Actinomycetes Potential Application for Biological Control of Banana Crop Disease

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A thesis submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Product Development Technology) with Honours

Faculty of Agro Based Industry

Universiti Malaysia Kelantan

2018

DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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Date:

I certify that the report of this final year project entitled "Actinomycetes Potential Application for Biological Control of Banana Crop Disease" by Noor Hafizah binti Ahmad Nasir, Matric number F15A0107 has been examined and all correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Product Development Technology) with Honours, Faculty of Agro-Based Industry, University Malaysia Kelantan.

Approved by:

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Supervisor

Name: Dr. Khomaizon binti Abdul Kadir Pahirul Zaman

Date:

ACKNOWLEDGEMENT

I would like to express my gratitude to all parties who assisted me in completing this final year project successfully. A special thanks to my supervisor, Dr. Khomaizon binti Abdul Kadir Pahirul Zaman whose contribution in stimulating suggestions and encouragement, helped me to coordinate my project. I would not have completed this research without her guidance.

Furthermore, I would like to acknowledge with much appreciation to the laboratory assistants, who gave the permission to use all required equipment, the necessary apparatus and for their time and knowledge shared with me. My appreciation also goes to University Malaysia Kelantan (UMK) especially the Faculty of Agro Based Industry (FIAT) for constantly providing guidance to complete this project.

Moreover, many thanks goes to my family whose have invested their full efforts in guiding me to achieve the goal, especially in terms of financial and moral support. Not to forget my fellow friends who are always there for me throughout my journey. Last but not least, I would like to give my huge appreciation to everyone who have helped me when I am in need.

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ABSTRACT

Sigatoka disease is one of the banana crop disease caused by *Mycosphaerella*, the pathogenic fungus. In order to control this disease, farmers usually spray their crop using fungicides. However, controlling approaches are not always well known or adopted by all farmers. This study aimed to isolate fungi from soil samples collected from infected banana plant. Besides, to determine antifungal activity of actinomycetes towards isolated fungi. Thirteen fungi were isolated from the soil samples and 10 potential fungi were proceed with antifungal assay. Five actinomycetes strains were picked as test microorganism where Strain 47, Strain 72 and Strain 108 showed positive antifungal activity against isolated fungi. However, Strain 5 and Strain 56 did not show any activities. Further study on identification of the fungi isolates by polymerase chain reaction (PCR) amplification and DNA sequencing should be carried out to confirm the species. Moreover, other microorganisms also can be used to test for antifungal activity towards the isolated fungi.

Keywords: *Mycosphaerella*, Sigatoka disease, actinomycetes, antifungal assay

Keupayaan Actinomycetes sebagai Kawalan Biologi terhadap Penyakit Pokok Pisang

ABSTRAK

Penyakit Sigatoka adalah salah satu penyakit pokok pisang yang disebabkan oleh *Mycosphaerella*, kulat patogenik. Untuk mengawal penyakit ini, petani biasanya merawat tanaman mereka menggunakan racun kulat. Walau bagaimanapun, pendekatan terhadap kaedah rawatan tidak selalu diketahui atau diguna pakai oleh semua petani. Kajian ini bertujuan untuk mengasingkan kulat dari sampel tanah yang diambil dari pokok pisang yang dijangkiti. Selain itu, untuk mengenalpasti aktiviti antikulat oleh actinomycetes terhadap kulat yang telah diasingkan. Tiga belas kulat telah diasingkan dari sampel tanah dan 10 kulat yang berpotensi telah diteruskan dengan ujian antikulat. Lima strain actinomycetes dipilih sebagai mikroorganisma kajian di mana Strain 47, Strain 72 dan Strain 108 menunjukkan aktiviti antikulat positif terhadap kulat yang diasingkan. Walau bagaimanapun, Strain 5 dan Strain 56 tidak menunjukkan apa-apa aktiviti. Kajian lanjut mengenai kulat yang telah diasingkan haruslah menggunakan amplifikasi rantai polimerase (PCR) dan jujukan DNA untuk mengesahkan spesies. Selain itu, mikroorganisma lain juga boleh digunakan untuk menguji aktiviti antikulat terhadap kulat yang diasingkan.

Kata kunci: *Mycosphaerella*, penyakit Sigatoka, actinomycetes, aktiviti antikulat

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LIST OF ABBREVIATIONS

g	gram
h	hour
L	litre
mL	millilitre
cm	centimetre
mm	millimetre
µg/mL	microgram per millilitre
G + C	Guanine plus cytosine
SDA	Sabouraud Dextrose Agar
NA	Nutrient agar
UMK	Universiti Malaysia Kelantan

LIST OF SYMBOLS

%	Percent
±	Plus-minus
°C	Degree Celcius
µ	micro
&	And

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Banana and plantain are ranked fourth after wheat, rice, and corn as it is among the world's most essential staple food crops (Arzanlou et al., 2007). Diseases of banana worrying all farmers as it is hard to cure, or even cannot be cured. Sigatoka disease is one of the severe cases amongst all. Usually farmers will spray their crops with fungicides to prevent the distribution of the disease. Nevertheless, fungicides is not environmental friendly.

Sigatoka disease caused by pathogenic fungus known as *Mycosphaerella*. *Mycosphaerella* species are harmful to the plant as it can reduce the plant growth and caused leaf spotting and necrosis (Aguín, Sainz, Ares, Otero, & Pedro Mansilla, 2013). This disease is a serious threats for banana production worldwide because photosynthetic capacity of plants is reduced, which lead to reduction of crop yield and fruit quality (Arzanlou et al., 2007). Hence, it is crucial to find effective novel antifungal compounds with broad-spectrum activity against the pathogens.

Actinomycetes are the most plentiful sources of antibiotics, antifungal and other specialized metabolites that can be used for agricultural, industrial and medical purposes (Wei, He, & Niu, 2018). Actinomycetes are very useful as it can be used as herbicides, pesticides, antiparasitic agents and many more (Benhadj, Gacemi-Kirane, Menasria, Guebla, & Ahmane, 2018). This research was done to identify the potential of actinomycetes to have activity against pathogenic fungi which can further be used to treat infected banana plant.

1.2 PROBLEM STATEMENT

Previous researches shows that Sigatoka disease is a worldwide problem that damage the production of bananas. This disease is very destructive if it is left uncontrolled. The fungus are easy to breed in damp, windy areas and location with poor drainage systems. In Malaysia, this disease is hard to control because of the high humidity. Most of the local farmers do not aware of this disease so this research is conducted to cater this problem faced by the farmers. Through this research, natural biological agent for the control of banana plant disease can be discovered.

1.3 HYPOTHESIS

1. Fungi that potentially cause the Sigatoka disease can be isolated from soil underneath infected banana plant
2. Actinomycetes can be a source for antifungal activity against isolated fungi

1.4 OBJECTIVE

1. To isolate the fungi from soil sample collected from infected banana plant
2. To determine antifungal activity of actinomycetes towards isolated fungi

1.5 SCOPE OF STUDY

This study consist of fungal isolation from soil underneath infected banana plant. The isolated fungi were then used to test actinomycetes that potentially has the antifungal activity.

1.6 SIGNIFICANCE OF STUDY

This study can help the local farmers to treat infected banana plant with natural biological agent. Study of soil sample infected by Sigatoka disease is essential as it is major cause of economic losses and the severity is worldwide. Besides, antifungal activity of actinomycetes can be identified.

1.7 LIMITATION OF STUDY

No specific fungal strain that cause Sigatoka disease can be used in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 BANANA

The herbaceous plants of the genus *Musa* is commonly known as banana and is important for the production of fruit thus it is largely cultivated (Tock, Lai, Lee, Tan, & Bhatia, 2010). Bananas required short time from planting to harvest (10 to 12 months) and the material needed for planting is easy to reach and cheap resulting the production in 120 countries is very common (Israeli & Lahav, 2016). In Malaysia, total planted area of banana reached 34,000 ha. in 2001 (Abdul Khalil et al., 2006).

The second highly consumed fruit in Malaysia is banana which is around 10 million banana trees are cut down yearly to meet the demand (Baharin, Fattah, Bakar, & Ariff, 2016). It is said to be the main source of carbohydrate consumers in rural and urban areas (Mobambo et al., 1993). It is familiar that most of developing countries treat bananas as the root of economies. Bananas is considered to be the world essential food crop that provides job opportunities and high source of income (Israeli & Lahav, 2017).

Banana has antibacterial and antioxidant activity that gives multiple health benefits such as immunostimulatory activity (Yang et al., 2019). Different food products that utilized banana as an ingredient produce a valuable effect on human health which improves some essential minerals (phosphorus, magnesium, potassium and calcium), total dietary fibre, total starch and resistant starch (Singh, Singh, Kaur, & Singh, 2016). Moreover, it helps in withstand diarrhoea and dysentery, useful in celiac disease, constipation and peptic ulcer (Karuppiah & Mustaffa, 2013).

2.2 FUNGAL INFECTION ON BANANA PLANT

Most fungi are able to produce both sexual spores (ascospores) and asexual spores (conidia) which spread through wind, rain, water and insects then landed on leaf surfaces causes the spores to germinate, sending infectious germ tubes through leaf stomata (Cousin, 2014). Numerous conidia are produced on the infected areas and spread to other plants at ease (Agrios, 2005). Besides, infection on banana plant provokes necrotic lesions on leaves and affect the quality of fruit which fail to ripen and may fall thus cause yield losses (Chillet, Abadie, Hubert, Chilin-Charles, & de Lapeyre de Bellaire, 2009). However, fungi spores would rather attached to the root tip of banana first then mycelia penetrate the epidermal cell wall directly before occupy and occlude the xylem vessels (Siamak & Zheng, 2018).

Despite the popularity of banana, there are limitations on the production which is fungal infection. Panama disease caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), is currently a serious threat to banana production worldwide (Maryani et al., 2019). *Fusarium* spp. has discovered to cause damage on banana plant in Malaysia (Zulkifli &

Zakaria, 2017). The disease is ready to spread within plantation even though separated by long distance and it kills infected plant quickly (Meldrum, Daly, Tran-Nguyen, & Aitken, 2013). Prevention of the fungal infection is vital as the fungus cannot be removed once introduced because of the absent of plant-applied chemical treatment or effective soil (Ploetz, 2015). This disease is hard to control due to limited knowledge on the distribution of the pathogenic fungi (Maryani et al., 2019).

Another fungal infection on banana is caused by *Colletotrichum musae*, that cause the most aggressive disease called Anthracnose (Williamson et al., 2008). The disease attacks during the postharvest of bananas and limits the fruit quality (Town, 2014). Infection by *C. musae* is inactive in the field during early stages of fruit development, therefore disease symptoms only appears on the peel of banana (sunken brown-black lesions) during fruit ripening (Vilaplana, Pazmiño, & Valencia-Chamorro, 2018). Lesions reduced fruit quality and also develop when fruit are injured thus the management should include lessen damage to fruit, refrigeration during shipping and fungicides treatment (Sivakumar & Bautista-Baños, 2014).

2.3 FUNGUS RELATED TO SIGATOKA DISEASE ON BANANA PLANT

Sigatoka disease caused by *Mycosphaerella musicola*, *Mycosphaerella fijiensis* and *Mycosphaerella eumusae* that causes yellow Sigatoka, black Sigatoka and eumusae leaf spot respectively (Surridge, Viljoen, Crous, & Wehner, 2003). The symptoms and life cycles of those three species are quite similar thus the exact dispersal and the epidemiology of the disease left uncertainly (Arzanlou et al., 2007). The disease was first

observed at Sigatoka Valley in the South Pacific island Fiji where the name of the disease comes from (Agrios, 2005).

2.3.1 Yellow sigatoka and black sigatoka

Mycosphaerella musicola caused yellow Sigatoka (Figure 2.1) on banana plant. It reduced photosynthesis and cause foliar necrotic lesions lead to loss of functional leaf area and diminished yield (Smith et al., 2018). Foliar necrotic lesions and reduced photosynthesis caused by yellow Sigatoka result in a reduction in bunch size, fruit number per bunch, and premature ripening in the field and post-harvest (Samuelian, Wright, & Vawdrey, 2016) thus making these bunches are unsaleable and harbour the banana fruit fly (Ramsey, Daniells, & Anderson, 1990).

Figure 2.1: Yellow Sigatoka of banana plant (Agrios, 2005)

Black Sigatoka (Figure 2.2) disease of bananas (also known as black leaf streak), caused by the fungal pathogen *Mycosphaerella fijiensis* is said to be disastrous foliar disease of banana (Landry et al., 2017). This hemibiotrophic filamentous fungus is a worldwide problem that cause the loss of banana fruit industry (Chuc-Uc et al., 2011).

The only way resistant cultivars could think of in those situations is viable means of control (Fullerton & Olsen, 1995). *M. fijiensis* spreads by windborne ascospores that are produced on senescent infected leaf tissues, ascospores and conidia germinate readily and penetrate the leaf surface through stomates at ease with high humidity and the leaf lamina surface turn tan and coalesce as the disease progresses lesions enlarge (Irish, Goenaga, Rios, Chavarria-Carvajal, & Ploetz, 2013).

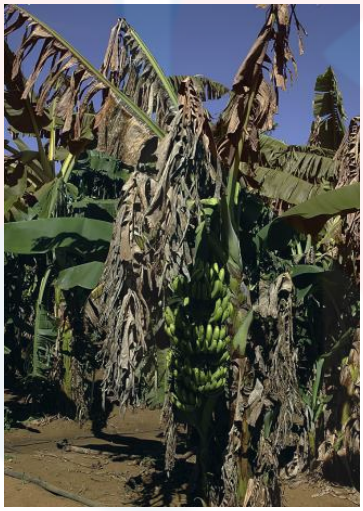


Figure 2.2: Black Sigatoka (black leaf streak) of banana plant (Irish et al., 2013)

The first stage of infections appears as small, light yellow spots or streaks parallel to the side veins of leaves that unfurled about a month earlier and become 1 to 2 centimetres long and turn brown with light grey centres after few days (Agrios, 2005). When the spots enlarge further, the tissue around them turns yellow and dies, and adjacent spots coalesce to form large, dead areas on the leaf and it takes few weeks for the entire leaves to die in severe infections (Meredith & Lawrence, 1970). When the mature leaves have been infected, there are only a few leaves to function thus produce immature fruit bunches and by the time the fruits are near to maturity during heavy infection, the flesh

ripens unevenly, individual bananas appear undersized and their flesh develops a buff pinkish colour (Ramsey et al., 1990).

Previous study about the conidial state of *M. musicola* were insufficient thus Meredith and Lawrence (1970) further research about morphology of *M. musicola* and relatively identify the differences between *M. musicola* and *M.fijiensis* since their symptoms and life cycle seems to be similar. In the mid-1970s, black Sigatoka replaced yellow Sigatoka within two years which the leaves spot occurred 8 to 10 days faster than yellow Sigatoka (Agrios, 2005). Previous studies have shown that there are consistent differences between symptoms caused by the two fungi after natural or experimental infection thus conclude that *M. musicola* and *M. fijiensis* are different species and not merely strains of the same species (Meredith & Lawrence, 1970).

2.3.2 Eumusae leaf spot

Middle of 1990s shows the new elector of the Sigatoka complex, *Mycosphaerella eumusae* (Figure 2.3) that caused Septoria leaf spot of banana appears to be in southern India, Sri Lanka, western Malaysia, Thailand, and Vietnam (Balint-Kurti, May, & Churchill, 2001). Southeast Asia is said to be the centre of origin for all these three species from the worldwide chronology of disease record and *M. eumusae* affects cultivars that are strongly resistant to both *M. musicola* and *M. fijiensis* (Arzanlou et al., 2007). *M. eumusae* attacks leaves resulting in the reduction of the leaf area, thus decreasing the photosynthetic capacity and affecting the growth and productivity of the plants, also affects bunch weight and fruit quality due to premature bunch maturation and results in yield losses (Thangavelu, Ganga Devi, Gopi, & Mustaffa, 2013). Infected plants

by *M. eumusae* showed faster symptom expression than *M. fijiensis* infected plants which is up to 1 week (Balint-Kurti et al., 2001)

2.4 ACTINOMYCETES AS A SOURCE FOR ANTIFUNGAL AGENT

Actinomycetes are aerobic spore forming gram-positive bacteria; containing high guanine plus cytosine (G + C) in their genome that secretes plant growth hormones, growth promoting compounds or helps the growth of beneficial microorganisms thus affects plant growth (Hozzein et al., 2019). Moreover, it is economically and biotechnologically most constructive microorganisms. In addition, well known for the production of wide range of secondary metabolites of various medical values like antibiotics, antifungal, antiprotozoal, antiviral, anticholesterol, antihelminth, anticancer and immunosuppressant (Bhatti, Haq, & Bhat, 2017).

Besides, actinomycetes are useful for many purposes such as agriculture and aquaculture as it is said to be used for biocontrol against fungal pathogen of plant and bacterial pathogen in aquaculture making it interesting to explore due to their potency to produce substances against drug resistance microbes (Nurkanto & Julistiono, 2014). Furthermore, it shows various implications including plant growth promotion, biocontrol agents, improvement in nutritional values and they roughly form 100 genera and 1000 species mainly occupying various types of soils including agricultural soils and have great potential of recycling natural minerals and organic substances in the natural world (Singh, Patil, Prabha, Yandigeri, & Prasad, 2018).

2.5 DISEASE CONTROLLING APPROACHES

A complete eradication of disease caused by fungi is relatively impossible. However, this may be controlled with several methods through cultural method and chemical method. Sigatoka disease can be managed effectively by chemical method such as the use of fungicides. For example dithiocarbamates, benzimidazoles, azoles and strobilurins (Thangavelu et al., 2013). Besides, Agrios (2005) stated that a combination of measures including sanitation, quarantine and application of fungicidal sprays can control Sigatoka disease where in acute areas it is required to apply airplane or ground sprays up to 12 days in a year while another areas with less severity once per 6 weeks is enough. Another examples of fungicides used are mancozeb, chlorothalonil, pyrimethanil, propiconazole, tebuconazole, epoxiconazole and difenoconazole, where those chemicals (except for chlorothalonil) are suggested to be mixed with petroleum-derived spray oil because it can retard the initial infection by the fungi and allow the fungicides to perform better (Samuelian et al., 2016). Furthermore, banana Fusarium wilt disease can be effectively controlled by ammonia fumigation with biofertilizer application (Shen et al., 2019). In addition, mycelia growth of *Colletotrichum musae* that caused Anthracnose disease of banana can be controlled by using thyme oil (Vilaplana et al., 2018)

The banana plant disease is highly depends on fungicide applications supported by cultural practices. Example of cultural method are providing right drainage and avoid water logging, avoid planting at close space and by destruction of diseased leaves but those methods does not provide satisfactory control and therefore chemical control is still required even though it is not environmental friendly (Samuelian et al., 2016). Moreover, the use of biological control (e.g. silicon amendments and companion planting with

Allium spp.), arbuscular mycorrhizal fungi (AMF), plant growth promoting rhizobacteria (PGPR) and crop rotation are another effective strategies to manage the banana disease (Siamak & Zheng, 2018). Furthermore, too much of fungicides gives bad impact towards banana plants and environment because of high amount of chemical residues accumulated. Therefore, another strategy in controlling banana Sigatoka disease is by natural plant products as evaluated by Thangavelu et al., (2013), to use various plant extracts as an alternative nonchemical integrated disease management (IDM) method.

2.6 FUNGAL ISOLATION

Isolation of fungi can be obtained using direct isolation or selective isolation. Various methods applied by the researcher to obtain desired fungi. Cruz-Lachica et al., (2018) obtained the fungi using a serial dilution method. Besides, hair bait technique were used where sterile human hairs were buried in the soil (Kachuei, Emami, Naeimi, & Diba, 2012; Deshmukh, Verekar, & Chavan, 2018). Moreover, surface strerilization method where the surface of fungi was sterilized with commercial bleach to kill adhering spores (Meldrum et al., 2013; Zakaria, Hidayah, & Rahman, 2011; Hu, Chen, Ye, & Hu, 2018). Another method is by using selective media to grow the desired fungi (Smithee, Tracy, Drescher, Pitz, & McDonald, 2014). Furthermore, fungal strains were isolated in a liquid solution (Aftab et al., 2017; Hernandez & Menéndez, 2018).

2.7 ANTIFUNGAL ASSAY

Antifungal activity of actinomycetes was tested by diffusion methods using either wells or disc techniques, double-layer activity test where Ouhdouch, Barakate, & Finance, 2001 suggested that well diffusion is better than disc diffusion technique. Inglin, Stevens, Meile, Lacroix, and Meile (2015) used agar-well diffusion method to perform antifungal assay of *Lactobacillus* species. Another researcher done the antifungal assay by evaluating the ability of the fungi fresh spores to form viable colonies on agar blocks that cut from clear zones formed in dual culture plates with highly antagonistic actinomycetes. After 5 days of incubation, inhibition zone between actinomycetes and fungi were measured (Jayasinghe & Parkinson, 2008). Moreover, disk diffusion method where the use of a disc shape filter paper (6 mm diameter) to absorb sample and release into agar seeded with test organism, was used by Semis, Nahmias, Lev, Frenkel, & Segal (2015). Furthermore, antifungal activity against *Aspergillus* spp. was tested using disk diffusion method (Martos et al., 2012). Chan, Ali, Salleh, Rosli, & Quah (2017) used disk-diffusion method to perform antifungal assay implying the effectiveness of an antifungal agent in the test is based on the size of inhibition zone.

CHAPTER 3

METHODOLOGY

3.1 MATERIALS

3.1.1 Chemical and reagents

The chemical and reagents needed are distilled water, trimethoprim, nutrient agar (NA), Sabouraud Dextrose Agar (SDA), ethanol.

3.1.2 Equipment / apparatus

Equipment and apparatus used were Duran bottle, spatula, petri dish, parafilm, 10 mL falcon tube, test tube rack, micro centrifuge tube, hockey stick, inoculation loop, cork borer, scalpel, 250 mL beaker, 50 mL beaker, pipette, pipette tips, 10 mL measuring cylinder, cotton swab, autoclave bag, bunsen burner, weighing scale, incubator.

3.1.3 Microbial strains

Fungal strains were isolated from soil collected underneath infected banana plant. Whereas, actinomycetes strains were provided by Dr. Khomaizon (UMK Jeli).

3.2 METHODS

3.2.1 Sample collection and pre-treatment process

The soil samples were collected from banana plant infected with Sigatoka disease at Kota Bharu, Kelantan. The soils were collected 10 cm below the soil surface and kept in a zip-lock plastic bag. Soil samples was sun dried for one day.

3.2.2 Fungal isolation

One gram of soil sample was transferred to 10 mL sterile distilled water and mixed for 1 min. The solution was serially diluted up to 10^{-3} with sterile distilled water and 0.1 mL each were inoculated onto Sabouraud Dextrose Agar (SDA) (4% dextrose, 1% peptone, 2% agar). Trimethoprim (25 µg/mL) was used as supplement to the SDA media to inhibit bacteria growth. All plates were incubated at 25 °C ± 2 °C for 7 days with daily observation.

3.2.3 Morphological selection of isolated fungi

Fungi colonies were selected from isolation plates and transferred onto fresh SDA by cutting a small piece of the fungi. The plates were then incubated for 1 to 2 weeks at 25 °C ± 2 °C and the morphological characteristics of the isolated fungi was observed based on its colour, form, surface, margin and mycelia texture. The morphologically different fungi were selected and used for antifungal assay.

3.2.4 Antifungal assay

The antifungal activities of selected actinomycetes were tested by agar plug method against isolated fungi. Thirteen actinomycetes were growth as a lawn on NA and incubated at 30 °C for 1 to 2 weeks. Agar plugs were prepared using a sterile 10 mm diameter cork borer. Three agar plugs of actinomycetes strains were transferred onto freshly prepared fungi plates and incubated at 25 °C ± 2 °C for 1 week with daily observation. The presence or absence of inhibition zone during the incubation period were observed and the diameter were recorded.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 ISOLATION OF FUNGI

The soil samples were first sieved where earthworms and plants are removed and root, granule and sand were separated to proceed for isolation. Each of the part from the soil (approximately 1 g) were then isolated by serially diluted each sample up to 10^{-3}. In another research, similar method were used where the isolation of fungi were obtained using 10^{-3} dilution factor (Cruz-Lachica et al., 2018; Hernandez & Menéndez, 2018).

In order to get only fungi, the media was supplemented with Trimethoprim (25 µg/mL) to inhibit bacteria growth. Similar concentration of antibiotic used by Palla *et al.* (2018), where 25 µg/mL of antibiotic was used as supplements to the nutrient media in order to prevent contamination. However, contamination from bacteria still occurred even though antibiotic was added in the media. Franková and Horecká (1995) also noted that some bacteria colonies grew up in the plates regardless the presence of antibiotic in the media. Nevertheless, repeated inoculation prevents the bacteria colonies to grow in the plates.

From 9 isolation plates, 13 potential fungi strains were cut and transferred to new SDA plates for further morphological characterisation. Based on the observation, fungi started to grow from the third day of incubation. Complete morphological characteristics of the fungi can be observed from day-10 of incubation. In order to avoid overlapping of microorganisms, daily observation is vital. The fungi were fully grow within 2 weeks of incubation. Only 10 fungi strains were picked for antifungal assay as another 3 strains possess the same characteristics among each other. The isolated fungi were then prepared as a lawn for antifungal assay.

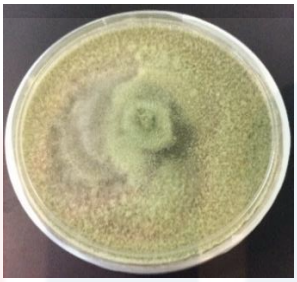
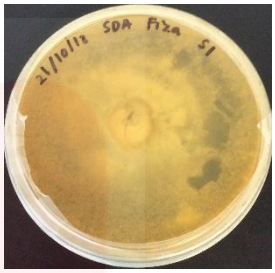
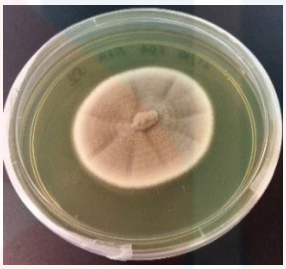
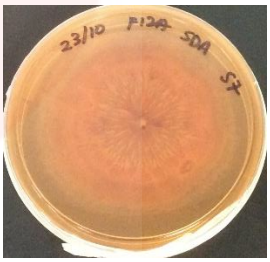
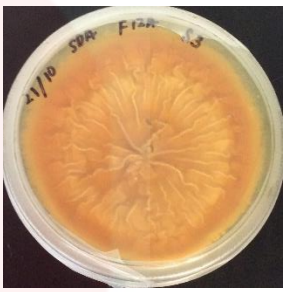
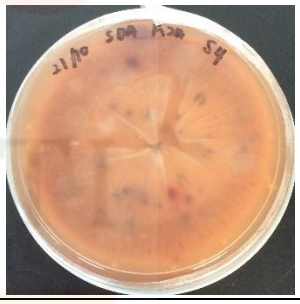
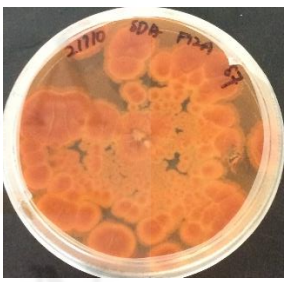
Isolate No.	Front view	Reverse view
1		
2		
3		
4		
5		

Figure 4.1: The isolated fungi from soil infected banana plant

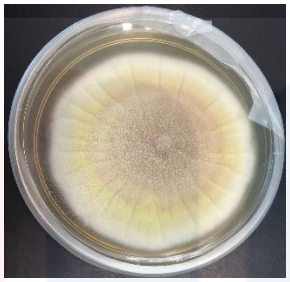
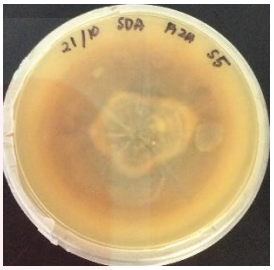
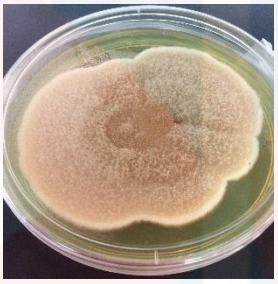
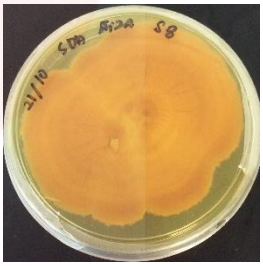
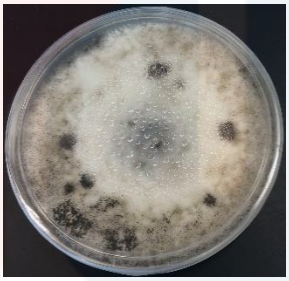
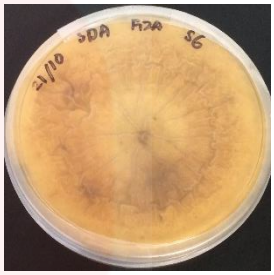
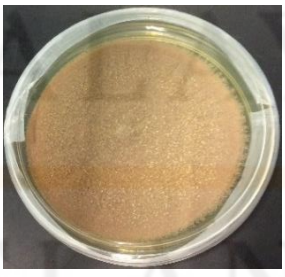
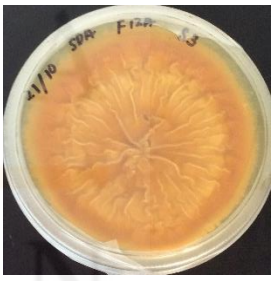
Isolate No.	Front view	Reverse view
6		
7		
8		
9		
10		

Figure 4.2: The isolated fungi from soil infected banana plant

4.2 MORPHOLOGICAL CHARACTERISTICS

Different types of fungi were isolated from infected banana plant. A total of 10 isolated fungi were identified based on morphological characteristics as described in Jogee, Ingle, & Rai, 2017; Ahmed et al., 2015; Davidson et al., 2009.

Table 4.1: Morphological characteristics of isolated fungi

Isolate No.	Form	Elevation	Margin	Pigmentation	Surface
1	Circular	Raised	Entire	Green	Glabrous
2	Circular	Raised	Entire	Buff	Glabrous
3	Rhizoid	Raised	Entire	Buff	Wrinkled
4	Filamentous	Raised	Filiform	Olivaceous	Wrinkled
5	Irregular	Ambonate	Slightly lobed	Green	Rough
6	Circular	Raised	Entire	Pale honey	Glabrous
7	Irregular	Flat	Slightly lobed	Buff	Glabrous
8	Circular	Flat	Entire	White grey	Glabrous
9	Circular	Flat	Entire	Black	Glabrous
10	Circular	Flat	Entire	Buff	Glabrous

Generally, most of the isolates were morphologically similar within most species of *Mycosphaerella* as the characteristics are quite similar with the description by Verkley, Crous, Groenewald, Braun, & Aptroot, 2004; Ahmed et al. (2015). *Mycosphaerella* sometimes appeared elevated mycelium in the centre, entire margin or slightly lobed, pale honey to olivaceous buff, colony surface glabrous or with appressed pure white aerial hyphae or conidiophores. In this study, most of the isolated are in buff and green colours. Isolates no. 1 produced green to white colours, circular form, elevation raised at the centre, colony surface glabrous and entire margin. Isolates no. 1 is quite similar with the genus *Aspergillus* as described by (Papagianni, Mattey, & Kristiansen, 1999). Isolates no. 2 shows colony with dark to light colours, glabrous surface, raised elevation with entire margin similar characteristics with isolates no. 7 but the margin is slightly lobed. Previous study stated that their isolates which produced isolates had dark to gray colonies with abundant pycnidia scattered throughout the radial colony were belongs to genus *Mycosphaerella* (Ahmed et al., 2015). Isolates no. 9 produced black pigmentation, glabrous surface, entire margin with circular form and elevation flat. Zulkifli & Zakaria (2017) noted that their finding which produced compact black colonies, globose to sub-globose vesicles and biseriate seriations were belongs to the genus *Aspergillus*.

4.3 ACTINOMYCETES SUB CULTURING

Actinomycetes pure cultures were obtained by streaking and subculture methods. Thirteen strains of actinomycetes were grown on NA plates. Each of the strain was streaked and subcultured using inoculating loop and sterile scalpel and left for incubation. The actinomycetes were revived for 2 to 3 weeks. Actinomycetes have different growth rate which is fast to slow. Fast grow actinomycetes can be seen after 2 days of incubation. Thus, only 5 strains were picked for antifungal assay because another 7 strains were the slow growth and some have been contaminated.

4.4 ANTIFUNGAL ACTIVITY OF SELECTED ACTINOMYCETES

The inhibitory activities of actinomycetes were tested by agar plug method. Previous research done by Palla *et al.* (2018) also stated that preliminary screening of selected actinomycetes by cross streak and agar overlay method showed a good antifungal producer since there were clear zone surrounding the wells on inoculated plates. In this study, actinomycetes were tested against 10 isolated fungi and 3 actinomycetes strains showed positive results while another 2 strains could not inhibit the fungi. The results was supported with previous research where there were only 10% of actinomycetes isolates have activity against pathogenic fungi (Nurkanto & Julistiono, 2014).

Absence or reduced growth of test pathogens in the vicinity of the growth of actinomycetes was considered as positive for antifungal activity. Based on the results, actinomycetes Strain 72 had the highest antifungal producer which inhibited 60% of the

isolated fungi. The diameter of inhibition zone observed were up to 14 mm. In addition, chitinase produced by actinomycetes was a biological agent which can effectively retard the growth and reproduction of fungal mycelia (Lu et al., 2018). In a study done by Kumar and Kannabiran (2010) stated that actinomycetes having antifungal activity against *Aspergillus* spp.

Moreover, actinomycetes Strain 5 and Strain 56 did not shows any activities since there were no inhibition zone produced. The actinomycetes did not released antifungal properties. Visual and microscopic observations indicated that there were no direct contacts between the actinomycete colonies and the inhibited fungal colonies indicating production of diffusible antifungal compounds. However, in some fungal–actinomycete interactions, there were no clear zones between actinomycete and fungal colonies (Jayasinghe & Parkinson, 2008).

Table 4.2: Antifungal activity of actinomycetes against isolated fungi

Fungi isolates	Actinomycetes zone of inhibition (mm)				
	Strain 5	Strain 47	Strain 56	Strain 72	Strain 108
1	-	15	-	-	-
2	-	11	-	11	22
3	-	-	-	12	-
4	-	-	-	-	16
5	-	-	-	5	11
6	-	-	-	13	-
7	-	-	-	-	-
8	-	9	-	14	20
9	-	11	-	11	9
10	-	-	-	-	-

Note: '-' indicates no inhibition zone

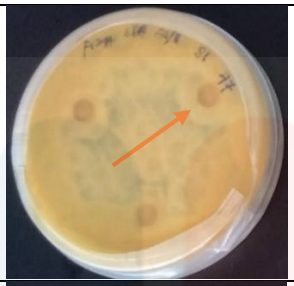
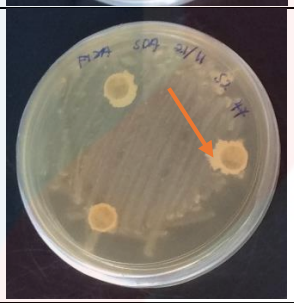
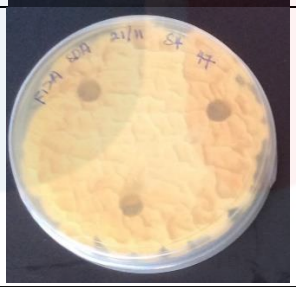
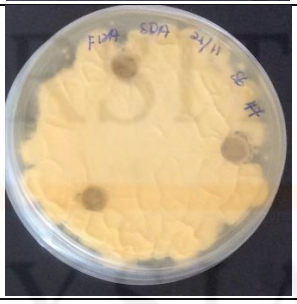
Isolate No.	Reverse View
1	
2	
3	
4	
5	

Figure 4.3: Antifungal assay of actinomycetes Strain 47 shows inhibitory zones observed against fungi Isolate no. 1 and 2 (red arrows)

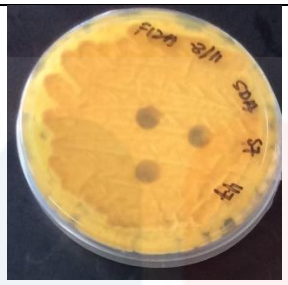
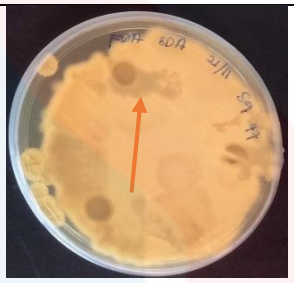
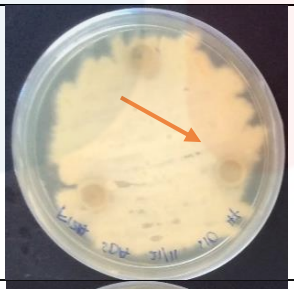
Isolate No.	Reverse View
6	
7	
8	
9	
10	

Figure 4.4: Antifungal assay of actinomycetes Strain 47 shows inhibitory zones observed against fungi Isolate no. 8 and 9 (red arrows)

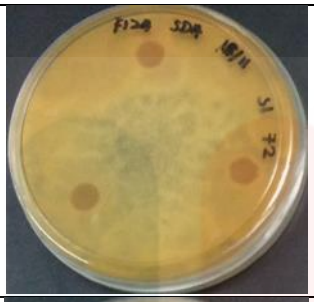
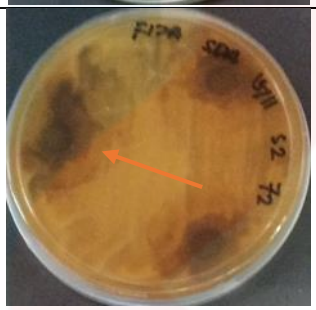
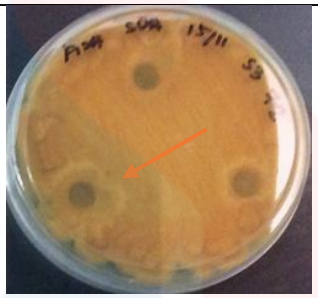
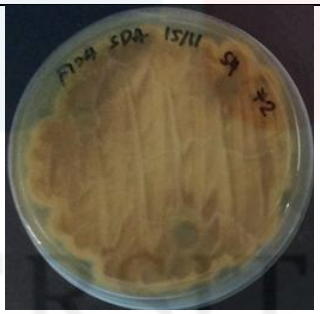
Isolate No.	Reverse View
1	
2	
3	
4	
5	

Figure 4.5: Antifungal assay of actinomycetes Strain 72 shows inhibitory zones observed against fungal Isolate no. 2, 3 and 5 (red arrows)

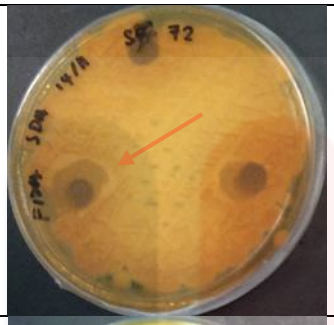
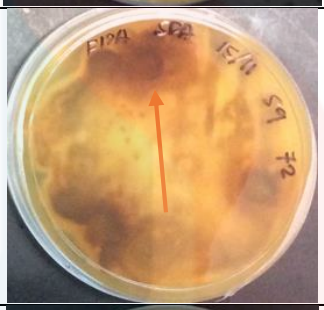
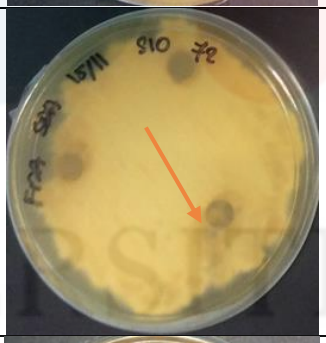
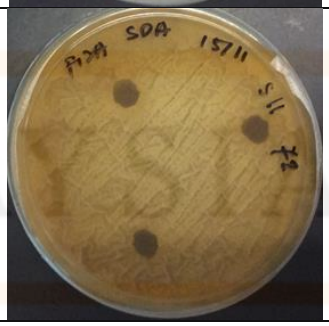
Isolate No.	Reverse View
6	
7	
8	
9	
10	

Figure 4.6: Antifungal assay of actinomycetes Strain 72 shows inhibitory zones observed against fungi Isolate no. 6, 8 and 9 (red arrows)

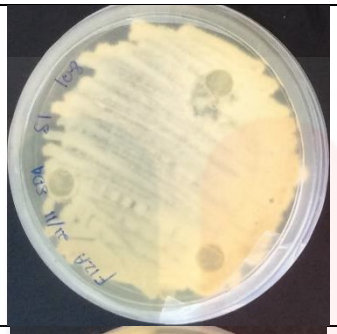
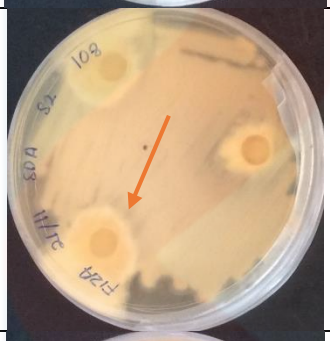
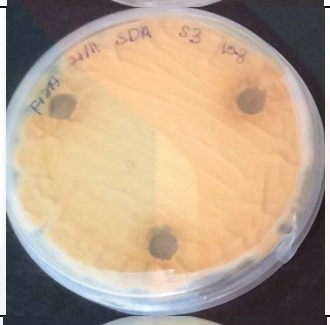
Isolate No.	Reverse View
1	
2	
3	
4	
5	

Figure 4.7: Antifungal assay of actinomycetes Strain 108 shows inhibitory zones observed against fungi Isolate no 2, 4 and 5 (red arrows)

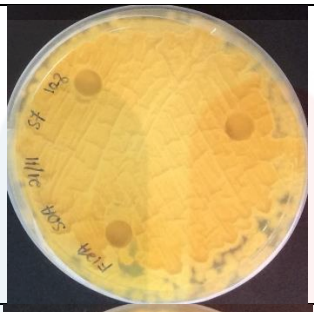
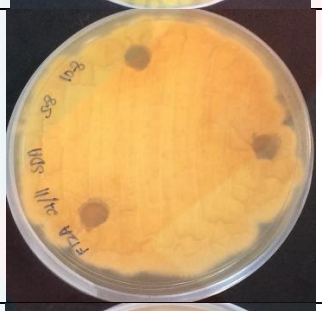
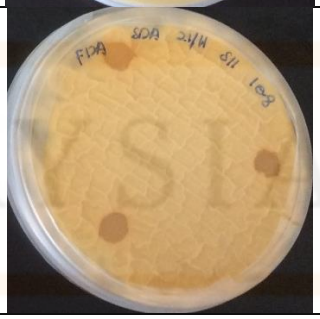
Isolate No.	Reverse View
6	
7	
8	
9	
10	

Figure 4.8: Antifungal assay of actinomycetes Strain 108 shows inhibitory zones observed against fungi Isolate no. 8 and 9 (red arrows)

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

Infected banana plant can be the potential source to isolate pathogenic fungi, *Mycosphaerella*. From the results, the granule part of the soil have more diversity of fungi compared to root and sand. There are 13 fungi isolated from the 1 g of soil samples. Morphological characteristics of isolated fungi were recorded by the observation of form, elevation, margin, surface and pigmentation of the fungi. Five out of 13 strains of actinomycetes were used as antifungal agent therefore only 3 strains were active against pathogenic fungi isolated. However, Strain 108 showed the largest inhibition zone which indicates high antifungal activity. Thus, it clearly shows that actinomycetes can effectively inhibit the growth and reproduction of fungal mycelia, implying its potential as biocontrol agent for the control of banana plant diseases.

Contamination is the biggest challenge faced in this project. Aseptic techniques need to be done before, during and after working with microorganisms. Moreover, further study on identification of the fungi isolates should be carried out by polymerase chain reaction (PCR) amplification and DNA sequencing to confirm the species. Morphological characteristics may not be sufficient as the microscopic and macroscopic characteristics for some species are similar. Other microorganisms also can be used to test for antifungal activity towards the isolated fungi.

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APPENDIX

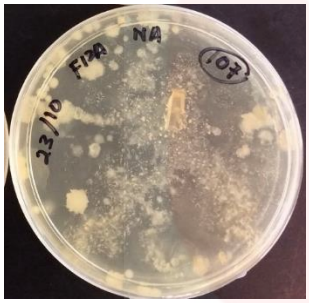


Figure A: Actinomycetes was contaminated with different morphology bacteria

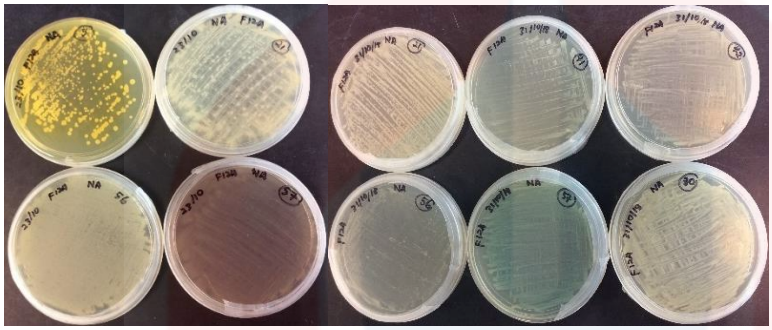


Figure B: Sub culturing of actinomycetes strains

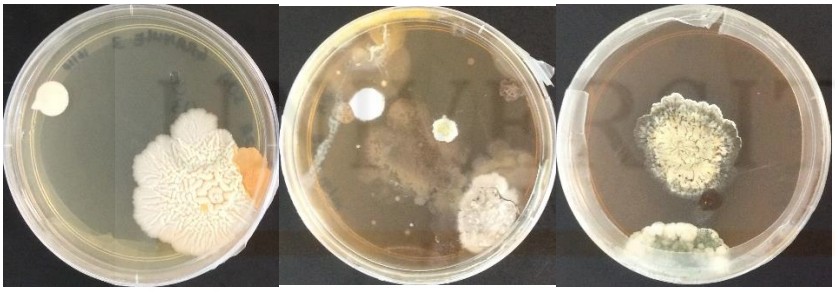
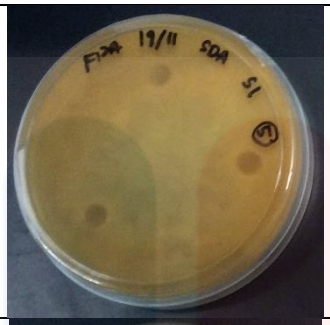
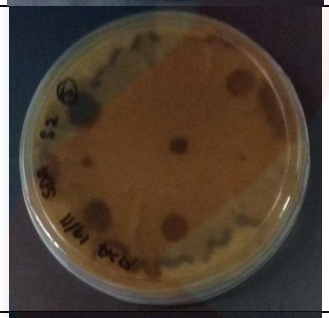


Figure C: Fungi isolation plates

Isolate No.	Reverse View
1	
2	
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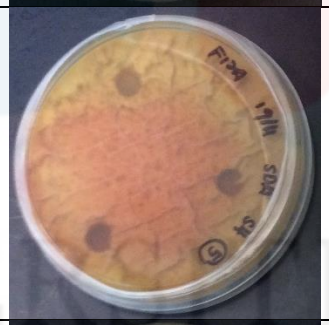
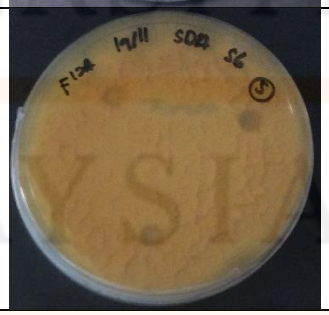
4	
5	

Figure D: Antifungal assay of actinomycetes Strain 5 against isolated fungi

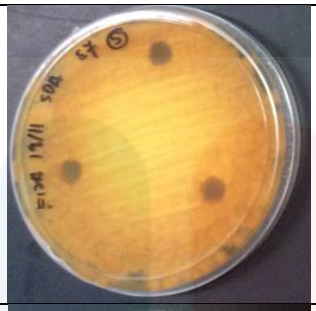
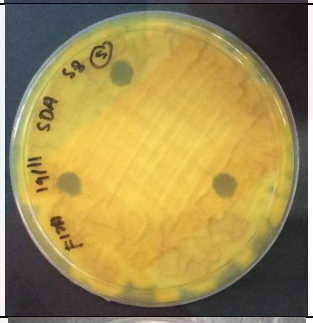
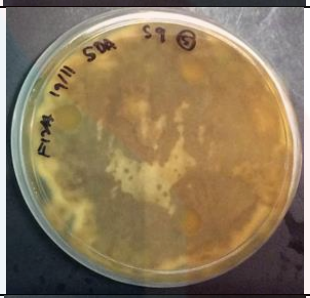
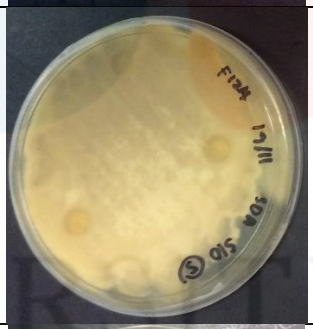
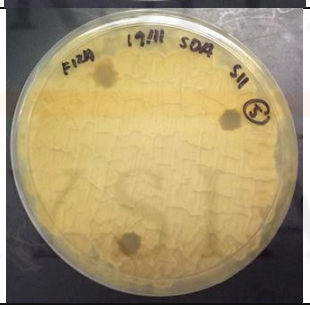
Isolate No.	Reverse View
6	
7	
8	
9	
10	

Figure E: Antifungal assay of actinomycetes Strain 5 against isolated fungi

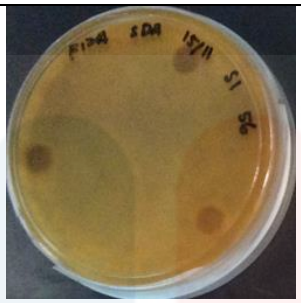
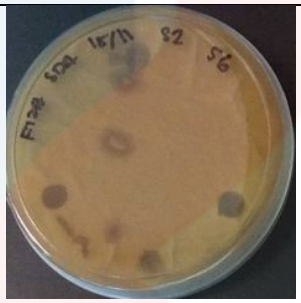
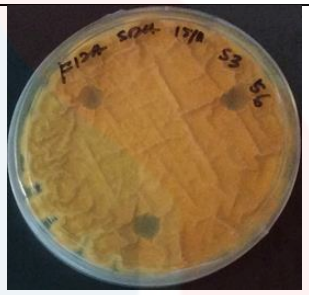
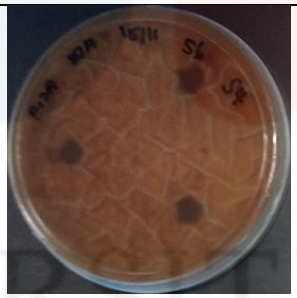
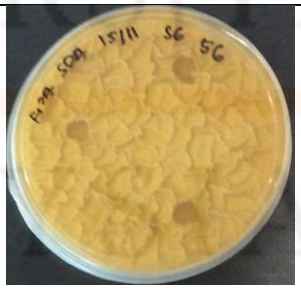
Isolate No.	Reverse View
1	
2	
3	
4	
5	

Figure F: Antifungal assay of actinomycetes Strain 56 against isolated fungi

Isolate No.	Reverse View
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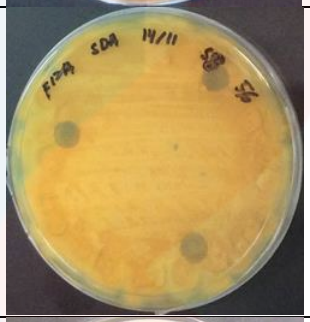
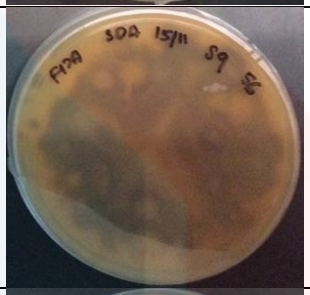
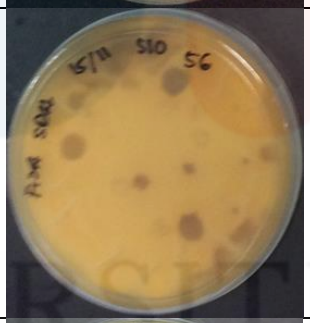
7	
8	
9	
10	

Figure G: Antifungal assay of actinomycetes Strain 56 against isolated fungi