+
SSCHA003	+
SSA004	-
SSA005	-
SSA006	-
SSA007	-
SSA008	-
SSA009	-
SSA010	-
SSA011	-
SSA012	-
SSA013	-
SSA014	-
SSA015	-
SSA016	-
SSA017	-
2. Angsila bridge, Chonburi province 1	
SSB001	-
SSB002	-
SSB003	-
SSB004	-
SSB005	-
SSB006	-
SSB007	-
SSB008	-
SSB009	-

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Table D-3 Chitinolytic bacteria of isolated bacteria from shrimp shells (Continued)

Bacterial Isolated	chitinolytic activity
2. Angsila bridge, Chonburi province 1	
SSB010	-
SSB011	-
SSB012	-
3. Angsila bridge, Chonburi province 2	
SSCHC001	+
SSCHC002	+
SSCHC003	+
SSCHC004	+
SSCHC005	+
SSCHC006	+
SSCHC007	+
SSC008	-
SSC009	-
SSC010	-
SSC011	-
SSC012	-
SSC013	-
SSC014	-
SSC015	-
SSC016	-
SSC017	-
SSC018	-
SSC019	-
SSC020	-
SSC021	-
SSC022	-
SSC023	-

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Table D-3 Chitinolytic bacteria of isolated bacteria from shrimp shells (Continued)

Bacterial Isolated	chitinolytic activity
4. Angsila bridge, Chonburi province 3	
SSD001	-
SSD002	-
SSD003	-
SSD004	-
SSD005	-
SSD006	-
SSD007	-
SSD008	-
SSD009	-
SSD010	-
SSD011	-
SSD012	-
SSD013	-
5. Angsila bridge, Chonburi province 3	
SSE001	-
SSE002	-
SSE003	-
SSE004	-
SSE005	-
SSE006	-
SSE007	-
SSE008	-
SSE009	-
SSE010	-
6. Angsila bridge, Chonburi province 4	
SSF001	-
SSF002	-

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Table D-3 Chitinolytic bacteria of isolated bacteria from shrimp shells (Continued)

Bacterial Isolated	chitinolytic activity
7. Angsila bridge, Chonburi province 5	
SSG001	-
SSG002	-
SSG003	-
SSG004	-
SSG005	-
8. Angsila bridge, Chonburi province 6	
SSH001	-
SSH002	-
SSH003	-
9. Angsila bridge, Chonburi province 7	
SSI001	-
SSI002	-
SSI003	-
SSI004	-
SSI005	-
SSI006	-
SSI007	-
10. Angsila bridge, Chonburi province 8	
SSJ001	-
SSJ002	-
SSJ003	-
SSJ004	-
SSJ005	-

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Table D-4 Chitinolytic bacteria of isolated bacteria from FBE

Bacterial Isolated	Level of Antagonistic	
	C. gloeosporioides	***S. rolfsii***
1. Company 1		
EMACH001	-	-
EMACH002	-	-
EMA003	-	-
EMA004	-	-
EMA005	-	-
EMA006	-	-
EMA007	-	-
EMA008	-	-
EMA009	-	-
EMA010	-	-
EMA011	-	-
EMA012	++	-
EMA013	-	-
2. Local 1		
EMB001	-	-
EMB002	-	-
EMB003	-	-
EMB004	-	-
EMB005	-	-
EMB006	-	-
EMB007	-	-
3. Local 2		
EMC001	-	-
EMC002	-	-
EMC003	++	-
EMC004	+++	+++

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Table D-4 Chitinolytic bacteria of isolated bacteria from FBE (Continued)

Bacterial Isolated	Level of Antagonistic	
	C. gloeosporioides	***S. rolfsii***
4. Company 2		
EMD001	-	-
EMD002	-	-
EMD003	-	-
EMD004	-	-
EMD005	-	-
EMD006	-	-
EMD007	-	-
5. Local 3		
EME001	-	-
EME002	-	-
6. Local 4		
EMF001	-	-
EMF002	-	-
EMF003	-	-
EMF004	-	-
EMF005	-	-
EMF006	-	-
EMF007	-	-
EMF008	-	-
7. Company 3		
EMG001	-	-
EMG002	-	-
EMG003	-	-
EMG004	+++	-
EMG005	-	-

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Table D-5 Chitinolytic bacteria of isolated bacteria from shrimp shells

Bacterial Isolated	**Level of Antagonistic**	
	C. gloeosporioides	***S. rolfsii***
1. Shrimp farm, Chachoengsao province 1		
SSCHA001	-	-
SSCHA002	-	-
SSCHA003	-	-
SSA004	-	-
SSA005	-	-
SSA006	-	-
SSA007	-	-
SSA008	-	-
SSA009	-	-
SSA010	-	-
SSA011	-	-
SSA012	-	-
SSA013	-	-
SSA014	-	-
SSA015	-	-
SSA016	-	-
SSA017	-	-
2. Angsila bridge, Chonburi province 1		
SSB1	-	-
SSB2	-	-
SSB3	-	-
SSB4	-	-
SSB5	-	-
SSB6	-	-
SSB7	-	-
SSB8	-	-
SSB9	-	-

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Table D-5 Chitinolytic bacteria of isolated bacteria from shrimp shells (Continued)

Bacterial Isolated	Level of Antagonistic	
	C. gloeosporioides	***S. rolfsii***
2. Angsila bridge, Chonburi province 1(Count.)		
SSB10	-	-
SSB11	-	-
SSB12	+++	-
3. Angsila bridge, Chonburi province 2		
SSCHC001	-	-
SSCHC002	-	-
SSCHC003	-	-
SSCHC004	+++	+++
SSCHC005	-	-
SSCHC006	-	-
SSCHC007	-	-
SSC008	+	-
SSC009	-	-
SSC010	-	-
SSC011	-	-
SSC012	+	-
SSC013	-	-
SSC014	-	-
SSC015	-	-
SSC016	-	-
SSC017	-	-
SSC018	-	-
SSC019	-	-
SSC020	-	-
SSC021	-	-
SSC022	-	-
SSC023	-	-

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Table D-5 Chitinolytic bacteria of isolated bacteria from shrimp shells (Continued)

Bacterial Isolated	**Level of Antagonistic**	
	C. gloeosporioides	***S. rolfsii***
4. Angsila bridge, Chonburi province 3		
SSD001	-	-
SSD002	-	-
SSD003	+++	-
SSD004	-	-
SSD005	-	-
SSD006	-	-
SSD007	-	-
SSD008	-	-
SSD009	-	-
SSD010	-	-
SSD011	-	-
SSD012	-	-
SSD013	-	-
5. Angsila bridge, Chonburi province 3		
SSE001	-	-
SSE002	-	-
SSE003	-	-
SSE004	+++	+++
SSE005	-	-
SSE006	-	-
SSE007	-	-
SSE008	+++	-
SSE009	-	-
SSE010	-	-

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Table D-5 Chitinolytic bacteria of isolated bacteria from shrimp shells (Continued)

Bacterial Isolated	Level of Antagonistic	
	C. gloeosporioides	***S. rolfsii***
6. Angsila bridge, Chonburi province 4		
SSF001	-	-
SSF002	-	-
7. Angsila bridge, Chonburi province 5		
SSG001	-	-
SSG002	-	-
SSG003	-	-
SSG004	-	-
SSG005	-	-
8. Angsila bridge, Chonburi province 6		
SSH001	-	-
SSH002	-	-
SSH003	-	-
9. Angsila bridge, Chonburi province 7		
SSI001	-	-
SSI002	-	-
SSI003	-	-
SSI004	-	-
SSI005	-	-
SSI006	-	-
SSI007	-	-
10. Angsila bridge, Chonburi province 8		
SSJ001	-	-
SSJ002	-	-
SSJ003	-	-
SSJ004	-	-
SSJ005	-	-

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3. Growth curve determination of selected bacteria

Table D-6 Growth curve of *A. salmonicida* SSCHC4

Peroid (hr)	*A. salmonicida* SSCHC4 Absorbance at 600 nm			Average	S.D.
	Rep. 1	Rep. 2	Rep. 3		
0	0.33	0.32	0.38	0.3433	0.0321
4	3.03	3.13	3.43	3.1967	0.2082
8	4.23	4.38	4.73	4.4467	0.2566
12	5.07	5.27	5.63	5.3233	0.2838
24	7.31	7.54	7.94	7.5967	0.3188
36	6.69	7.21	6.94	6.9467	0.2601
48	6.98	7.28	7.36	7.2067	0.2003
72	6.61	6.77	7.48	6.9533	0.4631

Table D-7 Growth curve of *B. subtilis* EMC4

Peroid (hr)	*B. subtilis* EMC4 Absorbance at 600 nm			Average	S.D.
	Rep. 1	Rep. 2	Rep. 3		
0	0.39	0.28	0.24	0.3633	0.0777
4	2.52	2.31	2.48	3.0267	0.1115
8	4.37	3.87	4.02	4.4933	0.2566
12	4.74	4.4	4.66	5.2133	0.1778
24	6.01	5.59	5.74	7.1633	0.2128
36	4.32	3.91	4.28	6.1567	0.2261
48	3.82	4.21	3.63	6.1533	0.2957
72	1.65	1.68	1.45	5.3000	0.1250

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Table D-8 Growth curve of *B. subtilis* SSE4

Peroid (hr)	*B. subtilis* SSE4			Average	S.D.
	Absorbance at 600 nm				
	Rep. 1	Rep. 2	Rep. 3		
0	0.35	0.27	0.3	0.3500	0.0404
4	1.75	1.72	1.8	2.7533	0.0404
8	3.57	3.39	3.47	4.3233	0.0902
12	4.14	4.22	3.99	4.9233	0.1168
24	6.05	5.91	5.7	6.5133	0.1762
36	3.6	4.12	3.66	5.0567	0.2845
48	3.55	4	3.56	5.1300	0.2570
72	1.6	1.83	2.04	3.6033	0.2201

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Figure D-2 Partial sequence of 16S rDNA of a potent antagonist SSCHC4

```
>emb|AM931169.1|  Aeromonas salmonicida partial 16S ribosomal RNA gene, strain
242-Hgo
Length=1461

 Score = 1092 bits (1210),  Expect = 0.0
 Identities = 644/661 (97%), Gaps = 11/661 (1%)
 Strand=Plus/Plus

Query  23   CTTTTGC-GGCGAGCG-CGGACGGGTGAGTAATGC-TGGGGATCTGCCCAGTCGAGGGGG  79
            ||||||| |||||||| |||||||||||||||||| ||||||||||||||||||||||||
Sbjct  58   CTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGG  117

Query  80   ATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGGAGGGGACCT  139
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  118  ATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGGAGGGGACCT  177

Query  140  TCGGGCCTTTCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGGGGTAATGGCT  199
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  178  TCGGGCCTTTCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGGGGTAATGGCT  237

Query  200  CACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGAC  259
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  238  CACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGAC  297

Query  260  ACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAA-CCTG  318
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  298  ACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTG  357

Query  319  ATGCAGCCATGCCGCGTGTGTGAAGAAGGNCTTCGGGTTGTAAAGCACTTTCAGCGAGGA  378
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  358  ATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGA  417

Query  379  GGAAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCT  438
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  418  GGAAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCT  477

Query  439  AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGG  498
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  478  AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGG  537

Query  499  CGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGG  558
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  538  CGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGG  597

Query  559  AA-TGCATTTAAAACTGTCCAGCTAGNNTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAG  617
            || |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  598  AATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAG  657

Query  618  CGGTGAAATGCGTAGAGATCTGGA-GAATACC---GGGGAAAGC-GCCNCCT-GACAAAG  671
            ||||||||||||||||||||||| ||||||||   || ||| || ||| ||| |||||||
Sbjct  658  CGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAG  717

Query  672  A  672
            |
Sbjct  718  A  718
```

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Figure D-3 Partial sequence of 16S rDNA of a potent antagonist EMC4

```
>gb|EF617316.1|  Bacillus subtilis strain SB2 16S ribosomal RNA gene, partial
sequence
Length=732

 Score = 1216 bits (1348),  Expect = 0.0
 Identities = 713/727 (98%), Gaps = 10/727 (1%)
 Strand=Plus/Plus

Query  4    GCGCGTGCTAT-CATGCAGTCGAGCGG-CAGATGGGAGCTTGCTCCCTGATGTTAGCGGC  61
            ||||||||||| ||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  1    GCGCGTGCTATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGC  60

Query  62   GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCG  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCG  120

Query  122  GGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCT  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCT  180

Query  182  ACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  ACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG  240

Query  242  CGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA  300

Query  302  GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC-  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| 
Sbjct  301  GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA  360

Query  361  ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAG-ACAAG  419
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||| 
Sbjct  361  ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAG  420

Query  420  TGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAA-CAGAAAGCCACGGCTAACTACG  478
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  421  TGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACG  480

Query  479  TGCCA-CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAG  537
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAG  540

Query  538  GGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCA  597
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCA  600

Query  598  TTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA  657
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  TTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAA  660

Query  658  ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAA-GNGACTCTCT-GTCTG-AACNG-CG  713
            |||||||||||||||||||||||||||||||||| | |||||||| ||||| ||| | ||
Sbjct  661  ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACG  720

Query  714  TAGAGGA  720
             |||||
Sbjct  721  CTGAGGA  727
```

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Figure D-4 Partial sequence of 16S rDNA of a potent antagonist SSE4

```
>gb|EU476016.1|  Bacterium MOBOSA51 16S ribosomal RNA gene, partial sequence
Length=1041

 Score = 1227 bits (1360),  Expect = 0.0
 Identities = 713/723 (98%), Gaps = 9/723 (1%)
 Strand=Plus/Plus

Query  3    TGCGCGTGCTATCATGCAAGTCGAGCGG-CAGATGGGAGCTTGCTCCCTGATGTTAGCGG  61
            ||||||| |||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  1    TGCGCGTCCTATCATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGG  60

Query  62   CGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC  120

Query  122  GGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGC  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGC  180

Query  182  TACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG  240

Query  242  GCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCC  300

Query  302  AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC  361
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Query  362  -ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA  420
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Sbjct  361  AACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAA  420

Query  421  GTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAG-AAGCCACGGCTAACTAC  479
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Sbjct  421  GTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTAC  480

Query  480  GTGCCAGCA-CCGCGGTAAT-CGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAA  537
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Query  538  GGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTC  597
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Sbjct  541  GGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTC  600

Query  598  ATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA  657
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Sbjct  601  ATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA  660

Query  658  AATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGG-GACTCTCT-GTCTG-AACTG-C  713
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Sbjct  661  AATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGAC  720

Query  714  GCT  716
            |||
Sbjct  721  GCT  723
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2. Chitinase activity of selected bacteria

Table D-9 Chitinase activity of *A. salmonicida/* SSCHC4

period (hr)	GlcNAc (ug/ml)	Total activity (U)	Total protein (mg/ml)	Specific activity (U/mg protein)
2-hr	1773.663	0.4009	0.077103	5.199553777
4-hr	1817.558	0.410822	0.130724	3.142659123
8-hr	1806.584	0.408341	0.037325	10.94022861
12-hr	1854.87	0.419255	0.017348	24.16717923
24-hr	1782.442	0.402885	0.037675	10.69361707
36-hr	1726.475	0.390234	0.030315	12.87246887
48-hr	1782.442	0.402885	0.043107	9.346047441
72-hr	1806.584	0.408341	0.032593	12.52832632

Table D-10 Chitinase activity of *B. subtilis* SSE4

period (hr)	GlcNAc (ug/ml)	Total activity (U)	Total protein (mg/ml)	Specific activity (U/mg protein)
2-hr	1736.351	0.392467	0.008411	46.65992967
4-hr	1815.364	0.410326	0.026285	15.61061802
8-hr	1757.202	0.39718	0.039778	9.984894913
12-hr	1792.318	0.405117	0.045561	8.891796848
24-hr	1840.604	0.416031	0.168925	2.462810404
36-hr	1769.273	0.399908	0.100409	3.982795213
48-hr	1725.377	0.389986	0.102161	3.817361257
72-hr	1830.727	0.413798	0.1316	3.144353821

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Table D-11 Chitinase activity of *B. subtilis* EMC4

period (hr)	GlcNAc (ug/ml)	Total activity (U)	Total protein (mg/ml)	Specific activity (U/mg protein)
2-hr	1793.416	0.405365	0.011565	35.0497376
4-hr	1765.981	0.399164	0.012442	32.08303034
8-hr	1739.643	0.393211	0.029965	13.12235747
12-hr	1787.929	0.404125	0.066589	6.06896099
24-hr	1868.038	0.422232	0.168224	2.509934122
36-hr	1809.877	0.409086	0.126519	3.233400552
48-hr	1789.026	0.404373	0.11618	3.480574174
72-hr	1818.656	0.41107	0.142465	2.885411079

5. Effects of culture filtrate on fungal radial growth

Table D-12 Statistic analytical of radial growth inhibition by exponential culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 after 3-day of incubation on *C. gloeosporioides*

SUMMARY

Groups	Count	Sum	Average	Variance
EMC4 exponential culture filtrate	3	1.65	0.55	0.0025
SSE4 exponential culture filtrate	3	1.5	0.5	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.00375	1	0.00375	3	0.158302	7.708647
Within Groups	0.005	4	0.00125			
Total	0.00875	5				

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Table D-13 Statistic analytical of radial growth inhibition by stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 after 3-day of incubation on *C. gloeosporioides*

SUMMARY

Groups	Count	Sum	Average	Variance
EMC4 stationary culture filtrate	3	0.6	0.2	1.16
SSE4 stationary culture filtrate	3	1.8	0.6	0.01

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.24	1	0.24	48	0.002278	7.708647
Within Groups	0.02	4	0.005			
Total	0.26	5				

Table D-14 Statistic analytical of radial growth inhibition by exponential culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 after 3-day of incubation on *S. rolfsii*

SUMMARY

Groups	Count	Sum	Average	Variance
EMC4 Exponential culture filtrate	3	7.65	2.55	0.0025
SSE4 Exponential culture filtrate	3	7.5	2.5	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.00375	1	0.00375	3	0.158302	7.708647
Within Groups	0.005	4	0.00125			
Total	0.00875	5				

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Table D-15 Statistic analytical of radial growth inhibition by stationary culture filtrates of *B. subtilis* EMC4 and *B. subtilis* SSE4 after 3-day of incubation on *S. rolfsii*

SUMMARY

Groups	Count	Sum	Average	Variance
EMC4 stationary culture filtrate	3	1.65	0.55	0.0025
SSE4 stationary culture filtrate	3	1.5	0.5	0

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.00375	1	0.00375	3	0.158302	7.708647
Within Groups	0.005	4	0.00125			
Total	0.00875	5				

6. Effects of heat and protinase K on the antifungal activity of extracellular antifungal metabolites

Table D-16 Statistic analytical of effects of heat and proteinase K on the antifungal activity of 20% (v/v) exponential culture filtrates of *B. subtilis* EMC4 to *C. gloeosporioides* at 3-day of incubation

SUMMARY

Groups	Count	Sum	Average	Variance
Untreat	7	24.4	3.485714	6.241429
Protinase K	7	23.1	3.3	5.803333
Heat	7	24.2	3.457143	6.20619

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Table D-16 Statistic analytical of effects of heat and proteinase K on the antifungal activity of 20% (v/v) exponential culture filtrates of *B. subtilis* EMC4 to *C. gloeosporioides* at 3-day of incubation (Continued)

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.14	2	0.07	0.011506	0.988567	3.554557
Within Groups	109.5057	18	6.083651			
Total	109.6457	20				

Table D-17 Statistic analytical of effects of heat and proteinase K on the antifungal activity of 20% (v/v) stationary culture filtrates of *B. subtilis* EMC4 to *C. gloeosporioides* at 3-day of incubation

SUMMARY

Groups	Count	Sum	Average	Variance
Untreat	7	7.5	1.071429	0.242381
Protinase K	7	7.1	1.014286	0.185595
Heat	7	7.3	1.042857	0.219524

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.011429	2	0.005714	0.026475	0.97391	3.554557
Within Groups	3.885	18	0.215833			
Total	3.896429	20				

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Table D-18 Statistic analytical of effects of heat and proteinase K on the antifungal activity of 20% (v/v) exponential culture filtrates of *B. subtilis* SSE4 to *C. gloeosporioides* at 3-day of incubation

SUMMARY

Groups	Count	Sum	Average	Variance
Untreat	7	24.5	3.5	6.7
Protinase K	7	23.7	3.385714	6.528095
Heat	7	24.3	3.471429	6.709048

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.049524	2	0.024762	0.003726	0.996282	3.554557
Within Groups	119.6229	18	6.645714			
Total	119.6724	20				

Table D-19 Statistic analytical of effects of heat and proteinase K on the antifungal activity of 20% (v/v) stationary culture filtrates of *B. subtilis* SSE4 to *C. gloeosporioides* at 3-day of incubation

SUMMARY

Groups	Count	Sum	Average	Variance
Untreat	7	7.5	1.071429	0.242381
Protinase K	7	6.45	0.921429	0.091548
Heat	7	7.2	1.028571	0.199048

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Table D-19 Statistic analytical of effects of heat and proteinase K on the antifungal activity of 20% (v/v) stationary culture filtrates of *B. subtilis* SSE4 to *C. gloeosporioides* at 3-day of incubation (Continued)

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.083571	2	0.041786	0.235202	0.792802	3.554557
Within Groups	3.197857	18	0.177659			
Total	3.281429	20				

Table D-20 Statistic analytical of Effects of heat and proteinase K on the antifungal activity of 20% (v/v) exponential culture filtrates of *B. subtilis* EMC4 to *S. rolfsii* at 3-day of incubation

SUMMARY

Groups	Count	Sum	Average	Variance
Untreat	5	14.5	2.9	5.755
Protinase K	5	13.3	2.66	5.473
Heat	5	14.1	2.82	5.637

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.149333	2	0.074667	0.013282	0.98682	3.885294
Within Groups	67.46	12	5.621667			
Total	67.60933	14				

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Table D-21 Statistic analytical of effects of heat and proteinase K on the antifungal activity of 20% (v/v) stationary culture filtrates of *B. subtilis* EMC4 to *S. rolfsii* at 3-day of incubation

SUMMARY

Groups	Count	Sum	Average	Variance
Untreat	5	5.7	1.14	0.398
Protinase K	5	5.7	1.14	0.368
Heat	5	5.6	1.12	0.367

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.001333	2	0.000667	0.001765	0.998237	3.885294
Within Groups	4.532	12	0.377667			
Total	4.533333	14				

Table D-22 Statistic analytical of effects of heat and proteinase K on the antifungal activity of 20% (v/v) exponential culture filtrates of *B. subtilis* SSE4 to *S. rolfsii* at 3-day of incubation

SUMMARY

Groups	Count	Sum	Average	Variance
Untreat	5	16.4	3.28	7.457
Protinase K	5	15.3	3.06	6.348
Heat	5	15.4	3.08	6.602

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Table D-22 Statistic analytical of effects of heat and proteinase K on the antifungal activity of 20% (v/v) exponential culture filtrates of *B. subtilis* SSE4 to *S. rolfsii* at 3-day of incubation (Continued)

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.148	2	0.074	0.010879	0.98919	3.885294
Within Groups	81.628	12	6.802333			
Total	81.776	14				

Table D-23 Statistic analytical of effects of heat and proteinase K on the antifungal activity of 20% (v/v) stationary culture filtrates of *B. subtilis* SSE4 to *S. rolfsii* at 3-day of incubation

SUMMARY

Groups	Count	Sum	Average	Variance
Untreat	5	5.2	1.04	0.348
Protinase K	5	5.1	1.02	0.342
Heat	5	5.2	1.04	0.348

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.001333	2	0.000667	0.001927	0.998075	3.885294
Within Groups	4.152	12	0.346			
Total	4.153333	14				

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7. Effects of antifungal potential of culture filtrate on fungal radial growth

Table D-24 Antifungal effects of exponential culture filtrate of *B. subtilis* EMC4 on the radial growth of *C. gloeosporioides*

Concentration of culture filtrate (v/v)	$\overline{X}$	SD	$\overline{D}$	$SD_{\overline{D}}$	t	Sig.
10%	1.8000	1.22923	0.3833	0.22509	4.171	0.009
20%	1.6833	1.17757	0.5000	0.27386	4.472	0.007
30%	1.2500	0.83187	0.9333	0.62583	3.653	0.015

Table D-25 Antifungal effects of stationary culture filtrate of *B. subtilis* EMC4 on the radial growth of *C. gloeosporioides*

Concentration of culture filtrate (v/v)	$\overline{X}$	SD	$\overline{D}$	$SD_{\overline{D}}$	t	Sig.
10%	0.4000	0.35777	1.78333	1.09072	4.005	0.010
20%	0.2750	0.24444	1.90833	1.20350	3.884	0.012
30%	0.1500	0.19748	2.03333	1.27070	3.920	0.011

Table D-26 Antifungal effects of exponential culture filtrate of *B. subtilis* SSE4 on the radial growth of *C. gloeosporioides*

Concentration of culture filtrate (v/v)	$\overline{X}$	SD	$\overline{D}$	$SD_{\overline{D}}$	t	Sig.
10%	1.7917	1.25595	0.39167	0.18819	5.098	0.004
20%	1.6917	1.23265	0.49167	0.21775	5.531	0.003
30%	1.6917	1.22532	0.49167	0.21755	5.531	0.003

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Table D-27 Antifungal effects of stationary culture filtrate of *B. subtilis* SSE4 on the radial growth of *C. gloeosporioides*

Concentration of culture filtrate (v/v)	$\overline{X}$	SD	$\overline{D}$	$SD_{\overline{D}}$	t	Sig.
10%	0.7083	0.55445	1.47500	0.89373	4.043	0.010
20%	0.3417	0.31371	1.84167	1.16121	3.885	0.012
30%	0.1417	0.15943	2.04167	1.29438	3.864	0.012

Table D-28 Antifungal effects of exponential culture filtrate of *B. subtilis* EMC4 on the radial growth of *S. rolfsii*

Concentration of culture filtrate (v/v)	$\overline{X}$	SD	$\overline{D}$	$SD_{\overline{D}}$	t	Sig.
10%	1.9375	1.54357	0.6125	0.31192	3.927	0.029
20%	1.4375	1.16931	1.1125	0.47793	4.755	0.018
30%	0.9625	0.86060	1.5875	0.75650	4.197	0.025

Table D-29 Antifungal effects of stationary culture filtrate of *B. subtilis* EMC4 on the radial growth of *S. rolfsii*

Concentration Of culture filtrate (v/v)	$\overline{X}$	SD	$\overline{D}$	$SD_{\overline{D}}$	t	Sig.
10%	0.4000	0.37639	2.1500	1.20899	3.557	0.038
20%	0.3375	0.31983	2.2125	1.25988	3.512	0.039
30%	0.2250	0.22174	2.3250	1.35739	3.426	0.042

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Table D-30 Antifungal effects of exponential culture filtrate of *B. subtilis* SSE4 on the radial growth of *S. rolfsii*

Concentration of culture filtrate (v/v)	$\overline{X}$	SD	$\overline{D}$	$SD_{\overline{D}}$	t	Sig.
10%	1.8625	1.49185	0.6876	0.30653	4.486	0.021
20%	1.6750	1.31814	0.8750	0.35707	4.901	0.016
30%	1.4625	1.2018	1.0875	0.46075	4.721	0.018

Table D-31 Antifungal effects of stationary culture filtrate of *B. subtilis* SSE4 on the radial growth of *S. rolfsii*

Concentration of culture filtrate (v/v)	$\overline{X}$	SD	$\overline{D}$	$SD_{\overline{D}}$	t	Sig.
10%	0.4000	0.39158	2.1500	1.20692	3.563	0.038
20%	0.2750	0.30957	2.2750	1.30224	3.494	0.040
30%	0.1000	0.14142	2.4500	1.45258	3.373	0.043

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