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Isolation, characterization and identification of actinomycetes from agriculture soils at Semongok, Sarawak

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A total of 62 isolates of actinomycetes were isolated from 7 soil samples collected from Agriculture Research Center Semongok, Sarawak. All 62 isolates exhibited a range of colony colours (dark grey, grey, dark brown, brownish, whitish and yellowish white). All the isolates were later purified and subjected to a few enzymatic screening. Results indicate that, 48, 46 and 41 isolates showed the ability to secrete cellulase, lipase and protease respectively. All 62 isolates were then subjected to antimicrobial test using selected phytopathogens as test strains and it was observed that 3, 25, 35 and 37 of the isolates showed antagonistic reaction with *Fusarium palmivora*, *Bacillus subtilis*, *Pantoae dipersa* and *Ralstonia solanacearum* respectively. Six of the most promising isolates were selected and identified using their 16S rRNA sequence. All six isolates were identified as *Streptomycetes* spp. Further study into the utilization of the actinomycetes for agriculture industry will be done to fully utilize these potential microbes for sustainable agriculture.

Key words: Actinomycetes, isolation, characterization, soil, Sarawak.

INTRODUCTION

Actinomycetes are gram-positive bacteria showing a filamentous growth like fungi. They are aerobic and widely spread in nature. Actinomycetes DNA are rich in G+C content with the GC % of 57 - 75% (Lo et al., 2002). They are predominant in dry alkaline soil.

Actinomycetes have been well known for the production of secondary metabolite. Many of the presently used antibiotics such as streptomycin, gentamicin, rifamycin and erythromycin are the product of actinomycetes. The actinomycetes are important not just to the pharmaceutical industries but also the agriculture. Previous study showed that actinomycetes isolated from Malaysia soil have the potential to inhibit the growth of several tested plant pathogens (Jeffrey et al., 2007). Oskay et al. (2004) also reported that the ability of actinomycetes isolated from Turkey's farming soil have the ability to inhibit *Erwinia amylovora* a bacteria that cause fireblight to apple and *agrobacterium tumefaciens* a casual agent of Crown Gall disease.

Identification of actinomycetes using microscopic techniques alone was not enough to ensure certainty. Biochemical methods would be the best method to iden-

tify actinomycetes to their species level. But this test consumes a lot of time and chemicals. With the advancement of technology in molecular study, primers had been developed by researchers to target specifically the 16S rRNA sequence of the actinomycetes (Schwieger and Tebbe, 1998; Wang et al., 1999). Identification of actionmycetes to genus level was made possible in a fast and accurate manner. This has been a great advancement in the area of identification as that the ability to obtain the genus of the actinomycetes in just a few hours is now possible.

In this study, actinomycetes from the farming soil of Agriculture Research Centre Semongok, Sarawak are isolated. The isolates were later tested for their bioactive compounds and selected isolates were later identified using primers targeting their 16S rRNA sequence.

METHODOLOGY

Soil samples collection

Soil samples were collected about 15 cm below the surface of the

soil. All the soil samples were collected randomly from Agriculture Research Center, Semongok, Sarawak. Soil sample were then packed in a ziplock bag and stored in a container with icepack (~ 4°C) during transportation from Agriculture Research Center, Sarawak to MARDI Serdang, Selangor.

Microbes' isolation and enumeration

Soil samples were air dried for 1 week prior isolation. This helps in decreasing the population of gram negative bacteria. Soil suspension method described by Oskay et al. (2004) was used, where 1 g of the soil sample were taken and mix with 100 ml of sterile distilled water (sdH$_2$O). The soil suspension was shaken vigorously under room temperature (25 ± 2°C) on an orbital shaker at 200 rpm for 1 h. 200 µl of the soil suspension were pipetted and lawn onto Starch Casein Agar (SCA) (Soluble starch, 10.0 g; Casein hydrolysate, 0.3 g; KNO$_3$, 2.0 g NaCl, 2.0 g; K$_2$HPO$_4$, 2.0 g; MgSO$_4$.7H$_2$O, 0.05 g; CaCO$_3$, 0.02 g; FeSO$_4$.7H$_2$O, 0.01 g; Agar, 18.0 g; distilled water, 1000 ml; cycloheximide, 100 µg/ml) at pH 7. A series of dilution of the suspension from 10^{-3} to 10^{-6} were done with duplicates. All the plates were incubated at 30°C for 1 - 2 weeks. Emerging actinomycetes were picked and streaked onto fresh SCA plates and incubated at 30°C for 1 week. Colony forming unit (cfu) per one gram of soil was determined for all the samples collected.

Enzymatic screening

All the isolates were screened for their cellulase producing ability using minimal medium agar (MMA) containing AZO-CM-Cellulose as substrate (Peptone, 1.0 g; yeast extract, 1.0 g; MgSO$_4$.7H$_2$O, 0.5 g; KH$_2$PO$_4$, 0.5 g; (NH$_4$)$_2$, 1.0 g; substrate (Megazyme), 1.0 g; agar, 15.0 g and distilled water, 1000 ml) at pH 7. Gelatin hydrolysis assay as described by Frazier (1926) was used in the screening of protease activity. Lipase activity was screened using method for determination of esterastic activity (Sierra, 1957) with little modifycation. Tween 80 used in the esterastic assay test was replaced with Tween 20. Formation of halo zone indicates positive reaction for the entire test conducted. Measurement of the halo zones were taken after 48 h of incubation.

Antimicrobial testing

Plate diffusion method (Bauer et al., 1966) with some modification as suggested by Barakate et al. (2002) was used for the antimicrobial testing against *Fusarium palmivora*, *Bacillus subtilis*, *Ralstonia solanacearum* and *Pantoae dispersa*. Isolates of actinomycetes were removed from their agar using a sterile cork bore (5 mm in diameter) and placed onto agar plate lawn with the pathogenic microbes. Formation of halo zone indicates positive reaction. Measurements of the halo zones were taken after 48 h.

Genomic DNA extraction

Genomic DNA was extracted from a few selected isolates using *BACTOZOL KIT* from Molecular Research Center, Inc. Isolation protocols are according to the manufacturer instruction (http://www.mrcgene.com/bactozol.htm).

Polymerase chain reaction amplifications

Amplifications were performed in a 25.0 µl mixture containing 16.3 µl of sdH$_2$O, 2.5 µl of 10X PCR buffer (Promega), 1.5 µl of 25 mM MgCl$_2$ (Promega), 0.5 µl of 10 mM dNTP's (Promega), 0.2 µl of *Taq* polymerase (Promega), 1.0 µl for both 0.05 mM of Com1

(5'CAGCAGCCGCGGTAATAC3') and 0.05 mM of Com2 (5'CCGTCAATTCCTTTGAGTTT3') primer (Schwieger and Tebbe, 1998) respectively, and 2.0 µl of genomic DNA. The reaction tube was then put into MJ Thermalcycler, which had been programmed to preheat at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s and elongation at 72°C for 45 s before a final extension of 72°C for 10 min. Product size estimated was 408 bp. Sterile distilled water which substitute template DNA was used as negative control. PCR products were later purified according to protocol suggested by the manufacturer of gel purification kit purchased from Invitrogen.

Sequencing of PCR products

Sequencing of the purified PCR products were conducted at facilities of First Base Laboratories Sdn. Bhd., Selangor using ABI PRISM ® 377 DNA Sequencer (Applied Biosystems). The obtained 16S rRNA sequences were compared to sequences in the NCBI genebank database with the Basic Alignment Search Tool (BLAST) (Altschul et al., 1990).

RESULTS

Microbes' isolation and enumeration

Colony forming unit per gram (cfu/g) of soil, showed the density of actinomycetes isolated form the soil was highest at 8.0 X 10^7 from the limau purut soil and the lowest was from the ciku soil with just 9.8 x 10^6. From all of the samples collected, a total of 62 isolates of actinomycetes were isolated (Table 1). Each of the isolates were later categorized accordingly their morphology colony colour ranging from dark grey, grey, dark brown, brownish, whitish and yellowish white (Figure 1).

Enzymatic screening

Enzymatic tests were conducted basically to determine the ability of actinomycetes to act as a degrader of organic compounds when applied for agricultural usage. Cellulose comprises the highest of the carbon sources in plants apart from lignin while lipid and protein are both present mostly in animal manure or other organic agrowaste but are least in plants. The study showed that 48, 46 and 41 isolates of actinomycetes produced cellulase, lipase and protease respectively.

Antimicrobial testing

Three strains of pathogenic microbes (*Fusarium palmivora*, *Bacillus subtilis*, *Ralstonia solanacearum* and *Pantoae dispersa*) were chosen as the test strains for the study. All the strains were chosen due to the reason that these microbes exhibited pathogenic effect towards certain commodity plants. Antimicrobial tests conducted showed that 3, 25, 37 and 35 isolates of actinomycetes produces antagonistic reaction for *F. palmivora*, *B. sub-*

Table 1. Colony colour and cfu/g for each soil sample.

Soil samples	Scientific name	Isolate number	Colony colour	cfu/g
Tongkat Ali	*Eurycoma longifolia Jack*	1, 2, 6 3, 5, 8 4, 7 9, 10	Dark grey Grey Brownish Whitish	4.1×10^7
Jambu air	*Syzygium samarangense*	11, 14, 16 12, 15, 17, 18 13, 19	Dark grey Grey Whitish	1.5×10^7
Isau	*Euphoria longana*	20, 27, 29 21, 22, 24, 25, 28 23, 26 30	Dark grey Grey Dark brown Yellowish white	3.3×10^7
Bunga Kantan	*Etlingera elatior*	31, 33, 36, 39 32, 34, 38 35 37, 40	Dark Grey Grey Brownish Yellowish white	1.1×10^7
Limau purut	*Citrus hystrix*	41, 43, 48 42, 45 44 46, 47, 49	Dark grey Grey Dark brown Brownish	8.0×10^7
Durian	*Durio zibethinus Murray*	50, 57 51, 52, 55 53, 54, 56	Dark grey Whitish Yellowish white	4.0×10^7
Ciku	*Manilkara zapota*	58, 60, 61 59 62	Grey Dark brown Whitish	9.8×10^6
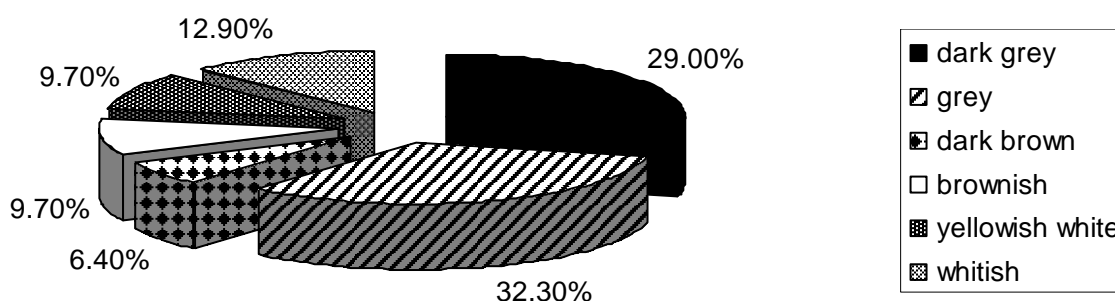
Figure 1. Percentage of colony colour observed on Starch Casien Agar.

tilis, *R. solanacearum* and *P. dispersa* respectively.

Identification of the actinomycetes

Identification of actinomycetes using molecular tools proved to be faster and least tedious compared to classical biochemical methods. The results obtained from the direct sequencing of purified PCR products showed that all the actinomycetes isolates belongs to the genus Streptomyces. Spore chain arrangements observed using research microscope at 1000X showed that of all the isolates bear spore chains of 3 or more and are non-motile. It was observed that isolate number 23 and 25 form a hook like structure which can only be found in the Streptomyces genus (Figure 2).

DISCUSSION

From the 7 farming soil samples collected from Agriculture Research Centre (ARC) Semongok, it was observed
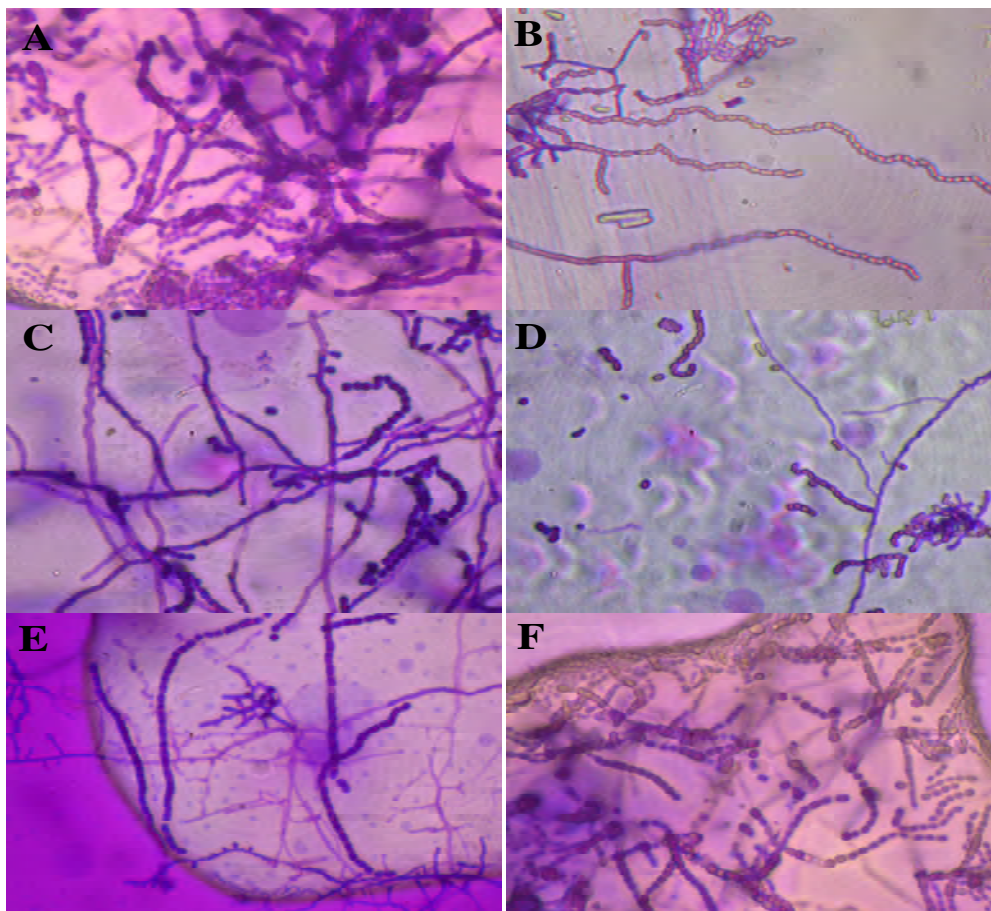
Figure 2. Spore chain arrangement observed at 1000X using research microscope. A) Isolate number 3, B) Isolate number 15, C) Isolate number 23, D) Isolate number 25, E) Isolate number 26 and F) Isolate number 27.

that the cfu/g of soil produce by each of the soil samples collected in this study were higher than the cfu/g of soil obtained from Ayer Keroh Recreational Park (2.8 - 5.6 x 10^6) obtained by Jeffrey et al. (2007). In the study conducted by Jeffrey and co-workers (2007), they observed that the highest cfu/g of soil for actinomycetes isolated from soils planted with ornamental plants were 1.57 x 10^3 which was very much lower than the count obtained in this study (0.98 - 8.0 x 10^7). Study done by Takizawa et al. (1993) and Lee and Hwang (2002), showed that cfu/g of soil for both Chesapeake Bay soil (1.8 x 10^2 - 1.4 x 10^5) and Korean vegetative soil (1.17 - 4.20 x 10^6) respectively, were also lower than the results obtained in this study. The reason for this maybe the differences in media, which influence the growth rate of the microorganisms and also the environment of the soil such as the humidity and pH which are noted to influence the microorganisms' growth rate (Athalye et al., 1981; Oskay et al., 2004).

Approximately 98.4% (61 isolates) of the isolate produced one or more enzymatic activity. From the total isolates 16.2% produced only one enzyme, while 54.8%

produces two types of enzymes and 27.4% produces all the tested enzymes. This indicates that actinomycetes possess the potential to secrete broad range enzymes, which maybe the results from natural selection of the microorganisms in order to survive in a competing environment.

A total of 3.23% of the actinomycetes produced antibacterial substances towards only gram positive bacteria, 37.1% towards only Gram negative bacteria and 38.8% against both gram positive and negative bacteria. Six (6) of the potential producers of antibacterial and enzymes that were selected based on their activity (clear zones produced) are 3, 15, 23, 25, 26 and 27. The most prominent secondary metabolite producer's is isolate number 27 which produces clear zone measuring 25.0 mm for cellulase, 18.0 mm for lipase, 31 mm for protease, 35.0 mm for *B. subtilis*, 35.0 mm for *R. solanacearum* and 38.0 mm for *P. dispersa* (Table 2).

Initial morphological characterization using light microscope showed that all the 6 isolates belong to the genus *Streptomyces* spp. Further identification using the actinomycetes 16S rRNA confirm the claim. None of the

Table 2. Bioactivities produce by 6 potential actinomycetes.

Isolate no.	ID	Enzymatic activity (mm)			Antimicrobial activity (mm)			
		Cellulase	Lipase	Protease	*F. palmivora*	*B. subtilis*	*R. solanacearum*	*P. dispersa*
3	*Streptomycetes* spp.	26.0	15.5	0	0	10.0	34.0	11.0
15	*Streptomyces aureofaciens*	23.5	27.5	33.0	0	17.0	38.5	27.0
23	*Streptomycetes* spp.	30.5	19.0	25.0	0	0	30.5	35.0
25	*Streptomycetes* spp.	13.0	15.0	0	0	28.0	31.0	36.5
26	*Streptomycetes* spp.	0	30.5	33.50	0	28.0	33.0	36.0
27	*Streptomycetes* spp.	25.0	18.5	31.0	0	35.0	35.0	38.0

actinomycetes produces both the antibacterial and antifungal substances observed in this study.

The reason for actinomycetes in producing higher antibacterial towards gram negative bacteria in comparison to gram positive bacteria and fungus tested maybe due to the cell wall for the Gram negative bacteria is much easier to break than those of the Gram positive and fungi. However this hypothesis does not concur with the finding by various researchers, where they observed that antagonistic reaction against the Gram positive bacteria were much higher than the Gram negative (Balagurunathan et al., 1996; Basilio et al., 2003; Oskay et al., 2004; Sacramento et al., 2004).

Pandey et al. (2005) showed that for the optimum production of antibiotics certain carbon and nitrogen sources are required. In that study the author and co-workers also suggested that pH might play an important factor for the production of antibiotics by actinomycetes. Study done by Vasavada et al. (2006) showed that the used of media, pH, salanity and carbon and nitrogen affect the growth and antibiotic production by actionmycetes.

The search for novel metabolites producer requires a large number of isolates. According to Oskay et al. (2004), actinomycetes diversity might be influence by the diversity of plants species grown on that particular soil. In was also noted that different plants produce different chemical metabolite which maybe useful for the microbes around it or *vice versa*. In order to survive in a threaten environment the microbes (in this case actinomycetes) need to adapt to the environment (Oskay et al., 2004).

Conclusion

Actinomycetes isolated from the soil of Agriculture Research Center Semongok, Sarawak showed that 48, 46 and 41 isolates of actinomycetes produced cellulase, lipase and protease respectively while for the antimicrobial tests conducted 3, 25, 37 and 35 isolates of actinomycetes produces antagonistic reaction for *F. palmivora*, *B. subtilis*, *R. solanacearum* and *P. dispersa* respectively. More intensive study should be conducted on the isolated actinomycetes to utilize potential actino-

mycetes either as biocontrol or bioremediation agents.

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