Screening of Antagonistic Bacteria for Controlling Fungal Contamination on Para Rubber Sheet

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ชื่อวิทยานิพนธ์ การคัดเลือกแบคทีเรียที่ยับยั้งเชื้อราที่ปนเปื้อนบนแผ่นยางพารา

ผู้เขียน นางสาวศิรินุช ด้วงสุข

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บทคัดย่อ

วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อคัดเลือกเชื้อแบคทีเรียปฏิปักษ์ในกลุ่มเชื้อบาซิลลัสและแอคติโนมัยสีทเพื่อนำมายับยั้งเชื้อราที่ปนเปื้อนบนแผ่นยางพารา โดยทำการแยกเชื้อบาซิลลัสจำนวน 206 ไอโซเลทและแอคติโนมัยสีท 151 ไอโซเลท จากตัวอย่างดินและตัวอย่างยางพาราแผ่น นำมาทดสอบความสามารถเบื้องต้นในการยับยั้งเชื้อราที่ปนเปื้อนบนแผ่นยางพาราในภาคใต้ของไทยจำนวน 6 ไอโซเลท ซึ่งประกอบด้วย *Aspergillus* spp. 2 ไอโซเลท, *Penicillium* sp. 1 ไอโซเลท, *Fusarium* sp. 1 ไอโซเลท, *Rhizopus* sp. 1 ไอโซเลท และ *Cladosporium* sp. 1 ไอโซเลท ด้วยวิธี dual culture technique พบว่า 85% ของเชื้อแอคติโนมัยสีทสามารถยับยั้งเชื้อราได้อย่างน้อย 1 ไอโซเลท ในขณะที่เชื้อบาซิลลัสเพียง 16% ที่แสดงฤทธิ์ต้านรา ดังนั้นจึงคัดเลือกเฉพาะเชื้อแอคติโนมัยสีทที่สามารถยับยั้งเชื้อราได้ดีที่สุดจำนวน 30 ไอโซเลท มาเพาะเลี้ยงในอาหารเหลว ISP-2 นำน้ำเลี้ยงเชื้อแอคติโนมัยสีทมาทดสอบฤทธิ์ต้านราด้วยวิธี agar dilution พบว่าน้ำเลี้ยงเชื้อแอคติโนมัยสีท AC41 และ AC51 สามารถยับยั้งเชื้อราทุกไอโซเลทได้มากกว่า 80 เปอร์เซ็นต์ น้ำเลี้ยงเชื้ออีกส่วนหนึ่งได้นำไปสกัดด้วย ethyl acetate แล้วนำสารสกัดหยาบมาหาค่า minimum inhibitory concentration (MIC) ด้วยวิธี broth microdilution สารสกัดจากเชื้อปฏิปักษ์แอคติโนมัยสีท 8 ไอโซเลท คือ แอคติโนมัยสีท

AC37, AC41, AC51, AC70, AC72, AC74, AC78 และ AC84 สามารถยับยั้งเชื้อราทดสอบได้ทั้ง 6 ไอโซเลท โดยมีค่า MIC อยู่ในช่วง 8-200 ไมโครกรัมต่อมิลลิลิตร ซึ่งเชื้อแอคติโนมัยสีท AC41 และ AC51 ให้ค่า MIC ดีที่สุดอยู่ในช่วง 16-64 ไมโครกรัมต่อมิลลิลิตร มีค่าใกล้เคียงกับสารต้านราพาราไนโตรฟีนอลที่ให้ค่า MIC ในช่วง 32-128 ไมโครกรัมต่อมิลลิลิตร เมื่อศึกษาสภาวะที่เหมาะสมในการสร้างสารต้านเชื้อราของแอคติโนมัยสีท AC41 และ AC51 โดยศึกษาปัจจัยที่เกี่ยวข้อง 3 ปัจจัย คือ การเขย่า พีเอช และอุณหภูมิ พบว่าสภาวะที่ดีที่สุดในการสร้างสารต้านเชื้อรา คือ เลี้ยงเชื้อแบบไม่เขย่า อาหารเลี้ยงเชื้อมีค่าพีเอชเริ่มต้น 7 และอุณหภูมิ 30 องศาเซลเซียส เชื้อปฏิปักษ์แอคติโนมัยสีททั้ง 8 ไอโซเลทที่สามารถยับยั้งเชื้อราทดสอบได้ทุกตัว ได้นำมาจำแนกด้วยวิธีทางสัณฐานวิทยาและวิธีทางชีวโมเลกุล พบว่าจัดอยู่ในจีนัส *Streptomyces*

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ABSTRACT

The aim of this study was to isolate *Bacillus* spp. and actinomycetes and screen for their antagonistic activity against fungi contaminated on para rubber sheet. Total of 206 *Bacillus* spp. and 151 actinomycetes from soils and para rubber sheets were screened for antagonistic activity by dual culture technique against six fungi (2 *Aspergillus* spp., 1 *Penicillium* sp., 1 *Fusarium* sp., 1 *Rhizopus* sp. and 1 *Cladosporium* sp.) commonly found on contaminated para rubber sheets in southern Thailand. Eighty-five percents of actinomycetes exhibited antifungal activity against at least one fungal isolate, whereas only 16% of *Bacillus* isolates was active. Thus, the top 30 actinomycetes having antifungal activity were selected for fermentation in ISP-2 broth. The culture filtrates were tested for antifungal activity by agar dilution. Actinomycetes isolates AC41 and AC51 showed >80% inhibitory activity against all tested fungi. The culture filtrates were also extracted with ethyl acetate and the crude extracts were tested for their minimal inhibitory concentrations (MICs) by broth microdilution. Extracts from 8 actinomycetes AC37, AC41, AC51, AC70, AC72, AC74, AC78 and AC84 exhibited antifungal activity against all tested fungi with MICs ranging from 8-200 µg/ml. The best MICs were in the range of 16-64 µg/ml by AC41 and AC51 which appeared to be comparable to *p*-nitrophenol, a control antifungal agent (32-128 µg/ml). The effect of agitation, initial pH and temperature on the production of antifungal metabolites by the isolates AC41 and AC51 was investigated. The optimum conditions for AC41 and AC51 were observed at the static condition, pH7 and temperature 30^{o}C. The top 8 antagonistic actinomycetes were identified by morphological characteristics and molecular technique (16S rDNA). All of these actinomycetes are in the genus *Streptomyces*.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	=	Degree Celsius
g	=	Gram
mg	=	Milligram
µ	=	Micro
µg	=	Microgram
µl	=	Microliter
ml	=	Milliliter
DMSO	=	Dimethyl sulfoxide
EtOAc	=	Ethyl acetate
Na_2SO_4	=	Sodium sulfate
RT	=	Room temperature

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Natural rubber is one of the most important economic polymers produced by plants because it is a strategic raw material used in more than 40,000 products, including tires, medical devices, surgical gloves, various engineering and consumer products (Hayashi, 2009) or other industrial uses that require elasticity, flexibility and resilience. Among over 2,500 rubber producing plant species *Hevea brasiliensis* (Para rubber tree) has been the only commercial source of natural rubber latex mainly because of its abundance in the tree, its quality and ease of harvesting (Oh *et al*., 1999). Global natural rubber production has grown by an average of 3.0% per year for the past 50 years, while consumption has increased by an average of 3.2% per year. Thus, demand and supply of natural rubber have almost always been in a tight balance. Various factors, like production capacity, processing costs as well as price differences with synthetic rubber, have influenced the supply of natural rubber. In recent years because of an active demand and escalating rubber prices, natural rubber production has been encouraged to expand and reached 9.62 million tons in 2006. The five largest consumers of natural rubber are China, USA, Japan, India and Malaysia. Natural rubber is produced in Southeast Asia (92%), Africa (6%) and Latin America (2%) (Hayashi, 2009). In Southeast Asia, Thailand is the largest producer and exporter of natural rubber and its products in the world total are more than 3 million tons per year (Rubber Research Institute of Thailand, 2009). The southern part of Thailand is the main source of natural rubber production.

Fungal contamination is a big problem for the production of para rubber sheet because it lowers the grade and price of the sheets. In addition, these fungi could be dangerous to the health of workers and producers. There are many causes of fungal contamination on para rubber sheet such as moisture, nutrients and temperature. High moisture promotes fungal growth. An incomplete washing during

the production of para rubber sheets allows for the retension of some proteins, sugar and other nutrients, that fungi can utilize, on the surface of the sheets. During para rubber sheet storage, the sheets are stacked together. The temperature inside the stacks of sheets is lower than the outside temperature and the moisture is higher and both favor fungal growth.

In the past, *p*-nitrophenol was used to control fungal contamination on para rubber sheets by adding it to the latex before coagulation or by simply soaking the fresh rubber sheets before drying (Fullerton, 1929). However, *p*-nitrophenol is toxic to those who handle the chemical as it enters the human body both by skin absorption and ingestion. Its use is now forbidden in para rubber sheet production. Biocontrol measures have been successfully used against plant pathogenic fungi and protection of post harvest farm products. This might be an alternative method to explore for controlling fungal contamination on para rubber sheet.

1.2 Review of the literature

1.2.1 Natural rubber

Natural rubber is *cis*-1,4 polyisoprene and is present as latex in a large variety of plants in many regions of the world. The most important source of latex is the rubber tree *Hevea brasiliensis*. Latex from the other sources suffer from disadvantages such as low rubber content, high resin content and difficulties in extraction (Mathew, 2001). In *H. brasiliensis*, rubber synthesis takes place on the surface of rubber particles suspended in the latex (the cytoplasm of laticifers). The laticifers are specialized vessels that are located adjacent to the phloem of the rubber tree (Oh *et al*., 1999). To extract latex from the tree, the vessels are opened by a process called tapping. Natural rubber latex is a milky colloid with a specific gravity of 0.96 to 0.98 and a pH in the range of 6.5 to 7.0. The dispersed phase is mainly rubber (rubber content between 30-40 per cent by weight) and the dispersion medium is water. However, in addition to rubber and water, latex contains small quantities of protein, resin including fats, fatty acids, other lipids, sterols and steroid esters, carbohydrates and mineral matter. The composition of fresh latex (latex as obtained from the tree) is given in Table 1 (Mathew, 2001). Any variations are due to factors such as the type of tree, age of the rubber tree, the tapping method, the soil condition and the season (Gazeley *et al*., 1990).

Table 1 Chemical composition of fresh latex

Constituent	Percentage
Rubber	30-40
Proteins	1-1.5
Resins	1.5-3.0
Minerals	0.7-0.9
Carbohydrates	0.8-1.0
Water	55-60

1.2.2 Components of natural rubber latex

Fresh latex can be separated into four main fractions by ultracentrifugation (Figure 1) comprising the top fraction of rubber particles, the orange-colored layer containing Frey-Wyssling particles, the aqueous phase of the latex or C-serum and the bottom fraction containing lutoid particles.

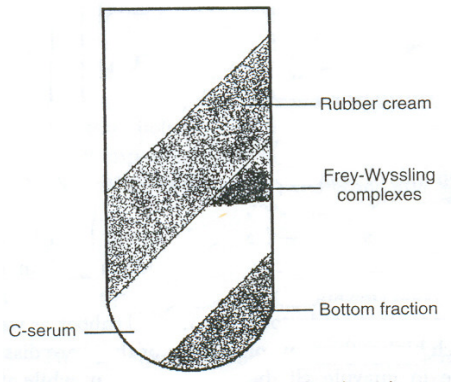


Figure 1 Fractions of centrifuged latex (*Hevea brasiliensis*)
Source : Ohya and Koyama (2001)

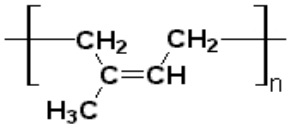
1.2.2.1 Rubber particles

The dominant particulate constituent of fresh latex is the rubber particles (Table 2). A rubber particle is a hydrocarbon compound with 5 carbon atoms and 8 hydrogen atoms $(C_5H_8)_n$ and its chemical name is polyisoprene (Figure 2).

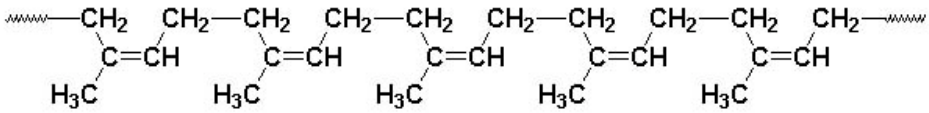
Table 2 Contents of dry rubber

Contents	Percent
Hydrocarbon rubber	86
Water in rubber particle	10
Protein	1
Lipid	3

Source: Kowuttikulrangsi (2003)



(a)



(b)

Figure 2 Structure of polyisoprene

a. Chemical structure of polyisoprene

b. Long chain structure of polyisoprene

Source : Michalovic (2007)

Rubber particle sizes range from 0.02 to 3.0 µm with a spherical shape and are strongly protected in suspension by a film of adsorbed proteins and phospholipids (Figure 3). The adsorbed proteins and the phospholipids on the rubber particles impart a net negative charge, thereby contributing to the colloidal stability of latex (Ohya and Koyama, 2001). The major proteins in fresh latex are α-globulin and hevin and the major phospholipid is α-lecithin.

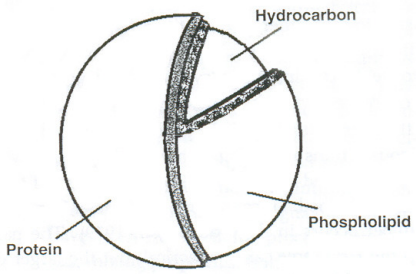


Figure 3 Schematic drawing of the rubber molecule surface

Source : Ohya and Koyama (2001)

1.2.2.2 Nonrubber content

The other particles in latex comprise lutoids and Frey-Wyssling particles and the most non-rubber part is the C-serum.

1.2.2.2.1 Lutoid particles and B-serum

The lutoids are subcellular membrane bound bodies ranging in size from 2 to 5 μm, containing a fluid serum known as B-serum, which is a destabiliser for rubber particles (Mathew, 2001). The B-serum has a pH of about 5.5 which consists of an acid serum enriched with divalent cations (Mg^{2+} and Ca^{2+}) and positively charged proteins (Webster and Baulkwill, 1989).

1.2.2.2.2 Frey-Wyssling particles

Frey-Wyssling particles are spherical, larger in size and are a yellow color due to the presence of carotenoids. These particles are mainly composed of lipid materials. Polyphenol oxidase and β-carotene are the classic markers for the Frey-Wyssling complexes (Mathew, 2001; Premakumari and Panikka, 1992).

1.2.2.2.3 C-serum

C-serum is the most abundant nonrubber part of the latex. It contains most of the soluble compounds such as inositols, carbohydrates, lipids, amino acids and proteins. Quebrachitol (methyl-l-inositol), sucrose and glucose are the major carbohydrates in the latex. Of the total protein content of fresh latex about 20% is adsorbed on the rubber particles, an equal quantity found in the B-serum and the remainder in the latex serum. Lipids in the fresh latex consist of fats, waxes, sterols, sterol esters and phospholipids. Lipids associated with the rubber and non-rubber particles in latex play a key role in the stability and colloidal behaviour of latex. Most of the normal L-amino acids have been found in latex. The nucleotides contained in latex are important as cofactors and are intermediates in the biosynthesis of rubber. Low molecular weight thiols such as glutathione, cysteine and ascorbic acid determine the redox potential of the latex (Mathew, 2001).

1.2.3 Para rubber sheet processing

Latex is sieved and collected in a large bulking tank. Sieving is necessary to remove contaminants such as bark shavings, leaves, sand and small clots of rubber. Stainless steel sieves of 40 and 60 mesh size are preferred. As it is necessary to determine the rubber content of latex for its further processing, dry rubber content (DRC) is measured quickly using a metrolac (hydrometer). Fresh latex after sieving and bulking, is diluted with water to 12.5 to 15% DRC. Dilution improves the color and transparency of the sheet and makes the sheeting operation easier, and allows any denser impurities in latex to sediment on standing for 10-15 min. The diluted latex is then transferred to coagulation tanks and coagulated with dilute formic or acetic acid. After a few hours, or the next day, the thick slab of coagulum is squeezed using a set of rollers to remove water and to produce a sheet of approximately 3 mm thickness. The final set of rollers is grooved to introduce ribbed markings on the sheets. The ribbed markings increase its surface area and facilitate drying (Mathew, 2001). The rubber sheets are dried in the sun for air dried sheets and smoke dried (the sheets are hung in the smokehouses and dried for a week at temperatures up to 60 ^{o}C) for ribbed smoked sheets (Figure 4).

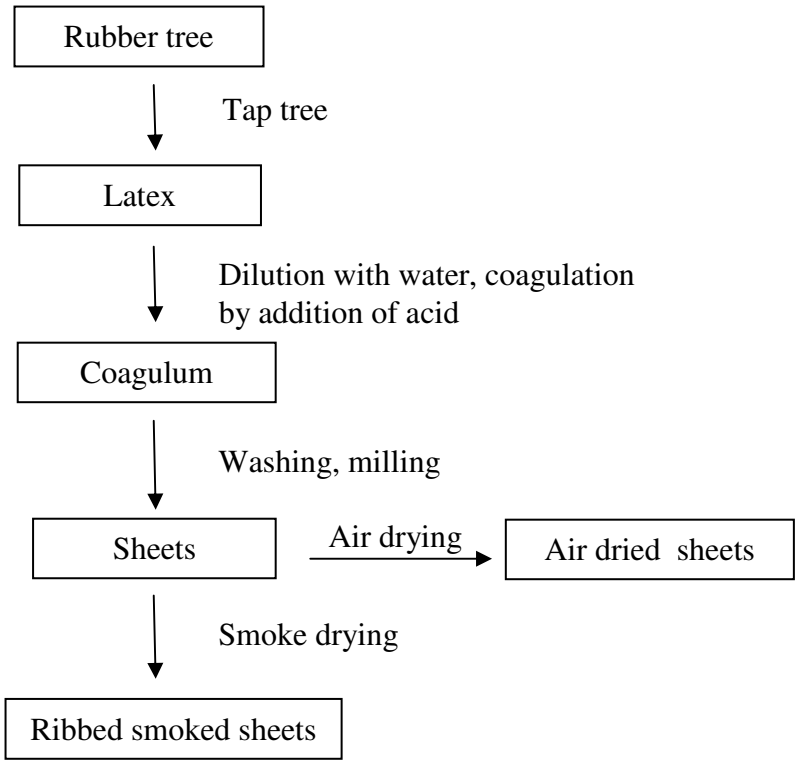


Figure 4 Processing of air dried rubber and ribbed smoked sheets

1.2.4. Types of para rubber sheet

Processing of latex to natural rubber in sheet form is the oldest and most popular first step as it is the simplest and easiest to produce on a small scale. Small holder's rubber latex in most of the countries is processed and marketed as sheet rubber. Two types of sheet rubbers are produced and marketed in the international market, namely the ribbed smoked sheets (RSS) and the air dried sheets (ADS). Among these two types, RSS is the most popular and is available for bulk consumption (http://www.pechsiam. com/rss%20ribbed%20smoked%20sheets.htm).

1.2.4.1 Ribbed smoked sheets (RSS)

Ribbed smoked sheets (RSS) are still widely used in different industries, especially the tyre business**.** The practice of smoke-drying the para rubber sheet is extremely beneficial in this respect, since a certain amount of antiseptic cerotic substances contained in the smoke are adsorbed by the sheets (Fullerton,

1929). The smoke is made by burning *Hevea* (rubber tree) wood and other organic materials such as coconut husks and this preserves the sheets. The specific smell of these sheets is caused by the materials used to produce the smoke (http://www.tis-gdv.de/tis_e/ ware/kautschuk/naturkautschuk/naturkautschuk.htm).

1.2.4.2 Air dried sheets (ADS)

Air dried sheets (ADS) are less common. They have an appearance similar to ribbed smoked sheets, but are more transparent, as they are manufactured in smoke-free rooms (http://www.tis-gdv.de/tis_e/ware/kautschuk/naturkautschuk/ naturkautschuk.htm).

1.2.5 The international grade descriptions for RSS

Ribbed smoked sheets are marketed based on a visual assessment of quality. To establish acceptable grades for commercial purposes, the International Rubber Quality and Packing conference, has stipulated the grade descriptions and the details are given in the Green Book. Only deliberately coagulated rubber latex processed into rubber sheets, properly dried and smoked can be used in making RSS. The following prohibitions also apply to the RSS grade. Wet, bleached, undercured and virgin rubber and rubber that is not completely visually dry at the time of buyer's inspection is not acceptable (except slightly undercured rubber as specified for RSS-No: 5). Skim rubber made of skim latex shall not be used in whole or in part of the batches as required under packing specifications. Prior to grading RSS, the sheets are separated, inspected and any blemishes are removed by cutting with a pair of scissors.

Grade description of RSS

No: IX RSS

The grade must be produced under conditions where all processes are carefully and uniformally controlled. Each bale must be packed free of mould but very slight traces of dry mould on wrappers or bale surfaces adjacent to the wrapper and found at the time of delivery will not be objected to, provided there is no penetration

of mould inside the bale. Oxidized spots or streaks, weak, heated, undercured, oversmoked, opaque and burnt sheets are not permissible. The rubber must be dry, clean, strong, sound and evenly smoked and free from blemishes, specks, rust, blisters, sand, dirty packing and any other foreign matter. Small pinhead bubbles if scattered, will not be objected to.

No: 1 RSS

Each bale must be packed free of mould but very slight traces of dry mould on the wrapper or bale surfaces adjacent to the wrapper found at the time of delivery will not be objected to, provided there is no penetration of mould inside the bale. Oxidized spots or streaks, weak, heated, undercured, oversmoked, opaque and burnt sheets are not permissible. The rubber must be dry, clean, strong, sound and free from blemishes, rust, blisters, sand, dirty packing and any other foreign matter, except for slight specks. Small pinhead bubbles, if scattered, will not be objected to.

No: 2 RSS

Slight rust, and a slight amount of dry mould on the wrappers, bale surfaces and interior sheets, found at the time of delivery will not be objected to, provided these conditions either singly or in combination, do not exist to an objectionable extent on and in more than 5% of the number of bales included in the delivery, lot or tender as determined by the number of bales inspected. Small bubbles and slight specks of bark, if scattered, will not be objected to. Oxidized spots or streaks, weak, heated, undercured, oversmoked, opaque and burnt are not permissible. The rubber must be dry, clean, strong, sound and free from blemishes, blisters, sand, dirty packing and all other foreign matter other than specified above as permissible.

No: 3 RSS

Rust and dry mould on wrappers, bale surfaces and interior sheets, found at the time of delivery will not be objected to, provided these conditions, either singly or in combination, do not exist to an objectionable extent on and in more than 10% of the number of bales included in the delivery, lot or tender as determined by the number of bales inspected. Slight blemishes in color, small bubbles and small specks of bark are permissible. Oxidized spots or streaks, weak, heated, undercured, oversmoked, opaque and burnt sheets are not permissible. The rubber must be dry,

strong and free of blemishes, blisters, sand, dirty packing and all other foreign matter other than specified above as being permissible.

No: 4 RSS

Rust, dry mould on wrappers, bale surfaces and interior sheets, found at time delivery will not be objected to, provided these conditions, either singly or in combination, do not exist to an objectionable extent on or in more than 20% of the number of bales included in the delivery, lot or tender as determined by the number of bales inspected. Medium sized bark particles, bubbles, translucent stains, slightly sticky and slightly over smoked rubber is permissible but should not be evident to a marked degree. Oxidized spots, or streaks, weak, heated, undercured opaque and burnt sheets are not permissible. The rubber must be dry, firm and free of blemishes, blisters, sand, dirty packing and all other foreign matter other than specified above as being permissible.

No: 5 RSS

Rust, dry mould on wrappers, bale surfaces and interior sheets, found at the time of delivery will not be objected to, provided these conditions, either singly or in combination, do not exist to an objectionable extent on or in more than 30% of the number of bales included in the delivery, lot or tender as determined by the number of bales inspected (http://www.pechsiam.com/rss%20ribbed%20smoked%20sheets.htm).

1.2.6. Types and effect of microorganisms found as contaminants of para rubber sheet

Natural rubber is a carbon source for microorganisms. Many microorganisms are able to grow in natural rubber sheet. Two types of microorganism are mainly found on para rubber sheet, fungi and bacteria.

1.2.6.1 Fungi

Fungal spores are present in abundance in the air. Environment factors including temperature, humidity and nutrients help to promote fungal growth on rubber sheet. Rubber sheet with more than 0.8% humidity favors fungal growth. Stacking rubber sheets in a humid environment or on a cement floor also promotes

fungal growth (Joseph *et al*., 2005). Various kinds of fungi have been found on para rubber sheet such as *Aspergillus, Fusarium, Penicillium*, *Paecilomyces* and *Trichoderma* (Linos and Steinbüchel, 2001). Three species of the genus *Aspergillus*; *A. fumigatus, A. flavus* and *A. aculeatus* were isolated from rubber sheet in Western Nigeria (Esuruoso, 1970). In Thailand, Chanduaykit (2008) reported eleven genera of fungi including *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium*, *Rhizopus*, *Mucor*, *Geotrichum*, *Trichoderma*, *Tritirachium*, *Daldinia eschscholzii* and *Schizophyllum commune* present on para rubber sheets collected from 14 provinces in the southern part of Thailand.

The presence of molds is a serious problem in natural rubber production. It affects the grading and the market value of rubber sheet (Table 3). Krisanasap and Krisanasap (1994) reported that 200,000 tons of RSS4 and RSS5 were produced in Thailand in 1994. This indicated that 20% of RSS products had low quality and price.

Table 3 Average prices of RSS in Thailand in 2010

Grade of RSS	Price per Kg (Baht)
RSS1-3	100.29
RSS4	99.25
RSS5	98.25

Source: Rubber Research Institute of Thailand (2010)

Fungi can act as agents of deterioration and degradation of natural rubber. In experiments with natural rubber smoked sheets as a sole carbon source (93% rubber content), inoculated with different *Penicillium* and *Aspergillus* species, an increase in the mold biomass of up to 6% of the initial rubber weight was detected and the final weight loss of natural rubber rose from 15.5% after 19 months to 30.9% after 5 years. In contrast, a noninoculated control showed negligible weight loss (Linos and Steinbüchel, 2001). Kwiatkowska *et al*. (1980) reported that the weight loss of natural rubber was up to 40% of the initial weight after 91 days inoculation

with *Fusariun solani*. More detailed investigations on the biodegradation of cis-1,4-polyisoprene by fungi were reported in 1982. The investigators inoculated spore suspensions of *Penicillium variabile* onto natural rubber smoked sheet in a humidity cabinet. This led to a successive increase of fungal biomass on the rubber sheet surfaces, as shown by determination of cell protein every 14 days, and was accompanied by a weight loss of the rubber strips of up to 13% after 56 days (Williams, 1982). In other reports, several rubber-deteriorating fungi were isolated from mineral agar plates containing powdered natural rubber as sole substrate and deteriorated tire material from soil dispersed in the agar as an inoculum. Liquid cultivation performed with isolated pure cultures for 20 days revealed the formation of a mycelial layer on the rubber surface, as well as losses in weight of up to 20% and an intrinsic viscosity of up to 35%. A relative reduction of the molecular weights of the rubber polymers was also detected in samples inoculated with *F. solani, Cladosporium cladosporioides* and *Paecilomyces lilacinus* (Borel *et al*., 1982: Linos and Steinbüchel, 2001).

Fungi contaminated on para rubber sheets not only lower the grade and the price of the sheets but can be also harmful to the health of workers and producers. Over the past few years it has become increasingly apparent that exposure to certain fungi or their spores can seriously impact the health of humans, pets and other animals. Although fungi are certainly not the only factors that detrimentally affect air quality, in many instances they have been identified as a primary contributor to air quality problems. Most fungi found on para rubber sheets as reported by Chanduaykit (2008) were airborne fungi. They can release many spores into the air. Spores are the primary means for dispersal and survival of fungi, and can remain dormant for months or even years and can withstand extremely adverse conditions, to germinate and flourish again when the environmental variables such as light, oxygen levels, temperature and nutrient availability again become favorable. The ubiquitous presence of fungi in the aerial environment is a potential health threat**.** Various strains of airborne fungi have been implicated as the cause of asthma and hypersensitivity pneumonitis. Chronic nose and sinus inflammation have been linked to airborne fungi (Shin *et al*., 2004).

1.2.6.2 Bacteria

Various kinds of bacteria are found on para rubber sheet such as actinomycetes; *Streptomyces, Micromonospora, Actinoplanes, Gordonia* and *Nocardia* (Linos *et al.*, 2000; Rifaat and Yosery, 2004), Gram-positive bacteria such as *Mycobacterium* sp. and *Bacillus* sp. (Linos *et al*., 2000) and Gram-negative bacteria such as *Xanthomonas* sp. and *Pseudomonas aeruginosa* (Rifaat and Yosery, 2004). Nette *et al*. (1959) reported a weight loss of purified rubber films by three actinomycetes (1 *Proactinomyces* and 2 *Actinomyces* strains) that ranged from between 25.8% and 43.2%, with a *Bacillus* sp. by 20.7%, and a *Mycobacterium* sp. by 17.2%.

1.2.7 The cause of fungal contamination on para rubber sheet

Many causes of fungal contamination on para rubber sheet include parameters such as moisture, incomplete washing during the production process, and insufficient dry smoking. High moisture promotes fungal growth. An incomplete washing during the production process allows some proteins, sugar and other nutrients to remain and support fungal growth. The very small amount of protein substances always present on the sheet rubber are also sufficiently rich in carbon and nitrogen to foster the growth of fungi. A certain amount of nitrogen as ammonia can be assimilated from the air. A short period of dry smoking is one cause of fungal growth on para rubber sheet (Fullerton, 1929). During para rubber sheet storage, the sheets are stacked together. The temperature inside the stacking sheet is lower than the outside temperature and the moisture is higher and both favor fungal growth.

1.2.8 Prevention of fungal growth on para rubber sheet

The fungal growth on para rubber sheet is a serious problem in para rubber sheet production because it lowers the grade and price of the sheets. Therefore, methods for prevention of fungal growth are required.

1.2.8.1 Chemicals

In the past *p*-nitrophenol was used to prevent fungal growth on para rubber sheet by 2 methods. In the first method, *p*-nitrophenol is added into the latex prior to the addition of the coagulant, or a mixture of the *p*-nitrophenol and coagulant may be added. In the second method, freshly prepared para rubber sheets are soaked in *p*-nitrophenol for half an hour. The sheets are then allowed to drip for a few hours before loading into the smoke house for drying (Fullerton, 1929). However, *p*-nitrophenol is toxic to those who handle the chemical as it enters the human body both by skin absorption and ingestion. Therefore use of *p*-nitrophenol is now forbidden in para rubber sheet production. Chanduaykit (2008) reported that sodium metabisulphite, potassium sorbate, potassium benzoate, acetic acid and smoked acid from bamboo are effective agents against fungal growth. In addition sodium metabisulphite at a concentration of 2x MIC could prevent fungal growth on para rubber sheet for more than 7 days. Baimark and Niamsa (2009) reported that wood vinegars from coconut shell, bamboo and *Eucalyptus* tree exhibited stronger antifungal activity against the main fungus *Penicillium griseofulvum* isolated from natural rubber sheet than acetic acid and formic acid, respectively.

1.2.8.2 Bacteria antagonistic to fungi

1.2.8.2.1 *Bacillus* spp.

The characteristics of *Bacillus* are Gram-positive rod-shaped cells, sometimes in chains, capable of producing cylindrical, ellipsoidal or spherical endospores, and are located either in the center of the cell, subterminally or terminally. Endospores of *Bacillus* are heat resistant (John, 1986). The temperature tolerance in the genus ranges from about -5 to 75 ^{o}C, tolerance to acidity from pH 2 to 8, and salt tolerance to as high as 25% NaCl. *Bacillus* are widely distributed in nature and are found in large numbers in most soil samples (Paul and Clark, 1989).

Bacillus are widely used as biological control agents because several species of *Bacillus* produce lytic enzymes and antibiotics (Paul and Clark, 1989). The ability of *Bacillus* to suppress a wide range of fungal plant pathogens has been widely studied. Leelasuphakul *et al.* (2006) reported that *Bacillus subtilis* NSRS 89-24 inhibited the growth of two rice pathogens *Pyricularia grisea* and *Rhizoctonia solani*

by producing β-1,3-glucanase and an antibiotic. Chitarra *et al*. (2003) also reported that many antifungal compounds are produced by *B. subtilis* such as alboleutin, botrycidin, clorotetain, fengycin, inturins and rhizocticins. These antifungal peptides inhibited the growth of *Aspergillus*, *Penicillium* and *Fusarium*. *Bacillus* strains GB-017 and GB-0356 produced antifungal substances that inhibited *Botrytis cinerea*, *Fusarium* sp., *Pythium* sp. and *R. solani*. Antifungal substances that have been separated and purified correspond to polyenes and lactones (Kim *et al*., 2003). *Bacillus brevis* inhibited the pigeon pea wilt pathogen (*Fusarium oxysporum* f. sp. *udum*) by producing an extracellular antagonistic substance that induced swelling of the pathogen's hyphal tips. The antagonistic substance also inhibited germination of conidia, and was fungicidal to the vegetative mycelia of the pathogen. When compared to the properties of this antagonistic substance with those of known antibiotics produced by *B. brevis* it was suggested that this antagonistic substance was a novel compound (Bapat and Shah, 2000). Bottone and Peluso (2003) reported a compound from *Bacillus pumilus* that inhibited *Mucor* and *Aspergillus* spore germination and aborted hyphal elongation. *Bacillus* sp. strain BC121 isolated from the rhizosphere of sorghum showed a high antagonistic activity against *Curvularia lunata*. The treated mycelia presented as being abnormal with condensation and deformation. There was swelling of the mycelial tips and the cells in between. SEM observations showed that the mycelia in the inhibition zone initially grew in a zigzag fashion (instead of growing in a straight line). This was followed by the occurrence of extensive malformations and damage to the mycelium (Basha and Ulaganathan, 2002). Pengnoo *et al*. (2006) reported that *Bacillus firmus* can inhibit the mycelial growth of *R. zolani*, a causal agent of the leaf blight of bambara groundnut.

In 2005, Joseph and coworkers isolated bacteria from dried cup lumps, scraps and sheet rubber and latex, and tested against *Penicillium* spp. and *Aspergillus* spp. isolated from sheet rubber in India. Two bacterial isolates Sc1 and Sc8 out of the 55 isolates tested prevented fungal growth even at 100% humidity. The antagonistic metabolites were found to be siderophores, HCN and salicylic acid. Their study indicated the potential of bacteria to be effective as biocontrol agents for fungal contaminants on RSS.

1.2.8.2.2 Actinomycetes

Actinomycetes are Gram-positive bacteria that comprise a group of branching unicellular microorganisms. They produce branching mycelia, substrate mycelium and aerial mycelium. Most actinomycete species are chemo-organotrophic, aerobic, mesophilic and grow optimally at a pH near neutrality (Williams and Wellington, 1982; Goodfellow and Williams, 1983). Actinomycetes are widespread in the environment and are found in large numbers in most soil samples. Among actinomycetes, *Streptomyces* are the dominant genus. About 90% of the actinomycetes isolated from soil can be assigned to the genus *Streptomyces*. (Paul and Clark, 1989) The important factors that control the abundance and activity of actinomycetes in the soil have been suggested to be the availability of nutrients, the nature and abundance of organic matter, salinity, relative moisture content, temperature, pH and soil vegetation (Goodfellow and Williams, 1983; McCarthy and Williams, 1990). Actinomycetes in general, prefer neutral to alkaline soils as a natural habitat (Flaig and Kutzner, 1960; Goodfellow and Williams, 1983).

Actinomycetes are potent producers of a wide variety of secondary metabolites with diverse biological activities including therapeutically and agriculturally important compounds. (Tanaka and Omura, 1993; Lange and Sanchez Lopez, 1996). Actinomycetes effectively inhibit a wide variety of plant pathogenic fungi, such as *Alternaria* (Chattopadhyay and Nandi, 1982), *Rhizoctonia* (Merriman *et al*., 1974; Rothrock and Gottlieb, 1984) *Verticillium* (Wadi and Easton, 1985), *Fusarium* (Cao *et al*., 2004) and *Macrophomia* spp. (Hussain *et al*., 1990). Among actinomycetes, the members of the genus *Streptomyces* are considered economically important because they alone produce 75% of total known bioactive molecules (Demain, 2000). The *Streptomyces* produce secondary metabolites such as enzyme inhibitors, herbicides and large number of antibiotics (Omura, 1992; Lange and Sanchez Lopez, 1996). In particular, approximately 60% of antibiotics developed for agricultural use were produced by *Streptomyces* (Tanaka and Omura, 1993). Various species of *Streptomyces* have antifungal activity. Apichaisataienchote *et al*. (2006) reported the antifungal antibiotic aerugine (4-hydroxymethyl-2-(2-hydroxyphenyl)-2-thiazoline) that was isolated from the culture filtrate of *S. fradiae* strain SU-1 and extracted by ethyl acetate. The extract strongly inhibited the radial growth of

Colletotrichum gloeosporioides and *Phytophthora parasitica* in an agar diffusion test. The minimum inhibitory concentrations of aerugine were 12.5 μg/ml against both *C. gloeosporioides* and *P. parasitica.* It completely inhibited conidial germination of *C. gloeosporioides* after 12 h of incubation and 100% inhibited encysted zoospore germination of *P. parasitica* after 24 h of incubation. Marten *et al*. (2001) reported that Rhizovit ^{R} from *Streptomyces rimosus* could inhibit a wide range of fungi such as *Pythium* spp., *Phytophthora* spp., *R. solani*, *Alternaria brassicola*, and *Botrytis* sp. Liu *et al*. (2004a) also reported that *S. rimosus* had a high antagonistic activity against *F. solani, F. oxysporium* f sp. *cucumarinum, Verticillium dahliae, R. solani, Fulvia fulva*, *Botrytis cinerea, A. alternata, Sclerotinia sclerotiorum* and *Bipolaris maydis*. An antifungal antibiotic, produced by *S. rimosus*, was purified by silica gel column chromatography. Its ultraviolet (UV) spectrum was consistent with that of a polyene macrolide (Liu *et al*., 2004b). The antifungal substances SH-1 and SH-2 were isolated from *Streptomyces humidus* strain S5-55 and identified as phenylacetic acid and sodium phenylacetate. SH-1 and SH-2 completely inhibited the growth of *Pythium ultimum, Phytophthora capsici, Rhizoctonia solani* and *Saccharomyces cerevisiae* at concentrations from 10 to 50 mg/ml (Hwang *et al*., 2001). There has been no report on actinomycetes against fungi isolated from sheet rubber.

1.3 Objectives

1) To isolate *Bacillus* spp. and actinomycetes from soils and other sources and screen for their antagonistic activity against fungi isolated from para rubber sheet.
2) To cultivate actinomycetes in broth medium for antifungal activity test and chemical extraction.
3) To test for antifungal activity of actinomycetes culture filtrates and their extracts against six fungi that contaminated on para rubber sheets.
4) To optimize the culture conditions for the production of antifungal metabolite by selected actinomycetes.
5) To identify the active actinomycetes.

CHAPTER 2

METERIALS AND METHODS

2.1 Materials

2.1.1 Microorganisms

- Tested fungi (Table 6)
- *Bacillus* spp. (Appendix 2: Table 17)

Three groups of *Bacillus* isolates were included in this study.

- 102 new isolates from soils from seven locations in southern Thailand (Table 4).
- 98 isolates provided by Dr.Metta Ongsakul, Department of Microbiology, Faculty of Science, Prince of Songkla University.
- 6 isolates provided by Assistant Professor Dr.Wichitra Leelasuphakul, Department of Biochemistry, Faculty of Science, Prince of Songkla University.

- Actinomycetes (Appendix 2: Table 18)
  - 77 new isolates from soils from seven locations in southern Thailand (Table 4).
  - 10 new isolates from rubber sheets (Table 5).
  - 64 isolates provided by Dr.Ampaithip Sukhoom, Department of Microbiology, Faculty of Science, Prince of Songkla University.

2.1.2 Media

- Yeast extract-malt extract agar (ISP-2) (Appendix 1)
- Yeast extract-malt extract broth (Appendix 1)
- Actinomycete Isolation Agar (AIA) (Difco)

- Potato dextrose agar (PDA) (Difco)
- Potato dextrose broth (PDB) (Difco)
- Potato dextrose broth (double strength) (Appendix 1)
- Nutrient agar (NA) (Difco)
- RPMI-1640 (Sigma Chemical Co., USA)

2.1.3 Chemicals

- Dimethyl sulfoxide (DMSO) (MERCK)
- *p*-Nitrophenol (UNIVAR)
- Amphotericin B (Bristol-Myer Squibb Co., USA)
- 1.8% resazurin (Sigma Chemical Co., USA)
- 0.85% NaCl, normal saline solution (NSS)

2.1.4 Equipment

- Autoclave (Tomy, SS-320)
- Hot air oven (Sanyo, MOV212)
- Centrifuge (HERMLE)
- Balance (DIETHELM & CO., LTD)
- pH meter (Beckman, 360)
- Laminar flow (Hotpack, 527044)
- Vortex mixer (LAB-Line, 1297)
- Microscope (Olympus, CX31)
- Stereo zoom microscope (Olympus, SZ40)
- Water bath (Memmert, W350)

2.2 Methods

2.2.1. Bacterial isolation

2.2.1.1 Isolation of *Bacillus* spp. from soil

Soil samples were collected from various places in southern Thailand (Table 4).

Table 4 Sources of soil samples for *Bacillus* and actinomycetes isolations

Collecting site	Province
Koh Samui	Suratthani
Koh Tan	Suratthani
Amphoe Kanjanadit	Suratthani
Amphoe Khanom	Nakhon Si Thammarat
Amphoe Muang	Krabi
Amphoe Muang	Satun
Amphoe Takbai	Narathiwat

Each 1 g of soil sample was suspended in 9 ml sterile 0.85% NSS. The samples were heated at 80 ^{o}C for 5 min in a water bath. The sample suspensions were then serially diluted in 0.85% NSS and the dilutions from 10^{-2} to 10^{-4} were spread on nutrient agar (NA) medium. Plates were incubated at 35 ^{o}C for 24 h. All the single *Bacillus*–like colonies were sub-cultured onto fresh plates of the same medium and were characterized by Gram-staining, cell shape and the presence of spores. *Bacillus* isolates (Gram-positive rods with spores) were kept on NA slants at RT until used.

2.2.1.2 Isolation of actinomycetes from soil

Each 1 g of the soil sample (Table 4) was suspended in 9 ml sterile distilled water and diluted to 10^{-4}. Then the dilutions 10^{-2} to 10^{-4} were spread on actinomycetes isolation agar (AIA) (Ouhdouch *et al*., 2001) and incubated at 30 ^{o}C for 7 days. Actinomycetes colonies were recognized on the basis of their morphological characteristics. Actinomycetes isolates were maintained on AIA at RT.

2.2.1.3 Isolation of actinomycetes from para rubber sheets

Ten grams of para rubber sheet collected from various places in southern Thailand (Table 5) were cut into small pieces (3x5 mm) and suspended in 90 ml sterile distilled water with 0.05% tween 80, then were diluted to 10^{-4}. The dilutions from 10^{-2} to 10^{-4} were spread on actinomycetes isolation agar.

Table 5 Sources of para rubber sheet samples for actinomycetes isolations

Collecting site	Province
Thai Muang	Phang Nga
Meuang	Phang Nga
Takua Thung	Phang Nga
Thai-Indo Rubber Co.,	Phatthalung
Phraek Ha	Phattahlung
Phabon	Phatthalung
Khok Changai	Phatthalung
Songkhla Rubber Research Center	Songkhla

2.2.2 Primary antifungal screening by the dual culture technique

2.2.2.1 Tested fungi

Six tested fungi isolated from para rubber sheets were kindly provided by Miss Supansa Chanduaykit (2008). The following strains (Table 6 and Figure 5) were used**.**

Table 6 Code and sources of tested fungi

Code	Test fungus	Sources
RSR12	*Rhizopus* sp.SR12	Songkla Rubber Research Center, Hat Yai, Songkhla
FSR2	*Fusarium* sp.SR2	Songkla Rubber Research Center, Hat Yai, Songkhla
ASR9	*Aspergillus* sp.SR9	Songkla Rubber Research Center, Hat Yai, Songkhla
ANY05	*Aspergillus* sp.NY05	Nayong, Trang
PPR02	*Penicillium* sp.PR02	Phraek Ha, Khuan Khanun, Phatthalung
CTT013	*Cladosporium* sp.TT013	Takua Thung, Phang Nga

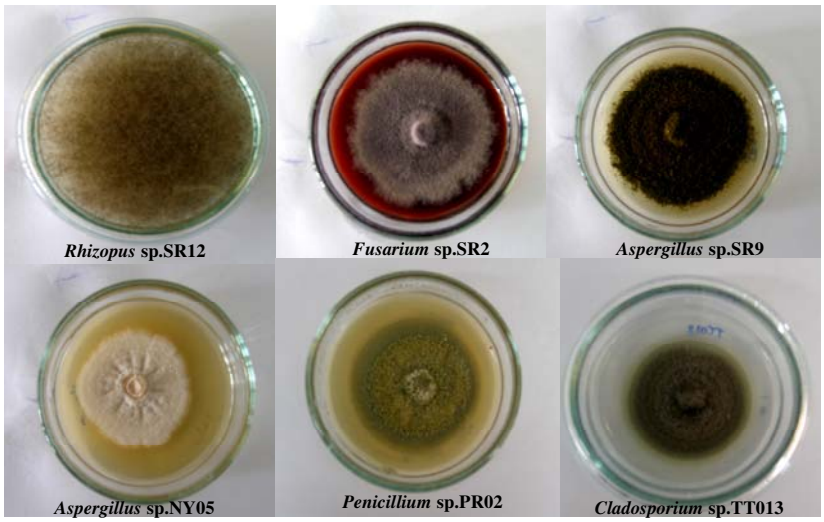


Figure 5 Tested fungi isolated from para rubber sheets

2.2.2.2 Hyphal growth inhibition by *Bacillus* spp. (adapted from Leelasuphakul *et al*., 2006)

A mycelial plug from the margin of a growing fungal colony was incubated centrally on a fresh Potato Dextrose Agar (PDA) plate and incubated at RT until the diameter of the colony was approximately 2 cm. The *Bacillus* was grown in Potato Dextrose Broth (PDB) and shaken at 180 rpm for 24 h, 30 ^{o}C. A loopful of the *Bacillus* broth culture was spotted or streaked 1 cm away from the test fungal colony (Figure 6) and incubated at RT. Test plates were observed for inhibition of fungal growth everyday for 7 days. The inhibition zone between the *Bacillus* streak and the edge of fungal colony was then measured. The test was performed in duplicate.

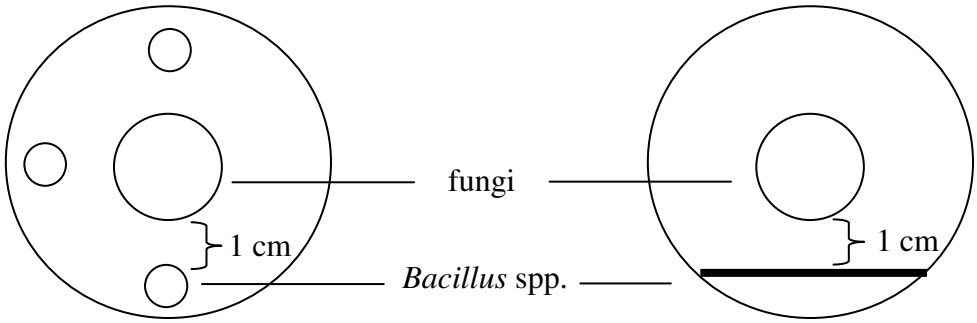


Figure 6 Diagram of fungal inhibition test for bacteria

The hyphal growth inhibition were classified into 3 levels :

+++	strong activity	: inhibition zone 6-10 mm.
++	moderate activity	: inhibition zone 1-5 mm.
+	weak activity	: fungal colony was close to the bacterial streak but could not grow across the streak.

2.2.2.3 Hyphal inhibition by actinomycetes (adapted from Jimenez-Esquilin and Roane, 2005)

Each actinomycetes isolate was streaked onto one half of an ISP-2 agar plate and incubated at room temperature until sporulation occurred. Then a mycelial plug from an actively growing fungal colony was placed about 1 cm from the edge of actinomycetes streak (Figure 7) and incubated at RT. Fungal inhibition was observed everyday for 7 days. The inhibition zone was measured as described in 2.2.2.2.

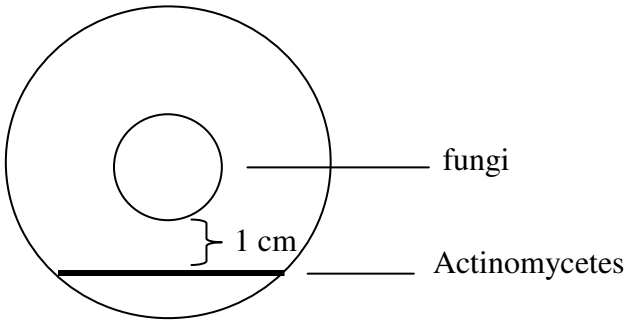


Figure 7 Diagram of fungal inhibition test by actinomycetes

2.2.3 Actinomycetes fermentation (modification of Taechowisan *et al*., 2005)

The top 30 active actinomycetes that inhibited all the tested fungi were selected for fermentation. A spore suspension of actinomycetes was prepared in distilled water from cultures grown on ISP-2 medium. The suspension was added to 100 ml ISP-2 broth in flat bottles to obtain about 10^6 spores/ml of the liquid medium and incubated at RT for one month (Figure 8).

Figure 8 Broth culture of actinomycetes in ISP-2 media

2.2.4 Hyphal growth inhibition by actinomycetes culture filtrates (Leelasuphakul *et al*., 2006)

The one month old cultures of actinomycetes were harvested by centrifugation at 5000 rpm for 30 min. The supernatants were collected and filtered through a 0.45 µm membrane filter under vacuum. Culture filtrates of the actinomycetes were mixed (1:1) with double strength melted PDA, then poured into a sterile plate. An agar plug of an actively growing fungal mycelium was placed on the center of the test plate and incubated at RT for 7 days. ISP-2 broth was used instead of a culture filtrate of actinomycetes in the control plates. The perpendicular diameters of the fungal colony on the culture filtrate mixed agar and control plate were measured. The percentage of hyphal growth inhibition was calculated using this formula :

$$\% \text{ inhibition} = 100-\left(\frac{R^2 \times 100}{r^2}\right) \quad \text{(Gamliel } et\ al.\text{, 1989)}$$

R = radius of treated colony

r = radius of the control colony

p-nitrophenol was used as chemical fungicide control.

2.2.5 Actinomycetes culture filtrate extraction

The culture filtrate of actinomycetes was extracted three times with an equal volume of ethyl acetate (EtOAc) in a separating funnel. The EtOAc layer was dried over anhydrous sodium sulfate (Na_2SO_4) and evaporated to dryness under reduced pressure at 45 ^oC using a rotary vacuum evaporator to obtain a crude extract (Figure 9).

Figure 9 Crude extracts from actinomycetes culture broth

2.2.6 Antifungal assays of the actinomycetes crude extract

2.2.6.1 Inoculum preparation

The tested fungi were grown on PDA at 25 ^oC for 7 days or until sporulation occurred. Conidia were harvested by adding sterile glass bead and 0.85% NSS and rocking gently over the colony. The conidial suspensions were transferred to a sterile tube. The conidia were counted using a haemacytometer and adjusted to the concentration in the range of $0.4x10^4$-$5x10^4$ conidia/ml.

2.2.6.2 Testing for antifungal activity (modification of CLSI M38-A, CLSI, 2002)

Crude extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solution of 100 mg/ml and stored at -4 ^oC until used. DMSO was used to dilute to 1:10 (10 mg/ml) and RPMI-1640 for future dilutions of 1:25 to obtain concentrations of 400 µg/ml. Triplicate 100 µl of crude extracts (400 µg/ml) were

pipetted into sterile 96 well microtiter plate. The final spore suspensions ($0.4x10^4$-$5x10^4$ conidia/ml) were added to each well, so that the final concentration of crude extract was 200 µg/ml. Amphotericin B and *p*-nitrophenol at the same concentration were used as antifungal agents for a positive inhibitory control and for comparison with the extracts. DMSO was used as solvent control. Microtiter plates were incubated at 25 ^oC for 1 day (RSR12), 2 days (FSR2, ASR9), 4 days (PPR02, CTT013) and 6 days (ANY05) for the growth of each fungal strain, then 10 µl resazurin indicator (0.18%) was added to each well and examined after incubation for one day at the same temperature for the completed reaction (modification of Drummond and Waigh, 2002). After incubation, if the solution turns pink, it indicates fungal growth or no inhibition (negative result). A blue or purple color indicates inhibition of fungal growth (positive result). Crude extracts that inhibited fungi were selected for determination of their minimal inhibitory concentrations (MIC) and minimal fungicidal concentrations (MFC).

2.2.6.3 Determination of minimal inhibitory concentration (MIC)

The MICs of crude extracts were determined by the broth microdilution method according to a modification of CLSI MA38-A (CLSI, 2002). The test was performed in the same manner for the screening test as described in 2.2.6.2. Crude extracts were diluted using serial 2-fold dilutions with final concentrations of 0.25-128 µg/ml, each tested in triplicate. After incubation under appropriate conditions, the lowest concentration of extract that inhibited growth (blue or purple color) was recorded as the MIC.

2.2.6.4 Determination of minimal fungicidal concentration (MFC)

After MIC determination, 10 µl of the concentrations of crude extract less dilute than the MIC and the MIC were dropped onto PDA plates. Plates were incubated under appropriate conditions and the lowest concentration of extract at which no growth occurred was recorded as the MFC.

2.2.7 Optimization of antifungal metabolite production (modification of Augustine *et al*., 2005)

2.2.7.1 Agitation : comparison between shaking and static conditions.

Actinomycetes inocula (described in 2.2.3) were added into flasks containing 100 ml of ISP-2 broth and incubated statically and on a rotary shaker at 200 rpm at 30 ^{o}C for 4 weeks. Culture filtrates were harvested every week and checked for antifungal activity by the agar dilution test (described in 2.2.4).

2.2.7.2 pH

The initial pH of the ISP-2 media was adjusted to 6, 7 and 8. Actinomycetes inocula (described in 2.2.3) were added to flat bottles containing 100 ml of ISP-2 broth and incubated under static conditions at 30 ^{o}C for 8 weeks and culture filtrates were harvested every week to check for antifungal activity by the agar dilution test (described in 2.2.4).

2.2.7.3 Temperature

Actinomycetes inocula (described in 2.2.3) were added to flat bottles containing 100 ml of ISP-2 broth, pH7 and incubated at different temperatures (25, 30 and 35^{o}C) under static condition for 8 weeks and culture filtrates were harvested every week to check for antifungal activity by the agar dilution test (described in 2.2.4).

2.2.8. Identification of actinomycetes

Actinomycetes were identified by morphological characteristics and molecular technique (16S rDNA).

2.2.8.1 Morphological characteristics

All morphological characters were observed on ISP-2 agar and were used for classification and differentiation adopted from Taddei *et al*. (2006) as follows:

2.2.8.1.1 Macroscopic morphology

The mass color of mature sporulating aerial mycelium was observed following growth on ISP-2 plates. The aerial mass was classified according to the Bergey's Manual of Systematic Bacteriology (Locci, 1989) in the following color series: gray, white, red, yellow, green, blue, and violet. Distinctive colors of the substrate mycelium were also recorded. The observed colors were: beige, black, blue, biscuit, brown, ivory, olive, orange, purple, pink, red, red-violet, tan, violet-purple, yellow, and yellow-greenish.

2.2.8.1.2 Microscopic morphology

According to the shape of the spore chains observed under light microscopy, the isolates were grouped as follows: Rectus-Flexibilis (RF), spores in straight or flexuous chains, and Spira (S), spore chains in the form of short gnarled or compact coils or extended, long and open coils.

2.2.8.2 Molecular identification

Selected potential isolates were identified based on the analysis of 16S rDNA sequences. DNA extraction, PCR amplification and DNA sequencing were done by KU Vector, Kasetsart University as the followings:

2.2.8.2.1 Actinomycetes DNA extraction

Actinomycetes were grown on an ISP-2 plate at RT for 3 to 4 weeks. The DNA of each isolate was extracted by suspending some colonies of actinomycetes in 400 µl of TE buffer, and 8 µl of lysozyme (50 mg/ml) in a microtube. The mixture was agitated and incubated at 37 ^{o}C for 30 minutes. Then 4 µl of proteinase K (20 mg/ml), 20 µl of 10%SDS and 4 µl of RNase A (100 mg/ml) were added. The mixture was mixed together and incubated at 37 ^{o}C for 30 minutes. After that, 70 µl of 5M NaCl, 55 µl of 10%CTAB (10%CTAB/0.7M NaCl) were added and incubated at 65 ^{o}C for 10 minutes. Then an equal volume of chloroform was added and centrifuged at 15,000 rpm, RT for 5 minutes. This step was repeated twice. The supernatant was transferred to a new microtube, added an equal volume of phenol/chloroform and centrifuged at 15,000 rpm, RT for 5 minutes. The supernatant was transferred to a

new microtube, then isopropanal was added and centrifuged at 8,000 rpm, RT for 2 minutes. DNA pellet was washed twice with 1 ml of 70% ethanol and centrifuged at 8,000 rpm for 1 minutes. After drying DNA pellet was resuspended in 20 µl of water or TE buffer for PCR amplification.

2.2.8.2.2 PCR amplification

Target region of the 16S rDNA was amplified using 27F and 1389 primers listed in Table 7.

Table 7 Primers used for the polymerase chain reaction (PCR) and DNA sequencing

Primers		Sequence (5'-3')
PCR	27F	AGA GTT TGA TCM TGG CTC AG
PCR	1389R	ACG GGC GGT GTG TAC AAG
DNA sequencing	520F	GT GCC AGC MGC CGC GG

Note: M represents A or C

PCR mixture

10 X *Ex-Taq* Buffer	1.5	µl
25 mM dNTP mix	1.2	µl
10 pmol/µl Forward primer (27F)	1.5	µl
10 pmol/µl Reverse primer (1398R)	1.5	µl
Ex-Taq polymerase	0.075	µl
Nanopure water	8.225	µl
DNA template (100 ng/µl)	1.0	µl
Total volume	15.0	µl

PCR profiles for amplification:

The PCR profile for primers 27F and 1389R

95 °C	1 minute	
95 °C	20 seconds	35 cycles
50 °C	30 seconds	
72 °C	2 minutes	
72 °C	4 minutes	

2.2.8.2.3 DNA sequencing and sequence alignment

Sequencing mixture

Nanopure water	9.0 µl
5X sequencing buffer	2.25 µl
520F primer	0.75 µl
Ready reaction mix	1.5 µl
DNA template	1.5 µl

The PCR profile for primer 520F

96 °C	1 minute	
96 °C	10 seconds	25 cycles
50 °C	5 seconds	
60 °C	4 minutes	

The 16S rDNA sequences were then compared with GenBank using BLASTN program (http://www.ncbinlm.nih.gov). Sequences of the selected actinomycetes and other sequences obtained from the GenBank database were aligned by ClustalW (Thompson *et al*., 1994). Manual gap adjustments were made to improve the alignments. The tree construction procedure was performed in PAUP* 4.0b10 in Window versions (Swofford, 2002). Maximum Parsimony (MP) was conducted using heuristic searches as implemented in PAUP* 4.0b10, with the default options method. Clade stability was assessed in a bootstrap analysis with 1,000 replicates, random sequence additions with maxtrees set to 1,000 and other default parameters as implemented in PAUP* 4.0b10. Neighbor joining (NJ) tree was constructed based on the total character differences and bootstrap values were calculated from 1,000 replicates using PAUP* 4.0b10.

Neighbour-joining: Neighbor-joining is based on the minimum-evolution criterion for phylogenetic trees, i.e. the topology that gives the least total branch length is preferred at each step of the algorithm. However, neighbor-joining may not find the true tree topology with least total branch length because it is a greedy algorithm that constructs the tree in a step-wise fashion. Even though it is sub-optimal in this sense, it has been extensively tested and usually finds a tree that is quite close to the optimal tree. Nevertheless, it has been largely superseded in phylogenetics by methods that do not rely on distance measures and offer superior accuracy under most conditions (http://en.wikipedia.org/wiki/Neighbor-joining).

Maximum parsimony: Maximum parsimony is a character-based method that infers a phylogenetic tree by minimizing the total number of evolutionary steps required to explain a given set of data, or in other words by minimizing the total tree length. The trees requiring the fewest character state changes are considered optimal. Individual characters are termed “informative” in maximum parsimony analysis when they require different numbers of changes on different trees. To be informative, an unpolarized character must have at least two character states and each state must be present in at least two taxa. Character state changes required for the tree is the length of the tree. As the number of taxa increases, the number of possible trees increases even faster, finding the most parsimonious trees (MPTs) for a given data set

can be a computationally intensive task to choose the tree that result in fewest character state changes.

Bootstrapping (Simpson, 2006)**:** Bootstrapping is a resampling tree evaluation method that works with distance, parsimony, likelihood and just about any other tree derivation method. The result of bootstrap analysis is typically a number associated with a particular branch in the phylogenetic tree that gives the proportion of bootstrap replicates that supports the monophyly of the clade. Bootstrap values greater than 70% correspond to a probability of greater than 95% that the true phylogeny has been found and greater than 50% will be an overestimate of accuracy. A high bootstrap value can make the right phylogeny.

Consistency index (CI) (Simpson, 2006)**:** One measure of the relative amount of homoplasy in the cladogram is the consistency index. The consistency index is equal to the ratio of minimum changes of character state or minimum possible tree length that must occur and the actual number of changes or tree length that do occur. A consistency index close to 1 indicates little to no homoplasy; a CI close to 0 is indicative of considerable homoplasy.

$$CI = \frac{\text{Minimum possible tree length}}{\text{Tree length}}$$

Retention index (RI) (Simpson, 2006)**:** The retention index is calculated as the ratio (g-s)/(g-m), where g is the maximum possible tree length that could occur on any conceivable tree, s and m are tree length and minimum possible tree length, respectively. Thus, the RI is influenced by the number of taxon in the study. A consistency index close to 1 indicates little to no homoplasy; a RI close to 0 is indicative of considerable homoplasy.

$$RI = \frac{\text{Maximum possible tree length - tree length}}{\text{Maximum possible tree length - minimum possible tree length}}$$

CHAPTER 3

RESULTS

3.1. Bacterial isolation

In this study, two groups of bacteria were isolated from soils and/or rubber sheets : *Bacillus* spp. and actinomycetes.

3.1.1 *Bacillus* spp.

One hundred and two cultures of *Bacillus* spp. were isolated from soils from seven locations in the southern part of Thailand (Table 8). Other sources of *Bacillus* spp. were those kindly provided by Dr.Metta Ongsakul (98 isolates) from the Department of Microbiology and Assist. Prof. Dr.Wichitra Leelasuphakul (6 isolates) from the Department of Biochemistry, Faculty of Science, Prince of Songkla University. A total of 206 *Bacillus* spp. isolates were included in this study.

Table 8 Numbers of *Bacillus* spp. isolated from soils from various locations

Locations	No. of isolates
Koh Samui, Suratthani	19
Koh Tan, Suratthani	31
Khanom, Nakhonsithammarat	14
Khanjanadit, Suratthani	13
Meuang, Satun	17
Takbai, Narathiwat	5
Meuang, Krabi	3
Total	**102**

3.1.2 Actinomycetes

A total of 151 actinomycetes isolates were included in this study. Seventy-seven isolates were isolated from soils (Table 9), ten isolates were from para rubber sheets (Table 10) and 64 isolates were kindly provided by Dr. Ampaithip Sukhoom, Department of Microbiology, Faculty of Science, Prince of Songkla University.

Table 9 Numbers of actinomycetes isolated from soils from various locations

Locations	No. of isolates
Koh Samui, Suratthani	12
Koh Tan, Suratthani	26
Khanom, Nakhonsithammarat	11
Khanjanadit, Suratthani	13
Meuang, Satun	5
Takbai, Narathiwat	4
Meuang, Krabi	6
Total	**77**

Table 10 Numbers of actinomycetes isolated from para rubber sheets from different sources

Sources	No. of isolates
Thai Muang, Phang Nga	1
Khok Changai, Phatthalung	2
Phraek Ha, Phattahlung	1
Meuang, Phang Nga	1
Thai-Indo Rubber Co., Phatthalung	1
Takua Thung, Phang Nga	1
Songkhla Rubber Research Center, Songkhla	2
Phabon, Phatthalung	1
Total	**10**

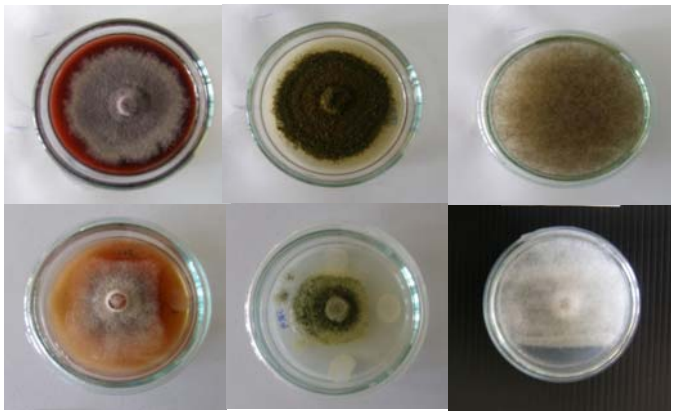
3.2. Primary antifungal screening by the dual culture technique

3.2.1 Hyphal growth inhibition by *Bacillus* spp.

A total of 206 *Bacillus* spp. were screened for antagonistic activity by the dual culture technique against six fungi (2 *Aspergillus* spp., 1 *Penicillium* sp., 1 *Fusarium* sp., 1 *Rhizopus* sp. and 1 *Cladosporium* sp.) commonly found on contaminated para rubber sheets in southern Thailand. Thirty three isolates (16%) of *Bacillus* spp. exhibited antifungal activity against at least one fungal isolate as indicated by the cresent shaped fungal growth around the *Bacillus* spot or by no growth across the *Bacillus* streak (Table 11 and Figure 10).

Table 11 Distribution of *Bacillus* spp. included in the study according to their antifungal activity

Origin of *Bacillus* spp.	**Active isolates/Total isolates tested (%)**
New isolates	19/102 (18.62)
Isolates having biosurfactant property (From Dr.Metta Ongsakul)	8/98 (8.16)
Isolates having antifungal activity against phytopathogenic fungi (From Dr.Wichitra Leelasuphakul)	6/6 (100.00)
Total	33/206 (16.02)



***Fusarium* sp. SR2** ***Aspergillus* sp. SR9** ***Rhizopus* sp.SR12**

Figure 10 Hyphal growth inhibition by *Bacillus* spp. tested by the dual culture method; upper row: control fungi and lower row: tested fungi

The highest number of antagonistic *Bacillus* spp. (18 isolates) were able to inhibit one tested fungus followed by 7, 3, 2, and 3 that inhibited 2, 3, 4, or 6 tested fungi (Figure 11). Only 3 isolates including MK007, 155, and B211 had a broad inhibititory activity against all 6 test fungi.

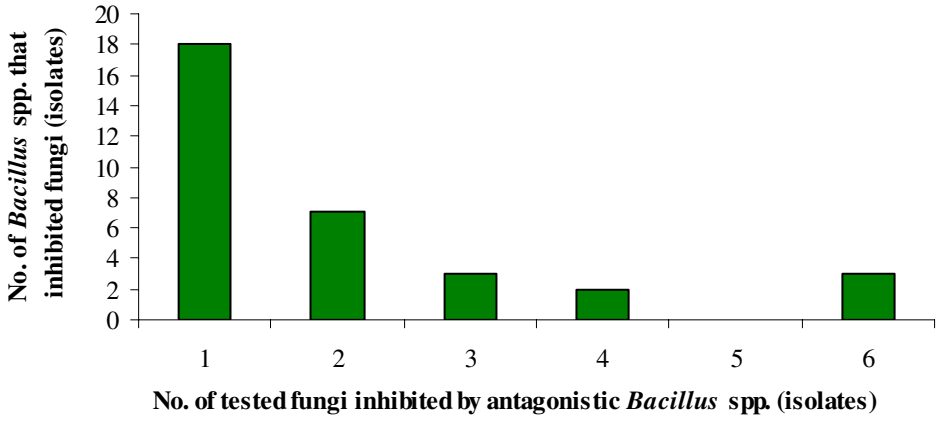


Figure 11 Number of antagonistic *Bacillus* spp. that inhibited tested fungi

In addition, it was found that the antagonistic *Bacillus* spp. tested in this study exhibited only weak (+) and moderate (++) antifungal activities (Figure 12). *Aspergillus* sp. NY05 was the most susceptible while *Rhizopus* SR12 was the most resistant to antagonistic *Bacillus* spp.

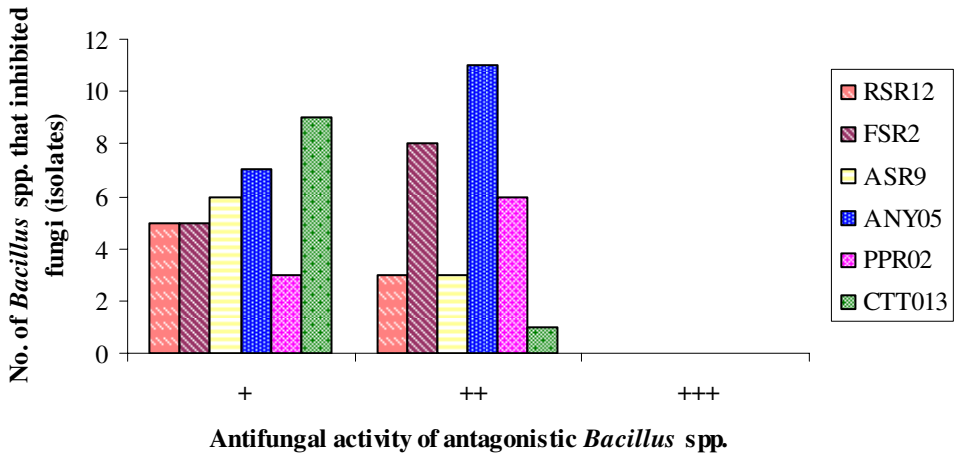


Figure 12 Antifungal activity of 33 antagonistic *Bacillus* spp.

+++	strong activity	: inhibition zone 6-10 mm.
++	moderate activity	: inhibition zone 1-5 mm.
+	weak activity	: fungal colony was close to the bacterial streak but could not grow across the streak.

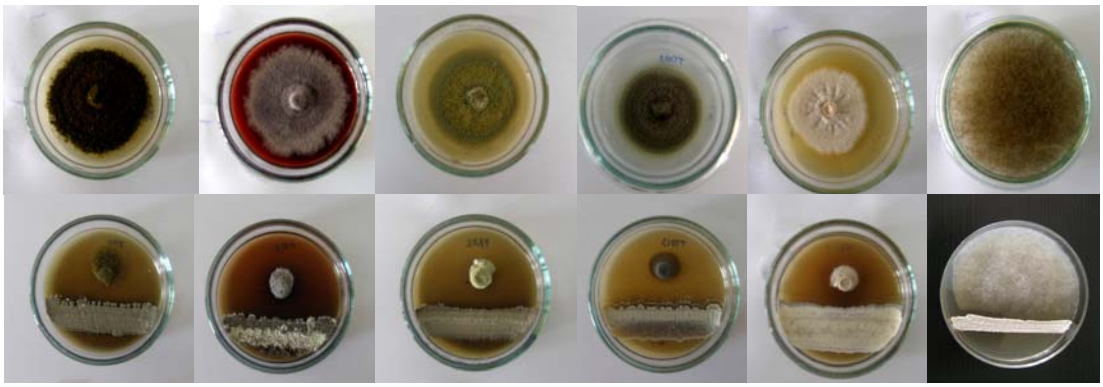
RSR12	: *Rhizopus* sp.SR12	FSR2	: *Fusarium* sp.SR2
ASR9	: *Aspergillus* sp.SR9	ANY05	: *Aspergillus* sp.NY05
PPR02	: *Penicillium* sp.PR02	CTT013	: *Cladosporium* sp. TT013

3.2.2 Hyphal growth inhibition by actinomycetes

One hundred and twenty nine out of 151 (85%) isolates of actinomycetes exhibited antifungal activity against at least one fungal isolate. Actinomycetes exhibited good antifungal activity (Table 12 and Figure 13). Most of the antagonistic actinomyctes (64 isolates) inhibited all the tested fungi (Figure 14).

Table 12 Distribution of actinomycetes included in the study according to their antifungal activity

Origin of actinomycetes	Active isolates/Total isolates tested (%)
New isolates from soil	61/77 (79.22)
New isolates from rubber sheets	4/10 (40.00)
Isolates having antifungal activity against phytopathogenic fungi (From Dr.Ampaithip Sukhoom)	64/64 (100.00)
Total	129/151 (85.43)



***Aspergillus* sp.SR9** ***Fusarium* sp.SR2** ***Penicillium* sp.PR02** ***Cladosporium* sp.TT013** ***Aspergillus* sp.NY05** ***Rhizopus* sp.SR12**

Figure 13 Hyphal growth inhibition by actinomycetes tested by dual culture method; upper row: control fungi and lower row: tested fungi

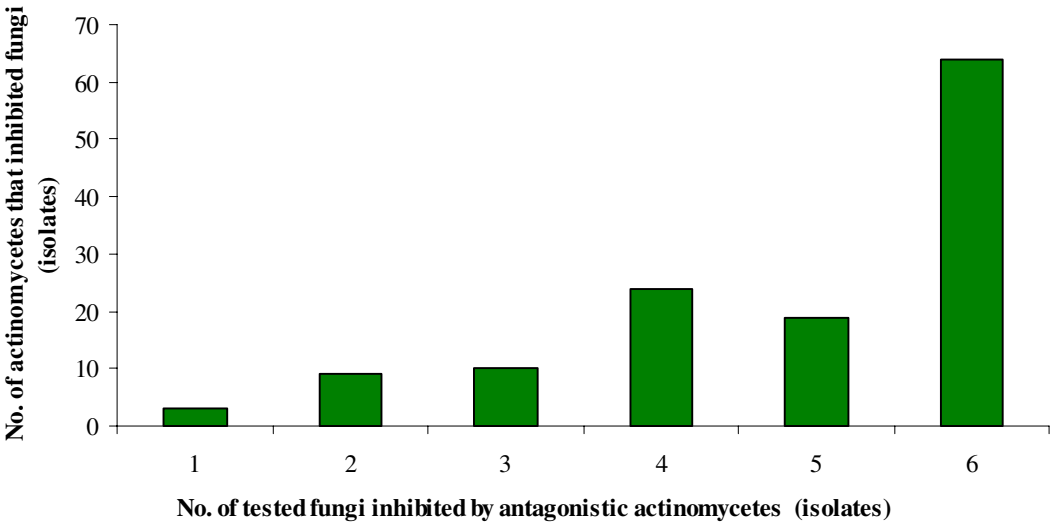


Figure 14 Number of antagonistic actinomycetes that inhibited tested fungi

Most of antagonistic actinomycetes exhibited moderate antifungal activity (62-90 isolates) while only 6- 23 and 12-17 isolates had weak and strong activities, respectively (Figure 15).

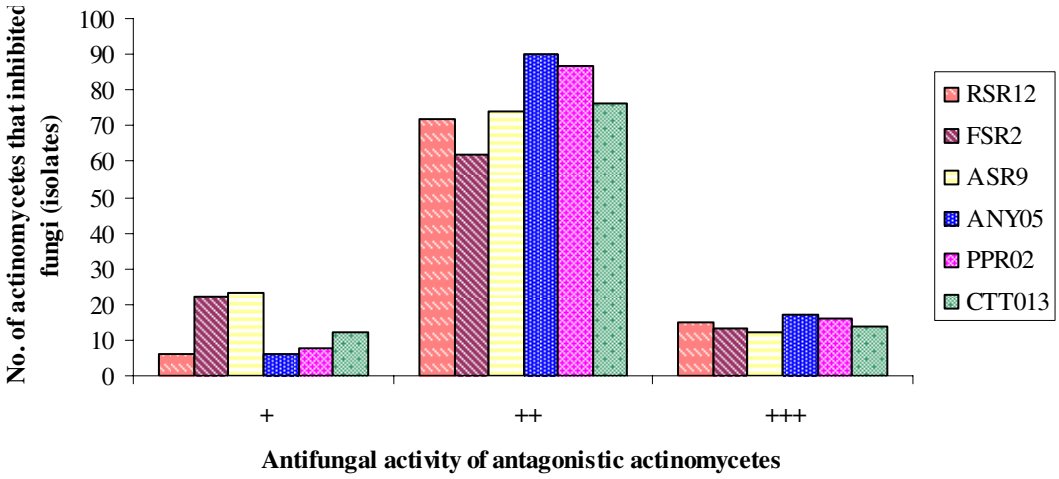


Figure 15 Antifungal activity of 129 antagonistic actinomycetes

+++	strong activity	: inhibition zone 6-10 mm.
++	moderate activity	: inhibition zone 1-5 mm.
+	weak activity	: fungal colony was close to the bacterial streak but could not grow across the streak.

RSR12	: *Rhizopus* sp.SR12	FSR2	: *Fusarium* sp.SR2
ASR9	: *Aspergillus* sp.SR9	ANY05	: *Aspergillus* sp.NY05
PPR02	: *Penicillium* sp.PR02	CTT013	: *Cladosporium* sp. TT013

From this study, it is clear that antagonistic actinomycetes had better antifungal activity than the *Bacillus* spp. Thus, the top 30 antagonistic actinomycetes (Table 13) were selected for further study.

Table 13 Top 30 antagonistic actinomycetes having antifungal activity against six tested fungi

Actinomycetes	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
AC27	++	++	++	+++	+++	+++
AC30	++	++	++	++	++	++
AC37	+++	++	++	++	++	++
AC40	+++	++	+++	+++	+++	++
AC41	++	++	+++	++	+++	++
AC43	++	++	+++	++	++	++
AC44	++	++	++	++	++	++
AC46	++	++	++	++	+++	++
AC49	++	++	++	++	++	++
AC50	++	++	++	++	++	++
AC51	+++	+++	+++	+++	+++	+++
AC52	+++	+++	++	+++	+++	+++
AC53	+++	+++	++	+++	+++	+++
AC54	+++	++	++	+++	++	+++
AC55	+++	+++	++	+++	+++	+++
AC59	+++	+++	+++	+++	+++	+++
AC62	+++	++	++	+++	++	++
AC70	++	+++	+++	+++	+++	+++
AC71	++	+++	++	+++	+++	+++
AC72	++	+++	+++	+++	+++	+++
AC73	++	+++	++	++	++	+++
AC74	++	+++	+++	+++	+++	+++
AC76	+++	+++	+++	+++	+++	++
AC78	+++	++	++	++	++	++
AC80	+++	++	++	+++	++	++
AC83	++	+++	++	++	++	+++
AC84	+++	+++	+++	+++	+++	+++
AC86	++	++	+++	+++	++	++
AC91	++	++	++	++	++	++
AC97	++	++	+++	++	++	++

+++ strong activity: inhibition zone 6-10 mm.;

++ moderate activity: inhibition zone 1-5 mm.

RSR12 : *Rhizopus* sp.SR12 FSR2 : *Fusarium* sp.SR2

ASR9 : *Aspergillus* sp.SR9 ANY05 : *Aspergillus* sp.NY05

PPR02 : *Penicillium* sp.PR02 CTT013 : *Cladosporium* sp. TT013

3.3 Hyphal growth inhibition by actinomycetes culture filtrates

The top 30 selected antagonistic actinomycetes were incubated in ISP-2 broth for one month. The culture filtrates were tested for antifungal activity against six tested fungi by the agar dilution method (Figure 16). The results are shown in Table 14. The culture filtrates of actinomycetes AC41 and AC51 showed the strongest antifungal activity against all tested fungi with more than 80% hyphal growth inhibition.

Figure 16 Hyphal growth inhibition by actinomycetes culture filtrate tested by agar dilution method; upper row: fungi grown with no culture filtrate and lower row: tested fungi

Table 14 Effect of culture filtrates of actinomycetes on hyphal growth

Actinomycetes	% Inhibition					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
AC27	99.35	95.36	23.91	81.05	56.20	80.32
AC30	99.11	88.56	30.69	44.31	55.77	82.92
AC37	99.79	94.06	79.57	85.45	71.22	86.36
AC40	97.16	86.54	36.68	73.10	86.11	75.05
AC41	**99.60**	**95.24**	**83.00**	**86.74**	**81.96**	**88.09**
AC43	42.89	31.72	55.20	52.12	38.27	8.41
AC44	95.64	56.81	22.69	62.68	28.91	30.10
AC46	86.51	79.62	83.87	68.54	37.95	73.58
AC49	88.59	45.18	46.87	27.38	38.78	51.69
AC50	92.38	86.93	53.59	41.52	48.40	73.03
AC51	**99.61**	**99.06**	**85.96**	**93.14**	**84.84**	**93.93**
AC52	94.09	59.59	71.63	32.80	57.08	52.17
AC53	96.28	67.42	45.69	41.84	58.85	70.99
AC54	96.79	56.81	49.36	82.00	62.68	64.27
AC55	88.10	10.10	36.26	24.03	28.07	34.50
AC59	89.90	47.62	58.56	6.57	50.57	12.03
AC62	99.06	88.30	41.64	78.52	39.72	56.04
AC70	99.04	83.12	69.38	83.29	87.73	90.52
AC71	91.91	53.98	63.98	15.37	34.72	36.95
AC72	93.61	79.21	73.62	58.97	62.44	59.84
AC73	58.16	37.39	21.56	45.72	26.77	37.71
AC74	76.68	64.59	49.42	54.87	32.17	54.07
AC76	96.17	76.81	82.49	33.37	78.10	83.07
AC78	86.20	99.14	84.05	71.59	23.98	81.99
AC80	88.10	60.18	30.16	63.10	44.31	45.26
AC83	99.51	95.86	41.42	78.10	46.06	51.81
AC84	99.69	96.26	94.03	85.45	64.00	76.67
AC86	91.84	80.52	85.74	76.34	70.97	90.09
AC91	91.03	86.25	79.89	80.25	85.72	83.47
AC97	90.90	86.05	87.02	60.79	83.71	85.87

RSR12 : *Rhizopus* sp.SR12 FSR2 : *Fusarium* sp.SR2

ASR9 : *Aspergillus* sp.SR9 ANY05 : *Aspergillus* sp.NY05

PPR02 : *Penicillium* sp.PR02 CTT013 : *Cladosporium* sp. TT013

3.4 Screening of actinomycetes crude extracts for antifungal activity

Culture filtrates of the top 30 antagonistic actinomycetes were extracted with ethyl acetate. Their crude extracts at a concentration of 200 µg/ml were primarily tested for antifungal activity against the tested fungi by the colorimetric microdilution method (Figure 17). The results are shown in Table 15. Only 27-53% of the extracts had antifungal activity. It was found that eight extracts comprising AC37BE, AC41BE, AC51BE, AC70BE, AC72BE, AC74BE, AC78BE and AC84BE exhibited antifungal activity against all the tested fungi (Table 16).

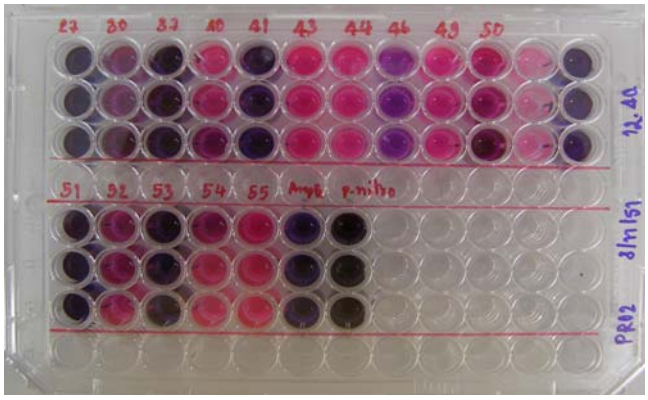


Figure 17 Primary antifungal screening against *Penicillium* sp. PR02 by the colorimetric broth microdilution method at 200 µg/ml (blue or violet color indicates an inhibitory result and pink color indicates growth of the tested strain)

Table 15 Antifungal activity of crude extracts of actinomycetes

	Tested fungi					
	RSR12	**FSR2**	**ASR9**	**ANY05**	**PPR02**	**CTT013**
Active extract (%) n=30	8 (26.67)	14 (46.67)	15 (50.00)	12 (40.00)	16 (53.33)	14 (46.67)
MIC (µg/ml)	8-128	32-200	32-200	64-200	16-200	64-200
MFC (µg/ml)	>128	>128	>128	>128	>128	>128

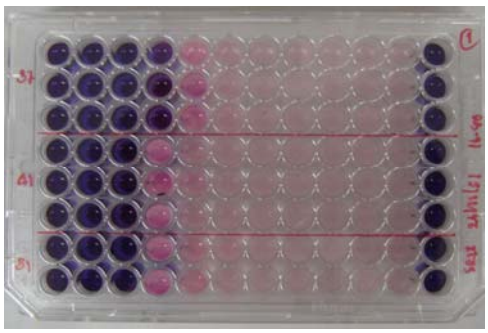
RSR12 : *Rhizopus* sp.SR12 FSR2 : *Fusarium* sp.SR2

ASR9 : *Aspergillus* sp.SR9 ANY05 : *Aspergillus* sp.NY05

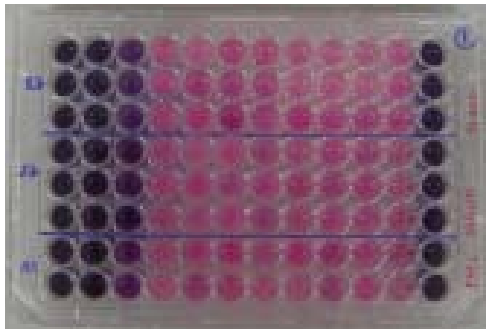
PPR02 : *Penicillium* sp.PR02 CTT013 : *Cladosporium* sp. TT013

3.5 Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

All extracts that showed inhibitory activity at 200 µg/ml were further assayed for their MICs (Figure 18) and MFCs by the colorimetric broth microdilution method. Data are shown in Table 19 in the Appendix 2.



a) *Rhizopus* sp. SR12



b) *Penicillium* sp. PR02

Figure 18 Example of the determination of MICs of crude extracts by the colorimetric broth microdilution method (blue or violet color indicates an inhibitory result and pink color indicates growth of the tested strain)

The MICs of the most active extracts against 6 tested fungi ranged from 8-200 µg/ml. The best MICs were in the range of 16-64 µg/ml by AC41BE and AC51BE which appeared to be comparable to *p*-nitrophenol, a control antifungal agent (32-128 µg/ml) (Table 16). All extracts had MFCs of > 128 µg/ml (Table 15).

Table 16 MIC values of actinomycetes extracts against six fungi isolated from para rubber sheet

Extract	MIC (µg/ml)					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
AC37BE	8	64	64	128	32	128
AC41BE	**16**	**64**	**64**	**64**	**32**	**64**
AC51BE	**16**	**32**	**32**	**64**	**32**	**64**
AC70BE	64	128	128	200	128	200
AC72BE	128	200	128	200	200	200
AC74BE	64	128	64	200	64	200
AC78BE	32	64	64	128	32	128
AC84BE	16	64	64	64	32	128
***p*-nitrophenol**	**64**	**128**	**64**	**32**	**32**	**128**

RSR12 : *Rhizopus* sp.SR12 FSR2 : *Fusarium* sp.SR2

ASR9 : *Aspergillus* sp.SR9 ANY05 : *Aspergillus* sp.NY05

PPR02 : *Penicillium* sp.PR02 CTT013 : *Cladosporium* sp. TT013

3.6 Optimization of culturing conditions of actinomycetes for the production of antifungal metabolite

3.6.1 Effect of agitation

In this study the antifungal metabolite productions of selected actinomycetes AC41 and AC51 were carried out under shaking (200 rpm) and static conditions at 30°C. The culture broths were withdrawn weekly for 4 weeks to investigate the inhibition activity against PPR02 and ASR9 fungi. Antifungal activity was measured in term of fungal growth inhibition. It was found that the culture broths cultured under the static condition of both actinomycetes showed stronger antifungal activity than those under the shaking condition. The best activity (≥80% of inhibition)

was shown after 4 weeks cultivation of both actinomycetes (Figure 19). However, only 60% inhibition was observed from actinomycetes AC41 after 1 week under shaking condition and was gradual decrease with time. While actinomycetes AC51 under shaking condition showed very low antifungal activity (<20%) throughout the 4 weeks fermentation.

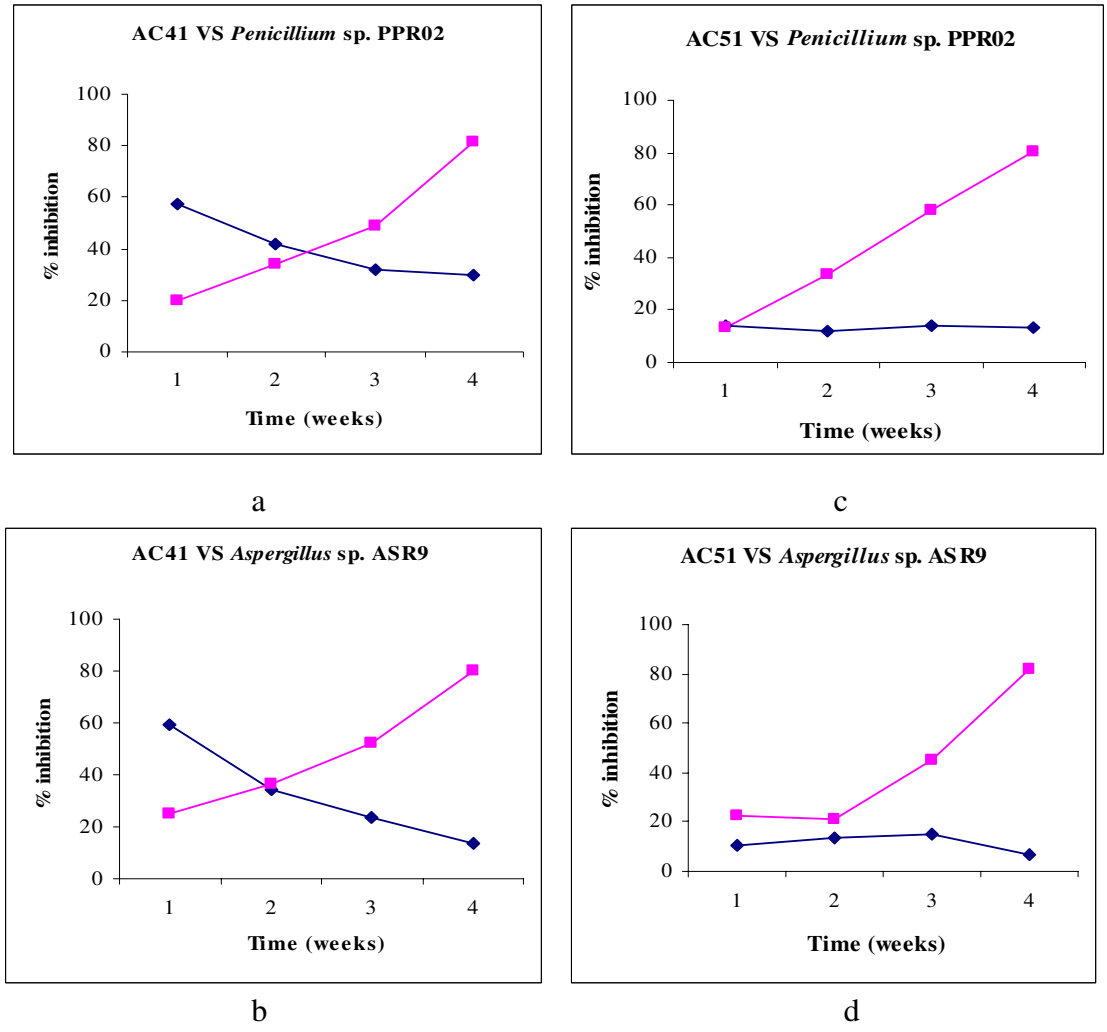


Figure 19 Effect of culture conditions on production of antifungal metabolites of actinomycetes AC41 (a and b) and AC51 (c and d). Symbols: ■, static condition; ◆, shaking condition.

3.6.2 Effect of the initial pH of ISP-2 medium

The initial pH of medium is important for growth and antifungal production of actinomycetes. In this study actinomycetes AC41 and AC51 were inoculated in ISP-2 medium at different initial pH (6, 7 and 8) at 30°C for 8 weeks. The best activity was observed in the medium with an initial pH 7 after 6 weeks of incubation by actinomycetes AC41 against both strains of tested fungi (85-87%). While the best activity for actinomycetes AC51 was observed at pH 7 and 8 after 3 weeks of incubation (81-82% against PP02 and 86-87% against ASR9) (Figure 20).

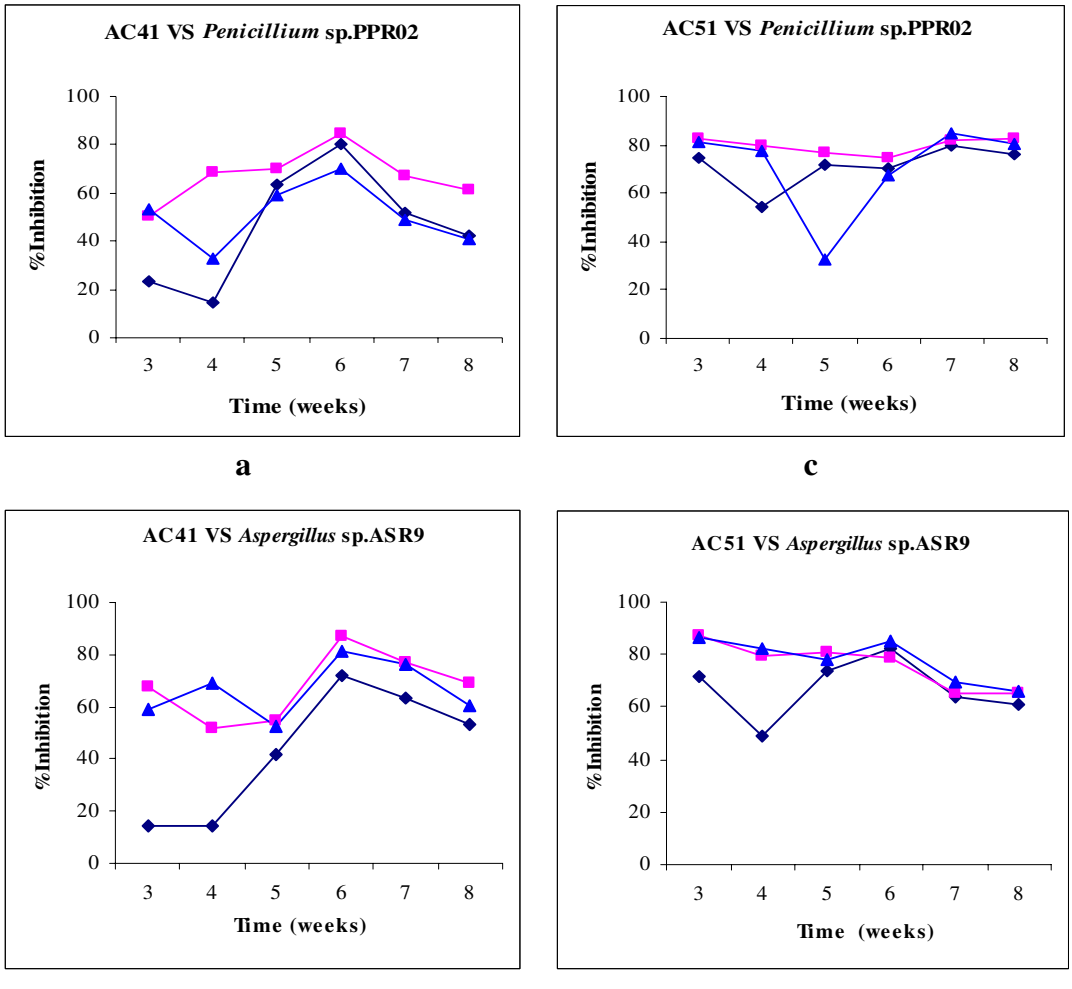


Figure 20 Effect of different initial pH of ISP-2 medium on antifungal metabolite production by actinomycetes AC41 (a and b) and AC51 (c and d). Symbols: ◆, pH 6; ■, pH 7; ▲, pH 8.

3.6.3 Effect of temperature

The temperature for growth and antifungal production of actinomycetes in this study was carried out at 25, 30 and 35 °C in ISP-2 medium pH 7 under static condition for 8 weeks. It was clearly shown that production of antifungal metabolite by actinomycetes AC41 was highest at 30°C on 6 weeks of incubation (Figure 21a, b). It inhibited PPR02 and ASR9 by 84.95 and 86.96%, respectively, and the second best was at 25°C. While actinomycetes AC51 exhibited highest activity against both test strains at 25°C and 30°C on week 3 (Figure 21c, d).

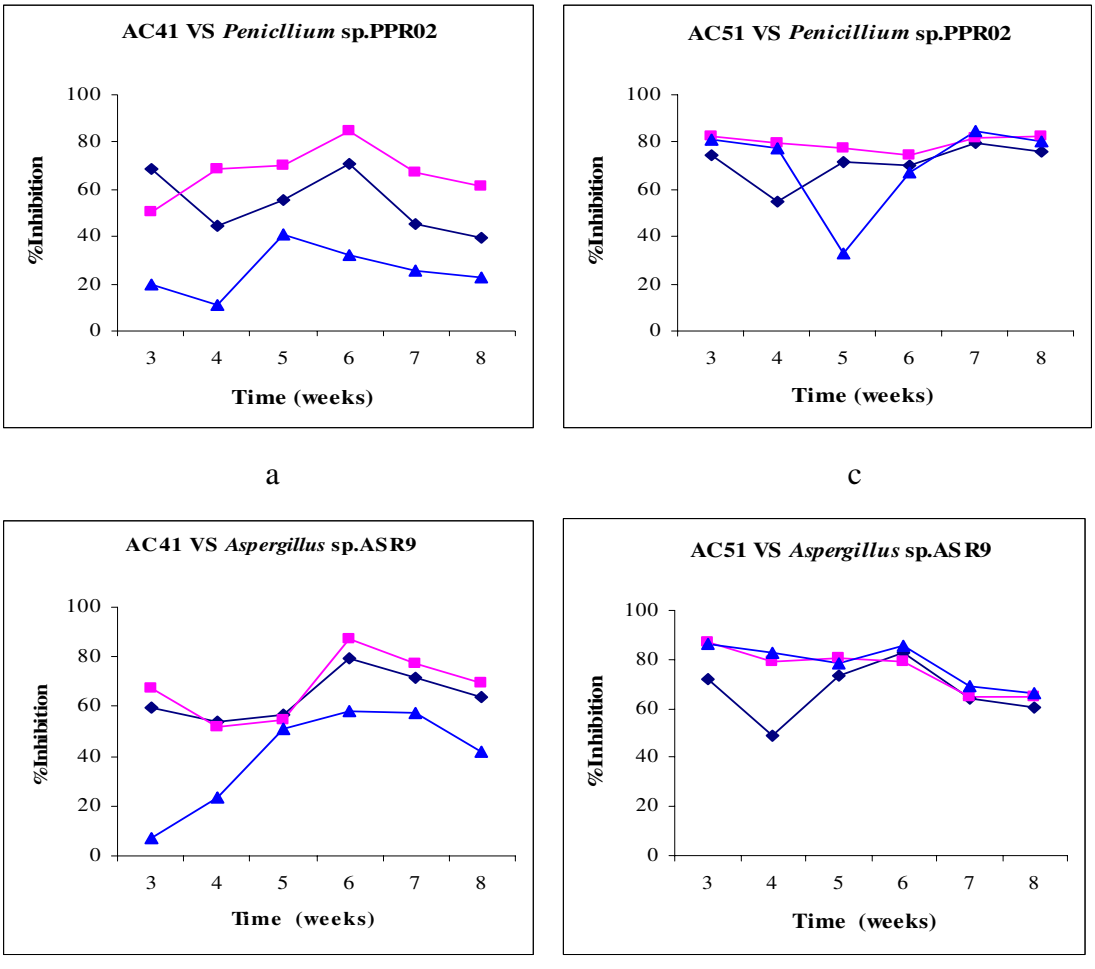


Figure 21 Effect of incubation temperature on antifungal metabolite production by actinomycetes AC41 (a and b) and AC51 (c and d). Symbol: ◆, 25 °C; ■, 30°C ; ▲, 35 °C .

3.7 Identification of actinomycetes

The top 8 antagonistic actinomycetes were identified by morphological characteristics and molecular technique (16S rDNA). All of these actinomycetes are in the genus *Streptomyces*.

3.7.1 Morphological characteristics

All of 8 antagonistic actinomycetes that grew on ISP-2 agar were slow growing, aerobic, glabrous or chalky and with gray aerial mycelia (Figure 22). All isolates possessed an earthy odour. The microscopic examinations of the selected actinomycetes showed that the spore chains are Spira as shown in Figure 23. These morphological characteristics suggest that the selected actinomycetes can be identified as *Streptomyces*.

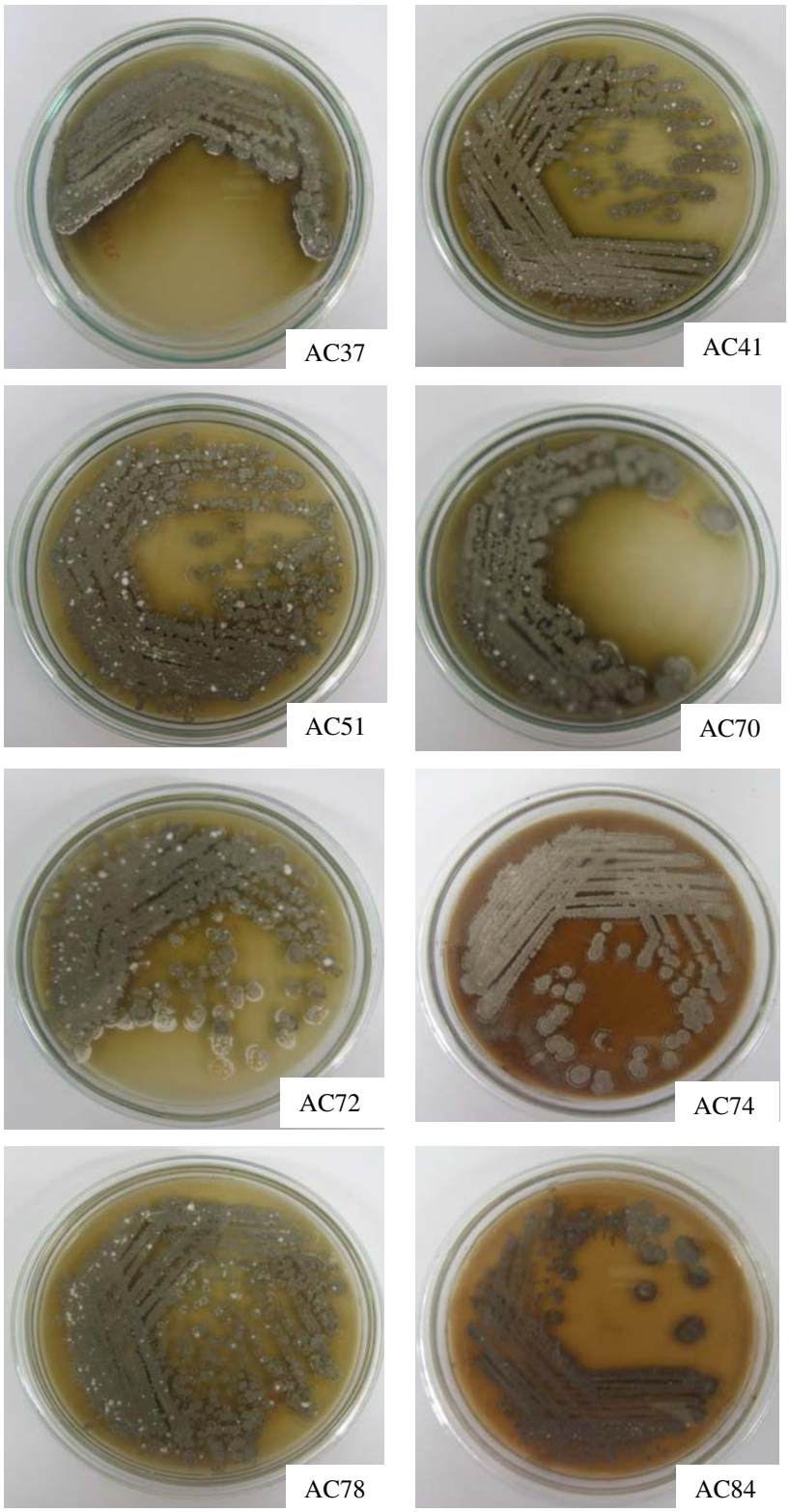


Figure 22 Morphology of 8 potential antagonistic actinomycetes growing on ISP-2 at 30 ^{o}C for 1-2 weeks

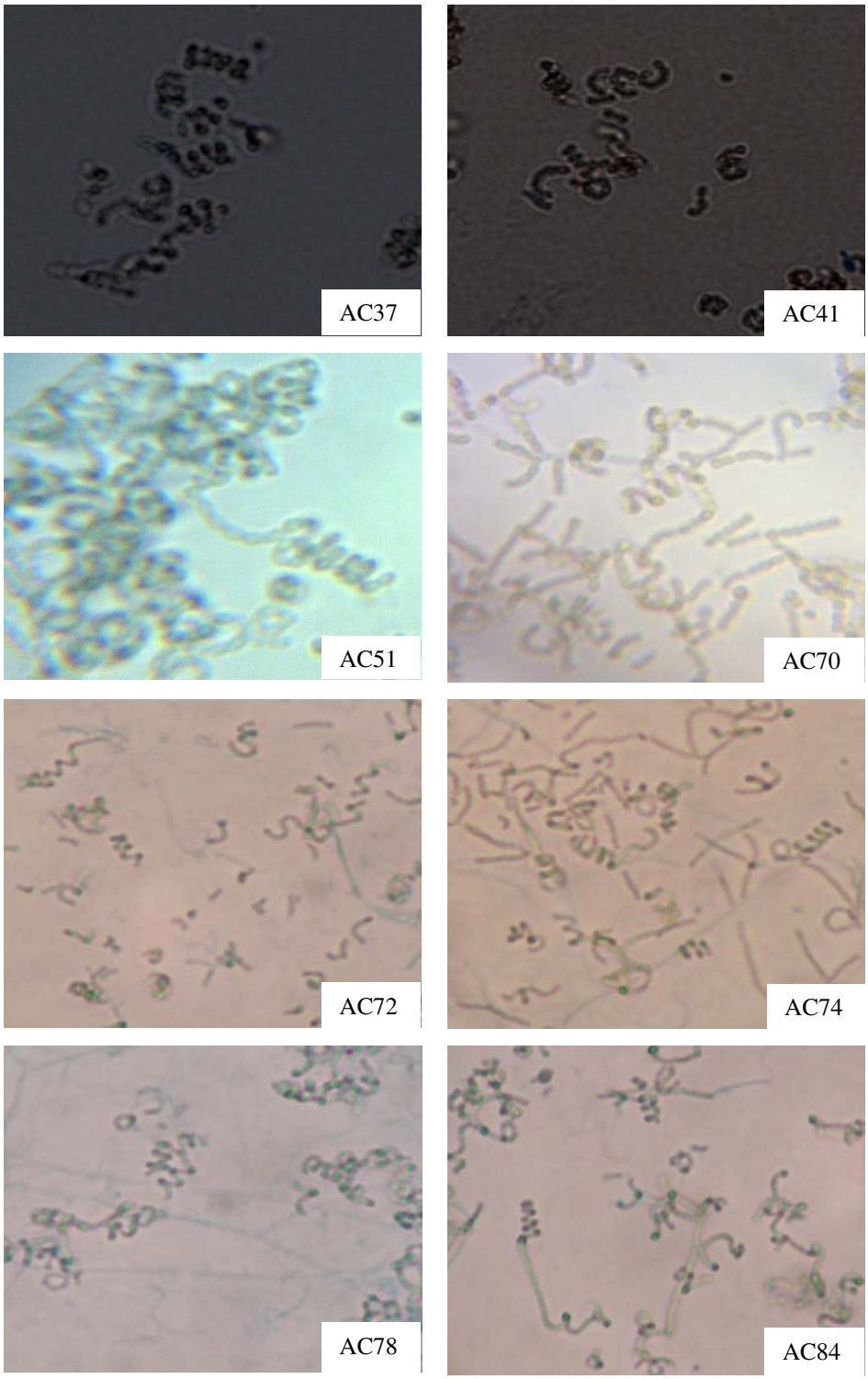


Figure 23 Microscopic morphology of 8 potential actinomycetes under light microscope (X100) showing spiral type of spore chains

3.7.1 Molecular technique (16S rDNA)

The 16S rDNA alignment consisted of 16 taxa in *Streptomyces*, with NR025783 *Kitasatospora terrestris* and NR 025784 *Kitasatospora peranensis* as an outgroup. The dataset comprised 543 characters, 527 of which were constant characters, 5 were uninformative characters and 11 were informative characters. Maximum parsimony analysis yielded 1 MPT. Tree as estimated by Kishino and Hasegawa (K-H) test is shown in Figure 24 with tree length, CI and RI of 17 steps, 1.0000 and 1.0000 respectively. The tree from NJ analysis had an identical topology to the tree from the MP analysis.

Actinomycetes AC37, AC41, AC51, AC70, AC72, AC74, AC78 and AC84 were placed in subclade A (Figure 24), and were closely related to *Streptomyces* sp. with short branch length and supported with 88% and 87% bootstrap values from the MP and NJ analyses respectively (Figure 24 and 25). The nucleotide identity between actinomycetes AC37, AC41, AC51, AC70, AC72, AC74, AC78, AC84 and taxa within this subclade A varied from 99.8% - 100%. *Streptomyces* sp. EU 490287, *Streptomyces pulveraceus* EU240417 and *Streptomyces* sp. DQ450946 were the most closely related with 100% sequence similarity. The sequence similarity of actinomycetes AC37, AC41, AC51, AC70, AC72, AC74, AC78 and AC84 was 100%.

Therefore, actinomycetes AC37, AC41, AC51, AC70, AC72, AC74, AC78 and AC84 were identified to the genus *Streptomyces*.

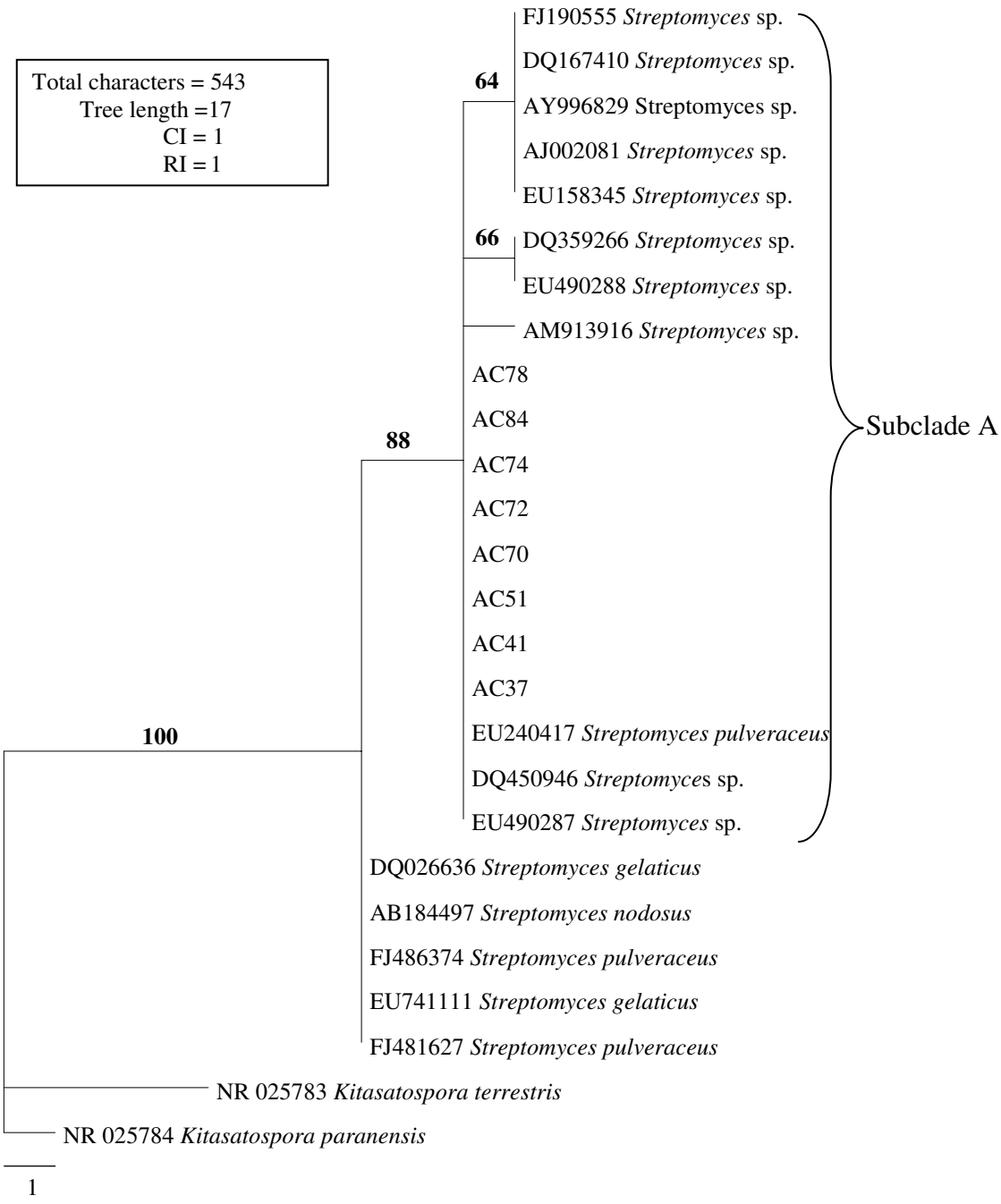


Figure 24 Phylogram obtained from 16S rDNA sequence analysis of actinomycetes AC37, AC41, AC51, AC70, AC72, AC74, AC78 and AC84 and related *Streptomyces* species from Maximum parsimony analysis

Figure 25 Phylogram obtained from 16S rDNA sequence analysis of actinomycetes AC37, AC41, AC51, AC70, AC72, AC74, AC78 and AC84 and related *Streptomyces* species from Neighbor-joining analysis

CHAPTER 4

DISCUSSION

Fungal contamination of para rubber sheet causes a big problem after production, because it lowers the grade and price of the sheets. In addition these fungi could be dangerous to the health of workers and producers. In the past *p*-nitrophenol was used to prevent fungal growth on para rubber sheet (Fullerton, 1929). However, *p*-nitrophenol is toxic and carcinogenic to anyone exposed to it. (http://www.jtbaker.com/msds/englishhtml/N6040.htm). Therefore *p*-nitrophenol is now forbidden for use in para rubber sheet production. At present, contamination by fungi cannot be solved. Chanduaykit (2008) reported that sodium metabisulphite, potassium sorbate, potassium benzoate, acetic acid and smoked acid from bamboo can effectively inhibit fungal growth. For bamboo smoked acid, a high concentration (100%) was needed to inhibit hyphal growth. Most other commercially available antifungal agents are highly toxic and not environmentally friendly. Therefore there is big incentive to identify natural antifungal agents with low toxicity.

This study aimed to isolate antagonistic bacteria that could control fungal contamination on para rubber sheets. The indicator fungi used comprised *Rhizopus* sp.SR12, *Fusarium* sp.SR2, *Aspergillus* sp. SR9, *Aspergillus* sp.NY05, *Penicillium* sp.PR02 and *Cladosporium* sp.TT013 all previously isolated from moldy para rubber sheets from 14 provinces in southern Thailand. All isolates were common airborne fungi and most were resistant to antifungal agents as reported by Chanduaykit (2008). Antagonistic bacteria could be an alternative source of antifungal agents used to control fungal contamination of para rubber sheets. This method has been successfully used to control plant pathogenic fungi with *Bacillus* spp. and actinomycete isolates being potential biocontrol agents (Crawford *et al*., 1993; Michereff *et al*., 1994; Korsten *et al*., 1995; Yuan and Crawford, 1995; Mari *et al*., 1996; Singh *et al*., 1999). Therefore these two groups of bacteria were screened for their ability to inhibit hyphal growth of the tested fungi mentioned above and all previously isolated from contaminated para rubber sheets.

4.1 Antagonistic *Bacillus* spp.

Bacillus spp. are well known soil organisms with many biological activities (Nicholson, 2002). In this study, a total of 206 isolates of *Bacillus* spp. were tested for their inhibitory activity against the test fungi. We newly isolated 102 *Bacillus* spp. from various types of soils from seven locations in the southern part of Thailand. Ninety eight isolates were provided by Dr.Metta Ongsakul and all having been previously isolated from soils and screened for producing biosurfactants (Akkosuwan, 2006; Pradubseang, 2006). *Bacillus subtilis* and *B. pulmilus* are reported to produce surfactin, one of the most potent biosurfactants (Mulligan, 2005) and shown to have antifungal activity (Thimon *et al*., 1992) against various fungi including *A. flavus* (Zhang *et al*., 2008; Mohammadipour *et al*., 2009). The other six *Bacillus* isolates were provided by Assistant Professor Dr.Wichitra Leelasuphakul and were known to have antifungal activity against various fungal plant pathogens. However, only 16% of all these isolates inhibited at least one test fungus using a dual culture method and most exhibited only weak (+) and moderate (++) antifungal activities. However, three *Bacillus* isolates including MK007, 155 and B211 did inhibit all the test fungi. They are from a *Bacillus* group well known for their antifungal activity. *Bacillus* spp. MK007 has been studied extensively. It was isolated from rice field soil from Phatthalung. It has exhibited antifungal activity against rice sheath blight *R. solani*, rice blast *Pyricularia grisea*, and other rice pathogens *Rhynchosporium oryzae*, *Curvularia lunata* and *Fusarium moniliformis*. Its antifungal compounds were identified to be surfactin and iturin A (Leelasuphakul *et al*., 1999). *Bacillus* are widely used as biological control agents of fungal plant diseases such as *Verticillium dahliae*, a cause of cotton *Verticillium* wilt (Zhu *et al*., 2009), *Alternaria solani*, *Botrytis cinerea*, *Monilia linhartiana*, *Phytophthora cryptogea*, *Rhizoctonia* sp. (Todorova and Kozhuharova, 2009) and *Fusarium oxysporum* (Gajbhiye *et al*., 2010). Chitarra *et al*., (2003) reported that *B. subtilis* strain YM10-20 produced antifungal peptides that can inhibit fungal growth of the common contaminating fungal species; *Aspergillus*, *Penicillium*, *Fusarium* and *Mucor* species. There is only one study of a bacterial antagonist, isolated from sheet rubber, in India. This isolate could inhibit *Penicillium* spp. and *Aspergillus* spp. (Joseph *et al*., 2005). They reported that the

antifungal metabolites isolated from two unidentified bacteria obtained from rubber products included siderophores, HCN and salicylic acid. However in this study only weak to moderate antifungal activities were observed from the many *Bacillus* spp. This indicated that the group of fungi isolated from contaminated rubber sheets were much harder to control than were plant pathogens. Therefore, no further studies were undertaken on these *Bacillus* spp.

4.2 Antagonistic actinomycetes

The actinomycetes are well-known antimicrobial producing organisms and are known to suppress the growth of a wide variety of fungal pathogens (Crawford *et al*., 1993; El-Abyad *et al*., 1993b; Yuan and Crawford, 1995; Trejo-Estrada *et al*., 1998). *Streptomyces* species have been used extensively for the biological control of several strains of *F. oxysporum*, that cause wilt disease in many plant species such as cotton (Reddi and Rao, 1971), carnation (Lahdenpera and Oy, 1987), asparagus (Smith *et al*., 1990), French bean (El-Abyad *et al.*, 1993a) and tomato (El-Shanshoury *et al*., 1996). Many antibiotics produced by actinomycetes have been used directly or have been assumed to be responsible for the biocontrol potential of the producing strain (Rothrock and Gottlieb, 1984; Smith *et al*., 1990; Trejo-Estrada *et al*., 1998).

Actinomycetes is a nontaxonomic term for a group of common soil microorganisms (Loynachan, 2008). Most of the actinomycetes used in this study were isolated from soils and comprised 77 new isolates and 64 isolates that had previously been shown to inhibit the growth of *R. solani*, a pathogen of Bambarra groundnut (*Vigna subterranea*) (Sukhoom and Chuenchitt, 2008). Only ten isolates used in this study had been isolated from rubber sheets. From the primary screening using the dual culture method against the six tested fungi, a high percentage (85%) of actinomycetes inhibited fungal growth (Table 12). The number of active isolates obtained from this study is very high. In the last ten years, there have been many studies that have screened for antifungal producing actinomycetes from soil. The results obtained varied widely from 8 to 86% of the isolates. Most of the studies on their effects on fungi have focused on human and plant pathogenic fungi. There have

been no reports on the antifungal activity of actinomycetes against fungi associated with para rubber sheets. Kitouni *et al*. (2005) found that 56% of their actinomycete isolates had antibacterial activity while only 8% had antifungal activty against potential human yeast pathogens (*Candida albicans* and *C. tropicalis*) and molds (*A. fumigatus*, *A. niger* and *F. oxysporum*). Ouhdouch *et al*. (2001) searched for non-polyenic antifungal antibiotics from 320 actinomycetes isolated from several Morrocan habitats such as soil from the rhizhosphere, mountain soils, Saharan sand, dung, sediments and manure and found that only 10% of the isolates showed strong activity against human fungal pathogens. In addition, Bonjar *et al*. (2005) assayed 110 soil actinomycetes for growth inhibitory activity against the phytopathogenic fungi *Verticillium dahliae*, *Alternaria solani*, *Fusarium solani* and *Geotrichum candidum*. Only 10 strains (9%) showed antifungal activity. In 2004, Iznaga *et al*. reported that 51% of actinomycetes isolated from Cuban soils produced antifungal compounds, mostly polyene macrolide antibiotics. Basilio *et al*. (2003) also reported a high prevalence (69%) of antimicrobial producing actinomycetes from soils and 23% showed antifungal activity against *A. fumigatus*, *C. albicans* and *Saccharomyces cerevisiae*. Augustine *et al*. (2005) found *Streptomyces rochei* AK39 that was isolated from soil produced metabolites active against only dermatophytes whereas yeast, *Aspergillus niger* and *Fusarium oxysporum* were resistant. In Thailand, Prapagdee *et al*. (2008) assessed some indigenous actinomycetes isolated from rhizosphere soils for antagonism against the plant pathogenic fungi *Colletotrichum gloeosporioides* and *Sclerotium rolfsii*. Among 146 strains tested, 86% inhibited the growth of *C. gloeosporioides*, 8% inhibited *S. rolfsii*, and 7% inhibited both pathogens. In the study described here, 64 isolates (31%) inhibited all the six tested fungi. All of them were from the actinomycete group that had been shown to produce antifungal activity. However, only 30 of the isolates with the highest inhibitory activity were selected for further studies.

Despite exhibiting moderate to strong activity by the dual culture assay, 1-month-old culture broths from these active actinomycetes inhibited hyphal growth of the test fungi to varying extents as follows: *Rhizopus* sp. SR12 (43-99%), *Fusarium* sp. SR2 (10-99%), *Aspergillus* sp. SR9 (22-94%), *Aspergillus* sp. NY05 (7-93%), *Penicillium* sp. PR02 (26-88%) and *Cladosporium* sp. TT013 (8-94%) (Table 14). In

the dual culture assay, each actinomycete isolate was first streaked on the agar surface until it produced spores. The sporulation times varied among actinomycetes, the slow growers may need 2 to 5 weeks to sporulate yet the fast growers require only 1 to 2 weeks. So the fast growers may produce more antifungal metabolites in the 1-month-old culture broths than those from the slow growers. However, the culture filtrates of strains AC41 and AC51 showed the strongest antifungal activity against all tested fungi with more than 80 % hyphal growth inhibition which agrees with the results from the dual culture assay. Both assays use the standard protocols that most investigators have used to detect antifungal activity against most filamentous fungi (Bonjar *et al*., 2005; Jiménez-Esquilín and Roane, 2005; Prapagdee *et al*., 2008). The results from both assays indicated that the active isolates released extracellular diffusible metabolites into the agar and broth media.

To study the secondary metabolites of actinomycete, many organic solvents have been used to extract them from culture broths such as ethyl acetate (Kathiresan *et al*., 2005; Singh *et al*., 2009), a mixture of chloroform-ethyl acetate (El-Mehalawy *et al*., 2005) and methanol (Basilio *et al*., 2003; Kavitha *et al*., 2010). In our study, culture filtrates of the top 30 antagonistic actinomycetes were extracted with ethyl acetate and tested for their antifungal activity using the microdilution broth test. Only 27 to 53% of the extracts showed inhibitory activity at the concentration of 200 µg/ml (Table 15). It has been mentioned previously that the assay methods that use the dual culture technique and hyphal growth inhibition of culture broth mainly determine diffusible and soluble metabolites but extracts obtained by ethyl acetate extraction contained most of the nonpolar compounds. In this study, eight extracts from AC37, AC41, AC51, AC70, AC72, AC74, AC78 and AC84 exhibited antifungal activity against all the tested fungi. Their MICs ranged from 8-200 µg/ml. The best MICs were from AC41 and AC51 (16-64 µg/ml) which were similar to those of the antifungal control agents, *p*-nitrophenol (32-128 µg/ml). The culture broths of these actinomycetes showed more than 50% fungal growth inhibition. A report from Frändberg *et al*. (2000) revealed that bafilomycins B1 and C1 compounds produced by *Streptomyces halstedii* K122 were equally active against the fungal species tested (*A. fumigatus*, *M. hiemalis*, *P. roqueforti*, *Paecilomyces variotii*, and *P. roqueforti*), with

MIC values in the range of <0.5-64 µg/ml. The MIC values and identities of the antifungal compounds from AC41 and AC51 will be determined in the future.

4.3 Optimization of the production of antifungal metabolites by actinomycetes AC41 and AC51 with potential to control fungal growth on para rubber sheets

Optimization of culture conditions is an essential step to obtain high yields of metabolites. Nutritional factors have been reported to play an important role during the metabolite synthesis process, such as media components and cultural conditions including aeration, agitation, pH, temperature and incubation time and these vary from organism to organism (Vilshes *et al*., 1990; Holmalahti *et al*., 1998; Yu *et al*., 2008). In this study, effects of agitation, pH of initial medium and incubation temperature on the production of antifungal compounds by *Streptomyces* AC41 and AC51 were investigated.

Complex media are routinely used in the fermentation industry. From our screening test by the dual culture method on ISP-2 agar, a high number of antifungal producing isolates were obtained. Therefore, ISP-2 broth was used in this study for seeding the fermentation media. ISP media were developed by Difco Laboratories for the International *Streptomyces* Project (ISP). ISP-2 is also referred to as Yeast Extract-Malt Extract agar and contains 0.4% yeast extract, 1% malt extract and 0.4% dextrose. Yeast extract and malt extract provide nitrogen, amino acids and vitamins, and dextrose is the main carbon source (http://www.bd. com/ds/technical Center/inserts/ISP_Medium_1_2 & 4_.pdf). It is the traditional medium used to determine the cultural characteristics of actinomycetes. However, it has also been used by many investigators for production of antimicrobial agents by actinomycetes (Taechowisan *et al*., 2005; Badji *et al*., 2006; Boudjella *et al*., 2006; Prapagdee *et al*., 2008). In addition, Badji *et al*. (2006) investigated the production of antibiotic substances from *Actinomadura* sp. AC104 using several culture media and found that the best antimicrobial activities were obtained on the ISP-2 medium.

The ability of streptomycete isolates to produce antibiotics is not a fixed property but can be greatly increased or reduced under different conditions of cultivation (Waksman, 1961). In this study, the production of antifungal agents by

Streptomyces AC41 and AC51 was carried out in static and shaken cultures. The better antifungal activity was obtained by both strains in static cultures rather than shaken cultures. Antibiotics are considered to be secondary metabolites produced at the idiophase rather than during the trophophase. It seems that a high degree of oxygen transfer during the exponential growth phase may ultimately lead to improved antibiotic production. Shake flask condition have been reported, by many investigators, to be an essential parameter for optimum antibiotic yields (Chandra and Nair, 1995; Devi and Sridhar, 1999; Bonjar *et al*., 2005; Kathiresen *et al*., 2005; Pragpadee *et al*., 2008; Yu *et al*., 2008; Singh *et al*., 2009; Thakur *et al*., 2009; Kavitha *et al*., 2010;). Rollins *et al*. (1988) reported that during the rapid growth phase of *Streptomyces clavuligerus*, in non shaking conditions the level of dissolved oxygen (DO) dropped to almost zero for a period of approximately 10 h and this resulted in delaying and lowering the production of cephamycin C. A 2.4-fold increase in cephamycin C production was observed when the DO was controlled at a saturation level throughout the fermentation (Yegneswaran *et al*., 1991). In contrast, other studies have reported that fermentation under static condition leads to a higher production of antimicrobial metabolites than in shake cultures (Hassan *et al*., 2001; El-Sersy and Abou-Elela, 2006; Al-Zahrani, 2007). The negative effect of shaking was also observed with *S. lividans* W25 in the production of deacetoxycephalosporin G (DAOG). With shaking, DAOG production at 5 h was 64 mg/l while under static conditions 106 mg/l was obtained (Gao *et al*., 2003). Furthermore, different compounds may be obtained using different culture conditions. Puder *et al*. (2001) isolated 3 compounds (streptazolin, streptazone A and 5-*O*-(*β*-D-xylopyranosyl) streptazolin) from *Streptomyces* strain A1 from shaking cultures and 5 compounds (streptazolin, streptazone A, 9-hydroxy-streptazolin, 13-hydroxystreptazolin and streptenol E) from fermentation in a stirred 50 L fermentor.

The pH of the growth medium is also important in influencing antibiotic production because the activity of several major enzymes that catalyze metabolic reactions of cell growth and antibiotic formation are affected by pH (Guimaraes *et al*., 2004; Liang *et al*., 2008). Streptomycetes are normally considered to prefer a neutral to alkaline environmental pH and a growth pH range from pH 6.5–8.0 (Locci, 1989) with an optimum pH around 7.0 (Jensen, 1930). However,

acidophilic and alkalophilic streptomycetes have also been found (Bok *et al*., 1984; Vyas *et al*., 1990). Among the common habitats of streptomycetes, soil has a low mean pH of 3.5–6.8 (Hagedorn 1976; Erviö *et al*. 1990). Acidophilic streptomycetes appear to be restricted to acidic soils and litter; neutrophilic species are most numerous in soils close to neutrality but also occur in low numbers in acidic soils (Williams and Mayfield, 1971). Acidophilic actinomycetes were distinguished from the neutrophilic ones on the basis of their reaction to a number of physiological tests. Acidophilic actinomycetes were presumably involved in decomposition process in acid soils and their exo-enzymes such as chitinase and amylase were more adapted to function at a low pH than those from neutrophiles (Williams and Flowers, 1978). Kim *et al*. (2008) reported that an acidic pH shock induces the expressions of a wide range of stress-response genes in *Streptomyces coelicolor*. From these observations, an acidic pH shock was considered to be one of the strongest stresses to affect a wide range of sigma factors and shock-related proteins including the general stress response proteins. The upregulation of the sigma factors and shock proteins was found to be related to actinorhodin biosynthesis and was considered to have contributed to enhance actinorhodin productivity by mediating the pH shock signal that regulates the biosynthesis genes for actinorhodin production. Kontro *et al*. (2005) studied the pH effects on growth and sporulation of ten *Streptomyces* spp. They found that the growth pH ranges and pH ranges for the optimal growth of those *Streptomyces* spp. were strongly dependent on the nutrient composition of the media. However the ability of *Streptomyces* spp. to sporulate were independent of the pH and medium composition. The strain-specific differences in sporulation were greater than those caused by pH. However, there were no clear differences in media composition that could be identified to explain the slight differences observed in the optimal values for their sporulation. In this study, the best antifungal activity from AC41 and AC51 was obtained from the ISP-2 medium with an initial pH of 7.

The growth temperature was found to regulate the biosynthesis of secondary metabolites by the culture. In our study, the highest antifungal activity of AC41 and AC51 was obtained at 30 ^{o}C. Kuznetsov *et al*. (1984) studied the regulation of the biosynthesis of secondary metabolites in *Streptomyces galbus* and found that the organism synthesized actinomycin at 28 ^{o}C, but it switched to the production of

melanoid pigments at 42 ^{o}C. This may be considered to be a protective reaction by the organism to an increase in the temperature of the environment and to protect against UV radiation that under natural conditions possibly increases as a consequence of temperature elevation. Liao *et al*. (2009) reported the effect of fermentation temperatures between 28 and 42 ^{o}C on production of the antifungal aminoglycoside antibitotic validamycin A (VAL-A) by *Streptomyces hygroscopicus* 5008. An interesting threshold of temperature for VAL- A biosynthesis was found between 35 and 37 ^{o}C. The result of this work indicated that a relatively high fermentation temperature brought about a vigorous pentose-phosphate pathway (PPP) metabolism, a higher protein synthesis rate and rapid increase of transcription level and consequently a high VAL-A productivity.

The environmental factors like incubation temperature, pH and incubation period were also found to have a profound influence on antibiotic production by *Streptomyces* (Srinivasan *et al*., 1991; Singh *et al*., 2009). The optimum culture conditions for producing antifungal agents from many studies, have varied. In 2009, Oskay reported that the optimum conditions for antimicrobial production by *Streptomyces* sp. KEH 23 was an initial pH of 7.5, a temperature of 30 ^{o}C under shaking condition. The optimum conditions for *Streptomyces tanashiensis* strain A2D was 28 ^{o}C, at a pH of 8 under shaking condition and this strain grew up to pH 9, and indicated that this strain was part of the alkaliphilic actinomycetes group (Singh *et al*., 2009). Thakur *et al*. (2009) reported the maximum production of bioactive metabolites from *Streptomyces* sp.20 was highest at 30 ^{o}C. This strain was able to grow in the pH range from 7-8 however, biosynthesis of antimicrobial agent was maximum at pH 7.5. A report by Augustine *et al*. (2005) revealed that *Streptomyces rochei* AK39 produced metabolites with anti-dermatophytes activity at the optimum conditions of pH 7, a temperature of 37 ^{o}C and agitation at 200 rpm. The maximum antimicrobial production by *Streptomyces* spp.ERI-3 was obtained at pH7 and a temperature of 28 ^{o}C (Arasu *et al*., 2009). Bhattacharyya *et al*. (1998) showed that 30 ^{o}C and a pH of 7.0 were the optimum temperature and pH for antibioltic production by *Streptomyces hygroscopicus* D1.5. Narayana and Vijayalakshmi (2008) studied the optimization of antimicrobial metabolites produced by *Streptomyces albidoflavus* and found that the optimum pH for biomass and antibiotic production was 7.0 and this strain also showed

high levels of biomass and antibiotic production when incubated at 35 ^{o}C . The strain was found to be strictly mesophilic for production of secondary metabolites Any extremes of pH and temperature were unfavorable for antibiotic production by any streptomycete. The optimum conditions for the production of antifungal agents by AC41 and AC51 were under static conditions, a pH of 7.0, at 30 ^{o}C for 6 weeks. This indicated that our two *Streptomyces* spp. are mesophilic and neutrophilic.

4.4 Identification of actinomycetes antagonistic to para rubber sheet fungi

The eight actinomycetes strains we used were identified to be in the genus *Streptomyces* based on their morphological and molecular characteristics. *Streptomyces* sp. EU490287, *Streptomyces pulveraceus* EU240417 and *Streptomyces* sp. DQ450946 were the most closely related with our actinomycetes AC37, AC41, AC51, AC70, AC72, AC74, AC78 and AC84 providing 100% sequence similarity. *Streptomyces* sp. EU490287 was isolated from soil in the Indonesian Black Water River Ecosystem. The data from GenBank do not indicate the isolation sources of *Streptomyces pulveraceus* EU240417 and *Streptomyces* sp. DQ450946. *Streptomyces* sp. DQ450946 showed antifungal activity (http://www.ncbi.nlm.nih.gov/nuccore/ 91221406) but *Streptomyces* sp. EU490287 and *Streptomyces pulveraceus* EU240417 had no biological activity. Isolates from Bambarra groundnut field soils have been shown to have antifungal activity against *R. solani*, a pathogen of Bambarra groundnut (*Vigna subterranea*) (Sukhoom and Chuenchitt, 2008). Crude extracts from our eight actinomycetes had high antifungal activity against all the tested fungi, previously isolated from contaminated para rubber sheets. These results indicate that some *Streptomyces* spp. have the potential to control fungal contamination on para rubber sheets.

CHAPTER 5

CONCLUSIONS

5.1 Conclusions

In this study, total of 206 *Bacillus* spp. and 151 actinomycetes were isolated and screened for their antifungal activity against six fungi commonly found on para rubber sheet. One hundred and twenty nine actinomycetes isolates (85%) and only 33 isolates of *Bacillus* spp. (16%) exhibited antifungal activity against at least one fungal isolate. The antagonistic *Bacillus* spp. in this study exhibited only weak and moderate antifungal activities. Actinomycetes exhibited strong antifungal activity and most of the antagonistic actinomycetes (64 isolates) inhibited all the tested fungi. From this study, it is clearly shown that antagonistic actinomycetes had better antifungal activity than the *Bacillus* spp. Thus, the top 30 active actinomycetes that can inhibit all the tested fungi were selected for fermentation in broth medium and chemical extraction. The culture filtrates from isolates AC41 and AC51 showed >80% inhibitory activity against all the tested fungi. The ethyl acetate crude extracts of the culture filtrates were determined for their MICs and MFCs by the colorimetric broth microdilution method. The MICs of the most active extracts against six tested fungi ranged from 8-200 µg/ml. The best MICs were in the range of 16-64 µg/ml by AC41BE and AC51BE which were comparable to *p*-nitrophenol, a control antifungal agent (32-128 µg/ml). All extracts had MFCs of >128 µg/ml.

Three factors including agitation, pH and temperatures were studied for the optimum production of antifungal metabolites by actinomycetes AC41 and AC51. The optimum conditions were observed to be under static condition, pH7 and temperature 30^{o}C for 6 weeks.

The top eight antagonistic actinomycetes were identified by morphological characteristics and molecular technique (16S rDNA) to be in the genus *Streptomyces*.

5.2 Suggestion for future work

1) Further testing of the most active crude extracts from *Streptomyces* sp. AC41 and AC51 on fungal challenged para rubber sheet.

2) Purification and structural identification of the bioactive compounds from the most active *Streptomyces* sp. AC41 and AC51.

3) Study mechanism of action of bioactive compounds from *Streptomyces* sp. AC41 and AC51.

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APPENDIX 1

Actinomycete Isolation Agar (AIA)	**1 litre**
Sodium caseinate	2.0 g
Asparagine	0.1 g
Sodium propionate	4.0 g
Dipotassium phosphate	0.5 g
Magnesium sulfate	0.1 g
Ferrous sulfate	0.001 g
Agar	15.0 g

Potato dextrose agar (PDA)	**1 litre**
Potato	200.0 g
Dextrose	20.0 g
Agar	15.0 g

Double strength potato dextrose agar	**1 litre**
Potato	400.0 g
Dextrose	40.0 g
Agar	30.0 g

Potato dextrose broth (PDB)	**1 litre**
Potato	200.0 g
Dextrose	20.0 g

Nutrient Agar (NA)	**1 litre**
Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g

Yeast extract-malt extract agar (ISP-2)	**1 litre**
Malt extract	10.0 g
Yeast extract	4.0 g
Glucose	4.0 g
Agar	20.0 g
pH	7

Yeast extract-malt extract broth (ISP-2)	**1 litre**
Malt extract	10.0 g
Yeast extract	4.0 g
Glucose	4.0 g
pH	7

1.8% resazurin

Add 1.8 g of resazurin dye to 100 ml of distilled water and mix thoroughly. Filter resarurin dye solution with membrane 0.45 µm and store in eppendorf wrapping with foil at 4 ^{o}C. Dilute 1.8% resazurin with sterile distilled water to 1:10 and mix thoroughly before using for antimicrobial test.

APPENDIX 2

Table 17 Code, source and antifungal activity of *Bacillus* spp.

Code of *Bacillus* isolate	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
New isolates from soil isolated by Sirinut Duangsook						
SM1/1	-	-	-	-	-	-
SM1/2	-	-	+	-	++	-
SM1/3	-	-	-	-	-	-
SM1/4	-	-	-	-	-	-
SM1/5	-	-	-	-	-	-
SM2/1	-	-	-	-	-	-
SM2/2	-	-	-	-	-	-
SM2/3	-	-	-	-	-	-
SM2/4	-	-	-	-	-	-
SM2/5	-	-	-	-	-	-
SM2/6	-	-	-	-	-	-
SA1/1	-	-	-	-	-	-
SA1/2	-	-	-	-	-	-
SA1/3	+	+	-	-	-	-
SA1/4	+	++	-	-	-	++
SA1/5	-	-	-	-	-	-
SA1/6	-	-	-	-	-	-
SA1/7	-	-	-	-	-	-
SA1/8	++	+	-	-	-	+
KN1/1	-	-	-	-	-	-
KN1/2	-	-	-	-	-	-

Table 17 (cont.) Code, source and antifungal activity of *Bacillus* spp

Code of *Bacillus* isolate	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
KN1/3	-	-	-	-	-	-
KN1/4	-	-	-	-	-	-
KN1/5	-	-	-	-	-	-
KN2/1	-	-	-	-	-	-
KN2/2	-	-	-	-	-	-
KN2/3	-	-	-	-	-	-
KN2/4	-	-	-	-	-	-
KN2/5	-	-	-	+	-	-
KN2/6	-	-	-	-	-	-
KN2/7	-	-	-	-	-	-
KN2/8	-	-	-	-	-	+
KN2/9	-	-	-	-	-	-
KT1/1	-	-	-	-	-	-
KT1/2	-	-	-	-	-	-
KT1/3	-	-	-	-	-	-
KT1/4	-	-	-	-	-	-
KT1/5	-	-	-	-	-	-
KT1/6	-	++	+	-	-	-
KT1/7	-	-	-	-	-	-
KT1/8	-	-	-	-	-	-
KT2/1	-	+	-	-	-	-
KT2/2	-	-	-	-	-	-
KT2/3	-	-	-	-	-	-
KT2/4	-	-	-	-	-	-

Table 17 (cont.) Code, source and antifungal activity of *Bacillus* spp.

Code of *Bacillus* isolate	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
KT2/5	-	-	-	-	-	-
KT2/6	-	-	-	-	-	-
KT3/1	-	-	-	-	-	-
KT3/2	-	-	-	++	-	-
KT3/3	-	-	-	-	-	-
KT3/4	-	++	-	-	-	-
KT3/5	-	-	-	-	-	-
KT4/1	-	-	-	-	-	-
KT4/2	+	++	-	-	-	-
KT4/3	-	-	-	-	-	-
KT4/4	-	-	-	-	-	-
KT5/1	-	-	-	-	-	-
KT5/2	-	-	-	-	-	-
KT5/3	-	-	-	-	-	-
KT5/4	-	-	-	-	-	-
KT5/5	-	-	-	-	-	-
KT5/6	-	-	-	-	-	-
KT5/7	-	-	-	-	-	-
KT5/8	-	-	-	-	-	-
KB1/1	-	-	-	-	-	-
KB1/2	-	-	-	-	-	-
KB1/3	+	++	+	++	-	-
PK1/1	-	-	-	-	-	-
PK1/2	-	-	-	-	-	-

Table 17 (cont.) Code, source and antifungal activity of *Bacillus* spp.

Code of *Bacillus* isolate	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
PK1/3	-	-	-	-	-	-
PK1/4	-	++	+	-	-	-
PK1/5	-	-	-	-	-	-
PK1/6	-	-	-	-	-	-
PK2/1	-	-	-	-	-	-
PK2/2	-	-	-	++	-	-
PK2/3	-	-	-	-	-	-
PK2/4	-	-	-	-	-	-
PK2/5	-	-	-	-	-	-
PK2/6	-	-	-	-	-	-
PK2/7	-	-	-	-	-	-
ST1/1	-	-	-	-	-	-
ST1/2	-	-	-	-	-	-
ST1/3	-	-	-	-	-	-
ST1/4	-	-	-	-	-	-
ST1/5	-	-	-	-	-	-
ST1/6	-	-	+	-	-	-
ST2/1	-	-	-	-	-	-
ST2/2	-	-	-	++	-	-
ST2/3	-	-	-	-	-	-
ST2/4	-	-	-	-	-	-
ST2/5	-	-	-	-	-	-
ST2/6	-	-	-	-	-	-
ST3/1	-	-	-	-	-	-

Table 17 (cont.) Code, source and antifungal activity of *Bacillus* spp.

Code of *Bacillus* isolate	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
ST3/2	-	-	-	+	-	-
ST3/3	-	-	-	-	+	-
ST3/4	-	-	-	-	-	-
ST3/5	-	-	-	-	-	-
NT1/1	-	-	-	-	-	-
NT1/2	-	-	-	-	-	-
NT1/3	-	-	-	-	-	-
NT1/4	-	-	-	-	+	-
NT1/5	-	-	-	-	-	-
Isolates having antifungal activity against phytopathogenic fungi provided by Dr.Wichitra Leelasuphakul						
MK007	++	++	++	++	++	+
155	++	++	++	++	++	+
AP042	-	-	-	-	+	+
M1	-	-	+	+	++	+
B211	+	+	++	++	++	+
BSUK	-	-	-	+	++	+

Table 17 (cont.) Code, source and antifungal activity of *Bacillus* spp.

Code of *Bacillus* isolate	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
Isolates having biosurfactant property provided by Dr.Metta Ongsakul						
OI3	-	-	-	-	-	-
O23	-	-	-	-	-	-
HI2	-	-	-	-	-	-
CD1	-	-	-	-	-	-
AW1	-	-	-	-	-	-
YN2	-	-	-	-	-	-
YI3	-	-	-	-	-	-
O22	-	-	-	-	-	-
YA1	-	-	-	-	-	-
HO3	-	-	-	-	-	-
YN1	-	-	-	-	-	-
OB3	-	-	-	-	-	-
HO1	-	-	-	-	-	-
YP3	-	-	-	-	-	-
RB3	-	-	-	-	-	-
HO2	-	-	-	-	-	-
CN1	-	-	-	-	-	-
RO3	-	-	-	-	-	-
SO1	-	-	-	-	-	-
ON1	-	-	-	-	-	-
HV1	-	-	-	-	-	-
OO2	-	-	-	-	-	-
KL2	-	-	-	-	-	-

Table 17 (cont.) Code, source and antifungal activity of *Bacillus* spp.

Code of *Bacillus* isolate	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
SN2	-	-	-	-	-	-
SN3	-	-	-	-	-	-
OZ1	-	-	-	-	-	-
RB1	-	-	-	-	-	-
HI3	-	-	-	-	-	-
OA2	-	-	-	-	-	-
HB1	-	-	-	-	-	-
RB2	-	-	-	-	-	-
SA2	-	-	-	-	-	-
RV1	-	-	-	-	-	-
HZ3	-	-	-	-	-	-
HI1	-	-	-	-	-	-
OO3	-	-	-	-	-	-
OW2	-	-	-	-	-	-
ON2	-	-	-	-	-	-
H21	-	-	-	-	-	-
YC1	-	-	-	-	-	-
VO2	-	-	-	-	-	-
SA1	-	-	-	-	-	-
OC1	-	-	-	-	-	-
CA4	-	-	-	-	-	-
EI3	-	-	-	-	-	-
OW1	-	-	-	-	-	-
RO1	-	-	-	-	-	-

Table 17 (cont.) Code, source and antifungal activity of *Bacillus* spp.

Code of *Bacillus* isolate	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
RX1	-	-	-	-	-	-
RV2	-	-	-	-	-	-
HZ2	-	-	-	-	-	-
OX1	-	-	-	-	-	-
RH1	-	-	-	-	-	-
VJ74	-	-	-	+	-	-
VJ108	-	-	-	++	-	-
VJ17	-	-	-	-	-	-
VJ4	-	-	-	-	-	-
VJ54	-	-	-	-	-	-
VJ29	-	-	-	++	-	-
VJ90	-	-	-	+	-	-
VJ44	-	-	-	-	-	-
VJ96	-	-	-	-	-	-
VJ69	-	-	-	-	-	-
VJ57	-	-	-	-	-	-
VJ73	-	-	-	-	-	-
VJ85	-	-	-	++	-	+
VJ81	-	-	-	-	-	-
VJ98	-	-	-	++	-	-
VJ101	-	-	-	+	-	-
VJ77	-	-	-	-	-	-
VJ41	-	-	-	-	-	-
VJ40	-	-	-	-	-	-

Table 17 (cont.) Code, source and antifungal activity of *Bacillus* spp.

Code of *Bacillus* isolate	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
VJ87	-	-	-	-	-	-
VJ80	-	-	-	-	-	-
VJ45	-	-	-	-	-	-
VJ32	-	-	-	-	-	-
VJ49	-	-	-	-	-	-
VJ58	-	-	-	-	-	-
VJ28	-	-	-	-	-	-
VJ1	-	-	-	-	-	-
VJ31	-	-	-	-	-	-
VJ23	-	-	-	-	-	-
VJ51	-	-	-	-	-	-
VJ13	-	-	-	-	-	-
QO3	-	-	-	-	-	-
YI2	-	-	-	-	-	-
RH2	-	-	-	-	-	-
OX3	-	-	-	-	-	-
XL3	-	-	-	-	-	-
SB1	-	-	-	-	-	-
OB1	-	-	-	-	-	-
VJ35	-	-	-	-	-	-
SO2	-	-	-	-	-	-
AI2	-	-	-	-	-	-
QO1	-	-	-	-	-	-
VJ68	-	-	-	-	-	-

Table 17 (cont.) Code, source and antifungal activity of *Bacillus* spp.

Code of *Bacillus* isolate	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
OO1	-	+	-	-	-	-
RO2	-	-	-	-	-	-
CH1	-	-	-	-	-	-

RSR12 : *Rhizopus* sp.SR12 FSR2 : *Fusarium* sp.SR2

ASR9 : *Aspergillus* sp.SR9 ANY05 : *Aspergillus* sp.NY05

PPR02 : *Penicillium* sp.PR02 CTT013 : *Cladosporium* sp. TT013

+++ strong activity : inhibition zone 6-10 mm.

++ moderate activity : inhibition zone 1-5 mm.

+ weak activity : fungal colony was close to the bacterial streak but could not grow across the streak.

- no activity

SM = Koh Samui, Suratthani

SA = Airport, Suratthani

KN = Khanom, Nakhonsithammarat

KT = Koh Tan, Suratthani

KB = Meuang, Krabi

PK = Khanjanadit, Suratthani

ST = Meuang, Satun

NT = Takbai, Narathiwat

Table 18 Code, source and antifungal activity of actinomycetes

Code of actinomycetes	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
New isolates from soil and para rubber sheet by Sirinut Duangsook						
SN1	-	-	-	-	-	-
SN2	-	-	-	-	-	-
SN3	-	-	++	-	-	-
SN4	-	-	-	-	-	-
SN5	-	-	-	-	-	-
SN6	+	-	-	-	++	++
SN7	-	-	++	++	++	++
SN8	-	-	-	-	-	-
SN9	-	-	-	-	-	-
SN10	-	+	+	+	+	+
SN11	++	-	++	++	-	++
SN12	++	++	++	++	-	++
SN13	-	-	-	-	-	-
SN14	-	-	-	-	-	-
SN15	++	+	++	++	++	++
SN16	++	-	-	-	-	-
SN17	-	-	-	-	-	-
SN18	-	-	-	++	++	++
SN19	++	++	++	++	++	++
SN20	-	++	+	++	++	++
SN21	++	++	++	-	++	++
SN22	++	++	++	++	++	++
SN23	++	++	+	++	+++	++

Table 18 (cont.) Code, source and antifungal activity of actinomycetes

Code of actinomycetes	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
SN24	++	++	++	++	++	++
SN25	++	++	++	++	++	++
SN26	++	++	++	++	++	++
SN27	++	++	++	++	++	++
SN28	++	-	-	++	-	-
SN29	+	-	++	-	++	-
SN30	++	-	++	++	-	++
SN31	++	-	++	++	++	-
SN32	-	+	-	-	-	+
SN33	-	-	-	-	-	-
SN34	-	-	++	++	++	-
SN35	++	-	++	++	++	-
SN36	-	-	-	-	-	-
SN37	+	+	+	++	++	++
SN38	++	++	-	++	++	-
SN39	+	-	++	-	++	-
SN40	-	-	-	++	++	++
SN41	-	++	-	++	++	++
SN42	++	-	-	++	-	-
SN43	++	++	++	++	++	++
SN44	-	++	-	++	++	++
SN45	-	+	+	-	+	+
SN46	+	-	+	++	++	++
SN47	-	++	++	++	++	++

Table 18 (cont.) Code, source and antifungal activity of actinomycetes

Code of actinomycetes	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
SN48	-	-	-	-	-	-
SN49	-	+	++	-	-	-
SN50	++	-	-	-	-	-
SN51	++	-	++	-	++	-
SN52	-	-	+	++	++	++
SN53	++	++	++	++	++	++
SN54	-	+	+	++	++	++
SN55	-	++	+	++	++	++
SN56	-	+	+	+	+	+
SN57	-	+	+	+	+	+
SN58	-	-	-	-	-	-
SN59	++	+	++	++	++	+
SN60	-	-	-	-	-	-
SN61	++	-	-	++	-	-
SN62	++	+	++	++	++	++
SN63	-	-	-	-	-	-
SN64	++	-	++	++	+	++
SN65	-	-	++	++	-	++
SN66	++	+	++	++	++	++
SN67	-	-	-	-	-	-
SN68	++	++	++	++	++	++
SN69	++	+	+	+	++	+
SN70	-	-	+	++	++	++
SN71	-	-	-	-	-	-

Table 18 (cont.) Code, source and antifungal activity of actinomycetes

Code of actinomycetes	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
SN72	-	+	+	-	+	+
SN73	++	++	++	++	++	++
SN74	++	++	++	++	++	++
SN75	-	+	+	++	++	++
SN76		-	-	-	-	-
SN77	-	+	+	-	-	+
SN78	++	-	-	++	-	-
SN79	-	-	-	-	-	-
SN80	-	+	+	++	++	+
SN81	-	+	++	-	-	-
SN82	-	+	+	-	+	+
SN83	++	++	++	++	++	++
SN84	++	++	++	++	++	++
SN85	-	-	-	-	-	-
SN86	-	-	-	-	-	-
SN87	-	-	-	-	-	-
Isolates from Bambara groundnut field having antifungal activity against *Rhizoctonia solani* provided by Dr.Ampaithip Sukhoom						
AC2	-	++	-	++	++	++
AC4	+++	++	+	++	++	-
AC6	-	++	-	++	++	++
AC11	++	++	++	++	++	++
AC12	++	++	++	++	++	-
AC13	++	-	++	++	++	-

Table 18 (cont.) Code, source and antifungal activity of actinomycetes

Code of actinomycetes	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
AC16	+	-	++	++	++	-
AC17	++	++	++	++	++	++
AC18	++	++	++	++	++	++
AC19	+++	-	++	++	++	-
AC20	++	++	-	++	++	-
AC22	++	++	++	++	++	++
AC25	++	++	++	++	++	-
AC27	++	++	++	+++	+++	+++
AC28	++	++	++	++	++	-
AC30	++	++	++	++	++	++
AC31	-	++	-	++	++	++
AC33	-	-	++	++	++	++
AC35	++	++	++	++	++	++
AC37	+++	++	++	++	++	++
AC40	+++	++	+++	+++	+++	++
AC41	++	++	+++	++	+++	++
AC42	++	++	++	++	++	++
AC43	++	++	+++	++	++	++
AC44	++	++	++	++	++	++
AC45	++	++	+	++	++	++
AC46	++	++	++	++	+++	++
AC49	++	++	++	++	++	++
AC50	++	++	++	++	++	++
AC51	+++	+++	+++	+++	+++	+++

Table 18 (cont.) Code, source and antifungal activity of actinomycetes

Code of actinomycetes	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
AC52	+++	+++	++	+++	+++	+++
AC53	+++	+++	++	+++	+++	+++
AC54	+++	++	++	+++	++	+++
AC55	+++	+++	++	+++	+++	+++
AC58	++	++	++	++	++	++
AC59	+++	+++	+++	+++	+++	+++
AC62	+++	++	++	+++	++	++
AC63	-	+	+	+	+	+
AC65	-	++	-	++	++	++
AC68	++	-	-	++	-	-
AC69	++	++	++	++	++	++
AC70	++	+++	+++	+++	+++	+++
AC71	++	+++	++	+++	+++	+++
AC72	++	+++	+++	+++	+++	+++
AC73	++	+++	++	++	++	+++
AC74	++	+++	+++	+++	+++	+++
AC76	+++	+++	+++	+++	+++	++
AC77	++	++	++	++	++	++
AC78	+++	++	++	++	++	++
AC80	+++	++	++	+++	++	++
AC81	++	++	++	++	++	++
AC83	++	+++	++	++	++	+++
AC84	+++	+++	+++	+++	+++	+++
AC85	++	++	++	++	++	++

Table 18 (cont.) Hyphal growth inhibition by actinomycetes

Code of actinomycetes	Antifungal activity against the tested fungi					
	RSR12	FSR2	ASR9	ANY05	PPR02	CTT013
AC86	++	++	+++	+++	++	++
AC88	++	++	++	++	++	++
AC89	-	+	+	++	++	++
AC91	++	++	++	++	++	++
AC94	-	+	++	-	-	-
AC96	++	++	++	++	++	++
AC97	++	++	+++	++	++	++
AC98	++	-	++	+	-	++
AC99	-	-	+	++	++	++
AC100	-	-	-	++	++	++

RSR12 : *Rhizopus* sp.SR12 FSR2 : *Fusarium* sp.SR2

ASR9 : *Aspergillus* sp.SR9 ANY05 : *Aspergillus* sp.NY05

PPR02 : *Penicillium* sp.PR02 CTT013 : *Cladosporium* sp. TT013

+++ strong activity : inhibition zone 6-10 mm.

++ moderate activity : inhibition zone 1-5 mm.

+ weak activity : fungal colony was close to the bacterial streak but could not grow across the streak.

- no activity

Table 19 SC, MIC and MFC of crude extracts from 30 active actinomycetes against tested fungi

Code	FSR2			RSR12			PPR02			SR9			CTT013			ANY05		
	SC	MIC	MFC	SC	MIC	MFC	SC	MIC	MFC	SC	MIC	MFC	SC	MIC	MFC	SC	MIC	MFC
AC27	+	128	>128	-			+	16	>128				+	64	>128	+	128	>128
AC30	-			-			-			-			-			-		
AC37	+	64	>128	+	8	>128	+	32	>128	+	64	>128	+	128	>128	+	128	>128
AC40	-			-			-			-			-			-		
AC41	+	64	>128	+	16	>128	+	32	>128	+	64	>128	+	64	>128	+	64	>128
AC43	-			-			-			-			-			-		
AC44	-			-			-			-			-			-		
AC46	+	200	>128	-			+	32	>128	+	200	>128	-			-		
AC49	-			-			-			-			-			-		
AC50	-			-			-			-			+	200	>128	-		
AC51	+	32	>128	+	16	>128	+	32	>128	+	32	>128	+	64	>128	+	64	>128
AC52	-			-			-			-			-			-		
AC53	+	128	>128	-			+	128	>128	+	64	>128	-			+	200	>128
AC54	-			-			-			-			-			-		
AC55	-			-			-			-			-			-		
Amphotericin B	+	0.5	1	+	0.25	4	+	2	128	+	2	64	+	8	64	+	0.5	32
p-nitrophenol	+	128	>128	+	64	>128	+	32	128	+	64	>128	+	128	>128	+	32	128

FSR2: *Fusarium* sp.SR2 RSR12: *Rhizopus* sp.SR12 PPR02: *Penicillium* sp.PR02

ASR9: *Aspergillus* sp.SR9 CTT013: *Cladosporium* sp. TT013 ANY05: *Aspergillus* sp.NY05

SC= Screening test (200µg/ml) MIC=minimum inhibitory concentration (µg/ml) MFC= minimum fungicidal concentration (µg/ml)

Table 19 (cont.) SC, MIC and MFC of crude extracts from 30 active actinomycetes against tested fungi

Code	FSR2			RSR12			PPR02			SR9			CTT013			ANY05		
	SC	MIC	MFC	SC	MIC	MFC	SC	MIC	MFC	SC	MIC	MFC	SC	MIC	MFC	SC	MIC	MFC
AC59	-			-			-			+	64	>128	+	200	>128	-		
AC62	+	64	>128	-			+	32	>128	-			+	128	>128	+	128	>128
AC70	+	128	>128	+	64	>128	+	128	>128	+	128	>128	+	200	>128	+	200	>128
AC71	-			-			-			-			-			-		
AC72	+	200	>128	+	128	>128	+	200	>128	+	128	>128	+	200	>128	+	200	>128
AC73	-			-			-			-			-			-		
AC74	+	128	>128	+	64	>128	+	64	>128	+	64	>128	+	200	>128	+	200	>128
AC76	-			-			-			-			-			-		
AC78	+	64	>128	+	32	>128	+	32	>128	+	64	>128	+	128	>128	+	128	>128
AC80	-			-			-			-			-			-		
AC83	+	64	>128	-			+	32	>128	+	32	>128	+	128	>128	+	64	>128
AC84	+	64	>128	+	16	>128	+	32	>128	+	64	>128	+	128	>128	+	64	>128
AC86	-			-			+	128	>128	+	200	>128	-			-		
AC91	-			-			+	64	>128	+	200	>128	-			-		
AC97	+	200	>128	-			+	128	>128	+	128	>128	+	200	>128	-		
Amphotericin B	+	0.5	1	+	0.25	4	+	2	128	+	2	64	+	8	64	+	0.5	32
p-nitrophenol	+	128	>128	+	64	>128	+	32	128	+	64	>128	+	128	>128	+	32	128

FSR2: *Fusarium* sp.SR2 RSR12: *Rhizopus* sp.SR12 PPR02: *Penicillium* sp.PR02

ASR9: *Aspergillus* sp.SR9 CTT013: *Cladosporium* sp. TT013 ANY05: *Aspergillus* sp.NY05

SC= Screening test (200µg/ml) MIC=minimum inhibitory concentration (µg/ml) MFC= minimum fungicidal concentration (µg/ml)

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List of Proceedings

Duangsook, S., Phongpaichit S. and H-Kittikun, A. 2009. Screening of antagonistic bacteria for controlling fungal contamination on para rubber sheet. Proceeding of 2nd KMITL Grad. Research Conference. King Mongkut's Institute of Technology Ladkrabang, April, 23-24, 2009. pp. 94.

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