

Full Length Research Paper

Biological active compounds from actinomycetes isolated from soil of Langkawi Island, Malaysia

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Actinomycetes which were categorised as beneficial microorganisms have long been studied for their potential in producing secondary metabolites either for pharmaceutical or agricultural industries. In this study, 160 isolates of actinomycetes had been isolated using soil suspension method. All the 160 isolates were later tested for their potential to secrete secondary metabolites such as cellulases, mannanases, xylanases, lipases, proteases and antifungal compounds. Prescreening of the 160 actinomycetes isolates showed that 73.1% of the actinomycetes produces xylanases, 69.4% produces cellulases, 65.0% proteases, 44.4% lipases and 9.4% mannanases. It was also observed that 43.1% (69/160) of actinomycetes showed antagonistic reaction towards *Colletotrichum capsici* while 18.8% (30/160) showed antagonistic reaction towards *Colletotrichum gloeosporioides*. Five (5) of the best producers of the bioactive compounds were identified using 16S rRNA primers. All of the isolates were identified to be originated from streptomyces genus. These potential actinomycetes need to be further tested for their application and have their potential fully characterized before being distributed or made known to interested industries.

Key words: Actinomycetes, anthracnose, bioactivity, biodiversity, Malaysia.

INTRODUCTION

Soil microbes which had been known to possess the ability to act as degradation and biocontrol agents have been widely studied by researchers around the world. One of these well-known soil microbes are the actinomycetes. Actinomycetes had been long known to be the main producer of antibiotics in the pharmaceutical industries. The used of actinomycetes for agricultural and medicinal purposes have also been studied by researchers in Malaysia as well as international (Johnson

1954; Vikineswary et al., 1997; Ismet et al., 2002; Lo et al., 2002; Jeffrey et al., 2011). According to Lo et al. (2002), there are about 100 genera of actinomycetes inhibiting in the soil. The ability of actinomycetes to act as the biodegradation of cellulosic and hemicellulosic compounds and biocontrol agents would have been favored by consumers globally due to the rising conscious of consumers towards synthetic chemical compounds that may polluted that environment as well as

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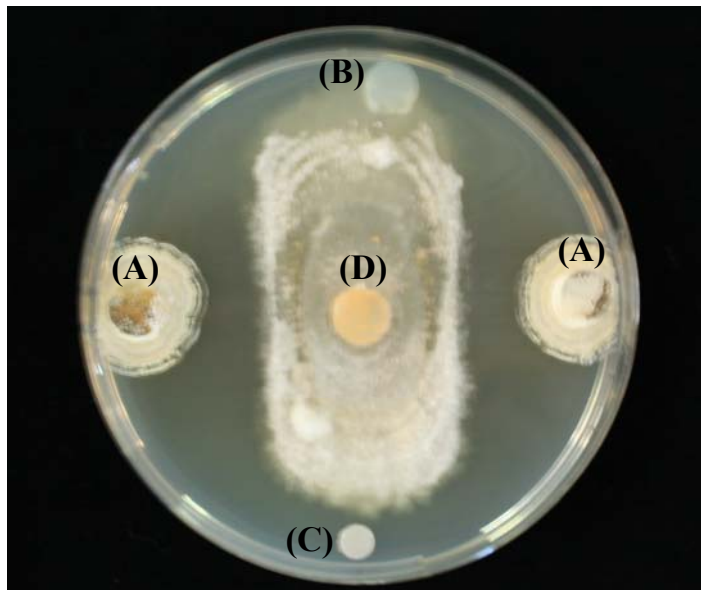


Figure 1. *Colletotrichum gloeosporioides*. screening against actinomycetes. (A) Actinomycetes, (B) negative control, (C) cycloheximide 50 mg/ml (positive control), D; *C. gloeosporioides*.

dangerous to human health. Studies have shown that *Streptomyces* spp. gave significant control towards pythium root rot of corn and sugarcane (Johnson, 1954) and cotton wilting (Arjunarao, 1971). Work done by Lee and Hwang (2002) on actinomycete isolated from Korean soil showed strong antifungal activity of actinomycetes against *Alternaria mali*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp. *cucumerinum* and *Rhizoctonia solani*. Managing pest and diseases using biological control would give long term advantages, such as cost saving and a sustainable agriculture although the effect is not immediately (Aghighi et al., 2004). All of these showed that, bioactive compounds isolated from actinomycetes were studied extensively by researchers through out the world. In this study, actinomycetes isolated from Langkawi Island soil were screened for their bioactivity. Potential isolates were later characterized and identified using molecular technique.

METHODOLOGY

Soil samples collection

Soil samples were collected randomly under the trees shrubs of few places at Langkawi Island. The soil was dug 15 cm from the surface and the soil samples were taken at that depth. The soil samples were put into a ziplock bag for transportation back to Serdang, Selangor.

Soil actinomycetes isolation and enumeration

The soil were left to air dried for about 7 to 14 days. Ten grams of

dried soil were grinded before being put into a conical flask added with 100 ml of sterile distilled water. The conical flasks were then put onto an orbital shaker and agitated at 220 rpm for 1 h at room temperature ($28\pm 2^\circ\text{C}$). Serial dilution was performed for each soil samples until the factor of 10^6 . A total of 150 μl of the soil suspension were pipetted and spread onto starch casien agar (SCA) plates supplemented with cycloheximide (50 $\mu\text{g/ml}$). Duplicate plates were prepared for each of the serial dilutions. The plates were then incubated at $28\pm 2^\circ\text{C}$ for about 10 days. The emerging colonies of actinomycetes were subcultured onto a fresh SCA plate.

Bioactivity testing for enzymatic activity

Plug of actinomycetes were inoculated onto Minimal Medium Agar (MMA) containing AZO-CM-Cellulose, AZO-Carob-Galactomannan or AZO-xylan (Oat) as substrate (Sahilah, 1991). Gelatin hydrolysis assay as described by Frazier (1926) was employed for the screening of protease activity. Lipase activity was conducted using method for determination of esteratic activity (Sierra, 1957) with modification. Tween 80 used in the esteratic assay test was replaced with Tween 20 for this purpose. Formation of halo zone indicates positive reaction for the entire test conducted. Measurement of the halo zones were taken at day 5 of the test. All the test were conducted under room temperature ($28\pm 2^\circ\text{C}$) condition.

Bioactivity testing of soil actinomycetes with for antifungal activity

Plug of actinomycetes was inoculated onto the test plate of potato dextrose agar (PDA). Cycloheximide 50 mg/ml was used as the positive control and plug of PDA was used as the negative control. Plug of tested plant pathogens (*C. gloeosporioides* and *Colletotrichum capsici*) was then inoculated at the middle of the test plate as shown as in Figure 1. Clear zone form was measured

Table 1. Inhibition profile produce by actinomycetes.

Pathogen	Isolates inhibition profile (x)			Total number of isolates which produce inhibition zone
	No inhibition (y= 0 mm)	Low inhibition (1 mm ≤ y ≤ 15 mm)	High inhibition (y > 15 mm)	
<i>Colletotrichum capsici</i>	91	45	24	69
<i>Colletotrichum gloeosporioides</i>	130	8	10	18

x was the number of isolates; y was the inhibition profile.

after seven days of the test and average size of each clear zone was calculated. All the tests were conducted under room temperature (28±2°C) condition. The inhibition profile was scored as no activity (0 mm), low antagonistic activity (1 to 15 mm) and high antagonistic activity (≥15 mm) (Table 1).

Identification of the best isolates

The isolates that gave the best results were chosen and molecular analysis was done. Deoxyribonucleic acid (DNA) was isolated using GF-1 bacterial extraction kit purchased from Vivantis. Protocol used was as suggested by the manufacturer (<http://www.vivantis.com/doc/1112006164042.pdf>). Polymerase chain reaction (PCR) was conducted using method stipulated by Jeffrey et al. (2008). PCR products were purified using kit obtained from Vivantis and protocol used was as suggested by manufacturer (<http://www.vivantis.com/doc/225200716542.pdf>). Purified PCR products were later sent to 1st Base sequencing for sequencing purposed. Sequencing results were later BLAST with database from NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

RESULTS

Isolation and enumeration of actinomycetes

From the soil samples collected, 160 isolates of actinomycetes were isolated. From the total number of the isolates, 36 isolates were observed to possess grey colour aerial mycelium, while 16 in yellow, 71 in white, 1 in green, 21 in yellowish white, 3 in brown, 8 in red, 2 in orange and 2 in dark brown. The average colony forming unit / gram (cfu/g) of dry soil for Langkawi Island soils were estimated to be 1.85×10^7 . Pigmentation was also observed from 12.5% (20/160) of the isolates. The highest colour of diffusible pigmentation observed was brown in colour (15 isolates), followed by yellow (four isolates) and green (one isolate).

Bioactivity testing for enzymatic and antifungal activity

From the 160 isolates of actinomycetes tested for enzymatic activities, 69.4% showed the ability to produce cellulases, 9.4% mannanases, 73.1% xylanases, 65.0% proteases and 44.4% lipases. While for the antifungal activities tested, 43.1% (69/160) showed antagonistic reaction towards *C. capsici* and 18.8% (30/160) showed

antagonistic reaction towards *C. gloeosporioides*.

Identification of actinomycetes

All the 5 isolates of actinomycetes with high bioactivity producing activity were identified to be from the genus of *Streptomyces*, with three from the species *Streptomyces humidus*, 1 from the species of *Streptomyces hygroscopicus* and 1 just identified as *Streptomyces* spp. only (Table 2).

DISCUSSION

Isolation and enumeration of actinomycetes

From the morphological characterization of the isolated actinomycetes conducted, it was observed that the highest aerial mycelium observed were from the white series. This finding however were different from the results obtained from studies conducted by researchers in Malaysia and overseas (Ndonde and Semu, 2001; Barakate et al., 2002; Jeffrey et al., 2008). The result of diffusible pigmentation was low comparing to the results obtained by Ndonde and Semu (2001). In their study, Ndonde and Semu (2001) observed that 65.0% of their isolates produced soluble pigmentation but in this study only 12.5% of the actinomycetes were seem to produce soluble pigmentation. From the colour of aerial mycelium and the production of diffusible pigment it was observed that the distribution of actinomycetes could be considered to be more diversified in this study compared to study done by Jeffrey et al. (2008) and Lo et al. (2002). The average cfu/g of dry soil obtained in this study is 1.85×10^7 which is much more higher compared to cfu/g of dry soil for actinomycetes obtained from some garden and agricultural soils isolated from selected areas in Selangors, Johor and Kuala Lumpur (Jeffrey et al., 2007). However, the cfu/g was lower than the cfu/g of actinomycetes isolated from Semongok soil which was 8.0×10^7 (Jeffrey et al., 2008). Study by Lee and Hwang (2002), on the diversity of actinomycetes isolated from various vegetative soils in Korea, showed that cfu/g for the actinomycetes isolated were between 1.17 to 4.2×10^6 which is much lower than the cfu/g obtained in this study.

Table 2. Bioactivity produced by 5 best actinomycetes isolates.

Isolate number	Isolate Id	Aerial mycelium colour	Pigmentation (if any)	Bioactivity produced (Clear zone size, mm)						
				Cellulases	Mannanases	Xylanases	Proteases	Lipases	<i>Colletotrichum capsici</i>	<i>Colletotrichum gloeosporioides</i>
DF11	<i>Streptomyces hygrosopicus</i>	Grey	Nil	22.5	16	15	14	20	18	Nil
LB06	<i>Streptomyces humidus</i>	White	Nil	30	20	27	11	14	0	16
K17	<i>Streptomyces humidus</i>	Yellow	Green	12	0	15	12	34	18	14
DF22	<i>Streptomyces humidus</i>	Yellow	Brown	16	16	20	0	12	10	19
N07	<i>Streptomyces</i> spp.	White	Nil	30	0	20	8	16	16	18

Bioactivity testing

Approximately 85.0% (136 isolates) of the isolate produced one or more enzymatic activity. From the total isolates, 15.0% (24 isolates) did not produce any enzyme, while only 1.9% (3 isolates) produces all the 5 types of enzymes. Most of the actinomycetes produces 3 to 4 enzymes with the percentage of 26.9% (43 isolates) and 31.3% (50 isolates), respectively. This indicates that actinomycetes possess the potential to secrete broad range enzymes, which maybe the results from natural selection of this microorganism in order for it to survive in a competing environment. In a study conducted by Arifuzzaman et al. (2010), soil content, soil types, pH and soil moisture played a role in determining the type of microbes and their population thus influencing the type of enzymes secreted. From the results obtained, it was observed that actinomycetes isolated from Langkawi Island did not show a high number for mannanases producing isolates. However, this is not the only study done in Malaysia that showed a low mannanases activity from actinomycetes. A few

studies conducted also showed that mannanases activities are low among soil actinomycetes compared to other polysaccharides produced (Jeffrey, 2006; Jeffrey et al., 2008). The low mannanases production by actinomycetes may indicate that actinomycetes isolated from Malaysian soils are not good producers for mannanase or the environment around these actinomycetes does not required for the production of mannanase from these microbes.

In a study conducted by Boontim and Lumyong (1999) on cellulases producing actinomycetes from Thailand, they observed that from a total of 125 isolates of actinomycetes isolated from Chiang Mai soil, only 1 isolate produces cellulase. This number however is lower than the percentage of actinomycetes producing cellulase obtained in this study (69.4%) and other study conducted by researchers in Malaysia (Jeffrey et al., 2007; Jeffrey, 2008). From the results obtained, it can be concluded that different demographic sample sites played its role in influencing the secretion of secondary metabolite by actinomycetes. For antifungal activities it was observed that a total of 3.1% of actinomycetes

produces both antifungal. Many antimicrobial activities had been detected from actinomycetes by researchers all around the world in the past few decades for medical purposes (Moncheva et al., 2002, Aghighi et al., 2004, Oskay et al., 2004, Jeffrey, 2006). Little emphasis was given to the utilization of actinomycetes as a biological control in agriculture sector in Malaysia as the used of biological control does not give immediate results to the farmers.

In recent years, it was observed that soil borne actinomycetes have the potential of controlling *C. gloeosporioides* in chilli (Suwan et al., 2012). Sacramento et al. (2004), showed that actinomycetes isolated from the Brazilian soils demonstrated antifungal activities against *Fusarium solani* with the inhibition zone of about 30 mm. Studies on the infection of *C. gloeosporioides* on orchid had been done by Prapagdee et al. (2008). Known antifungal from *Streptomyces* spp. had been isolated and identified by Intra and Panbangred (2008), showing high inhibitory activity towards *C. capsici* and *C. gloeosporioides*. These examples showed that actinomycetes had the potential to produce

antifungal compounds although the novelty of the compounds maybe unknown. In this study, we observed that actinomycetes isolated were able to secrete antifungal for both *C. capsici* and *C. gloeosporioides*. As we observed, some of the actinomycetes produces antifungal for both the *C. capsici* and *C. gloeosporioides* while other may be towards a specific species only. This maybe due to the reason that antifungal compounds secreted were non specific antifungal (broadrange antifungal) while other maybe specific antifungal (narrow range antifungal). The production of enzymes such as glucanases, proteases and β -1,3-glucanases might be the another factor influencing the inhibitory activity of the actinomycetes towards *C. gloeosporioides* (Anitha and Rebeeth, 2010).

The production of antifungal compounds by actinomycetes in this study need to be studied more extensively as the production of antibiotics are often influenced by few factors such as chemicals added to the agar media (salt, carbon and nitrogen sources), temperature and pH (Vasavada et al., 2006). By changing the composition of media used we may be able to increase or decrease the production of antibiotics form on these microorganisms. It is also possible that other useful antimicrobial compounds were produced and not screened in this study (Barakate et al., 2002). Porter (1971) hypothesized that all actinomycetes possessed some antimicrobial properties and can be assessed when cultured and study under suitable condition. Kokare et al. (2004) had demonstrated that antimicrobial activity by actinomycetes was dependent upon the media used for its cultivation.

Conclusion

Preliminary screening from 160 actinomycetes isolated from Langkawi Island showed that actinomycetes isolated from Langkawi Island were potent producers of bioactive compounds. Five (5) potential producers of bioactivities were identified as *S. humidus* (3 isolates), *S. hygrosopicus* (1 isolate) and *Streptomyces* spp (1 isolate). These potential actinomycetes should be further utilized in various industries such as agriculture and food for the benefit of human race.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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