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Isolation, Screening and Characterization of Antibiotic-Producing Actinomycetes from Rhizosphere Region of Different Plants from a Farm of Sungai Ramal Luar, Malaysia

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ABSTRACT

A total of 25 soil samples were collected from rhizospehere regions of different plants from a farm in sungai ramal luar, malaysia. These samples were divided into two sets for the isolation of actinomycetes: one receiving the treatment with calcium carbonate and other set without calcium carbonate. A total of 300 actinomycetes isolates with different morphology were obtained. Of 50 fast-growing isolates, four potential antibiotic producing isolates were obtained by employing primary and secondary screening. The antibacterial activity of crude compounds extracted from the actinomycetes was tested against bacillus subtilis, staphylococcus aureus, salmonella sp. And serratia sp. Two actinomycete cultures, lm1a and mg1a that antagonized most bacteria with largest inhibition zones (b. Subtilis: 20.5 mm, salmonella sp.: 13.0 mm, serratia sp.: 13.0 mm, s. Aureus: 19.0 mm) during screening were selected for further study. Both of the isolates were found to be growing at ph and temperature ranges of 5.0-9.0 and 30-37°c respectively and tolerated nacl concentrations as high as 7%. Further, the isolates were presumed as streptomyces sp.

Keywords: actinomycetes, antibiotic, isolation, screening, antagonistic, antibacterial.

1. Introduction

The discovery and application of antibiotics in the treatment of bacterial diseases had been a noteworthy medical success of the 20th century. However, gradual emergence and spreading of antibiotics resistance among bacterial population due to misuse or overuse of antibiotics has had led to the development of public health problems. Antibiotic resistance in bacterial isolates was recorded since the first use of antibacterial agents. Penicillin-resistant *Escherichia coli* were the first to be discovered in 1940 to possess penicillinases that inactivated the drug penicillin, followed by discovery of penicillin-resistant *Staphylococcus aureus* in 1944. In 2008, the NDM-1 gene, encoding novel beta-lactamase enzyme capable of hydrolyzing penicillins, cephalosporins and carbapenems was discovered in *Klebsiella pneumoniae*. Bacteria possessing the gene were found to be resistant for most of the tested antibacterial agents (Moellering, 2010). Although there are advances in drug discovery and development in recent years, the world is not keeping pace with bacterial ability in adapting

and resisting antibiotics. In addition, many bacteria gain resistance to the newly launched drugs that were modifications of the existing antibiotics. Hence, it is highly essential to search for new antimicrobial compounds particularly from microorganisms to combat the threat of increasing population of antibiotic-resistant bacteria. Actinomycetes are filamentous bacteria that belong to the phyla actinobacteria and the order actinomycetales (Waksman, 1959a). Actinomycetes are known as the most invaluable prokaryotes in medical and biotechnology industries due to their ability in producing a vast number of bioactive molecules, particularly of the antibiotic compounds. *Streptomyces*, a representative genus of actinomycetes that is mainly of terrestrial soil origin, has accounted for the production of 60% of antibiotics which are useful in agricultural industries (Mellouli et al., 2003; Fguira et al., 2005; Singh et al., 2006; Thakur et al., 2007). The wide distribution of *Streptomyces* in soil and their proven ability to produce novel antibiotics and non-antibiotic lead molecules had caused these bacteria to be targeted in drug screening programme. Discovery of novel antibiotics from actinomycetes is important in helping to cope with the growing proportion of antibiotic-resistant bacterial infections that become untreatable. Hence, this investigation was conducted with the aim of isolating and screening for antibiotic-producing actinomycetes from rhizosphere soil. Selected antibiotic-producing actinomycetes were identified and effects of pH, temperature and concentration of sodium chloride on the growth of actinomycetes were also determined.

2. Methodology

2.Materials And Methods

2.1.Soil samples

A total of 25 soil samples, each weighing approximately 100 g was collected from rhizosphere regions of twelve different plants, including brinjal, banana, cassava, sapodilla, guava, ladyfinger, lemon, mango, papaya, rose apple, star-fruit and sour sop fruit. All soil samples were collected from a depth of 15 cm from a farm located at Sungai Ramal Luar, Selangor, Malaysia in December 2009. These samples were kept in plastic bags and air-dried at room temperature for 10 days and processed employing standard microbiological methods.

2.2.Culture media

Isolation of actinomycetes was carried out using starch casein agar (SCA) (Küster and Williams, 1964). Nutrient agar (Merck, Germany) was used for primary screening of antibiotic producing actinomycetes by cross-streak method. Further, antibiotic production by the actinomycetes was checked in submerged culture using starch casein broth (SCB) with the pH adjusted to 7.2.

2.3.Test bacteria

Two Gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus) and two Gram-negative bacteria (Salmonella sp. and Serratia sp.) were employed for screening of antibiotic producing actinomycetes. All test bacteria were obtained from Microbiology Laboratory, UCSI University and handled as per standard procedures.

2.4.Calcium carbonate treatment of soil samples

2.5 g of CaCO3 as added with 25 g of each soil sample and mixed in a pestle in a sterile mortar and pestle. The mixture was transferred to a sterile Petri dish and incubated at 30°C

for 7 days. Another set of each 25 g of soil was kept in sterile Petri dishes and used for isolation of actinomycetes.

2.5.Isolation and enumeration of actinomycetes

One gram of soil was taken from each CaCO3 treated and untreated soil samples and mixed with 9 ml of sterile physiological saline (9 g/l of NaCl). The mixture was allowed to settle and serial dilutions were prepared. Precisely, 100 µL of 10-3, 10-4, and 10-5 dilutions was pipetted and spread over the surface of SCA supplemented with nystatin (50 mg/ml) and rifampicin (50 mg/ml) in order to inhibit fungal and bacterial contamination respectively (Williams and Davies, 1965; Pisano et al., 1959). All the plates were incubated at 30°C for 7-21 days and the isolation was carried out in triplicates. Actinomycetes colonies were identified morphologically and colony forming units per gram (cfu/g) of soil were calculated. All isolated colonies were subcultured onto SCA without supplementing any antibiotics for 3 times with different period of incubation: The 1st and 2nd subcultures were carried out for 14 days followed by the 3rd subculture for 7 days. Based on the growth observed in the 3rd subculture, the fast-growing strains were selected for screening of antibiotic production.

2.6.Primary screening by cross-streak method

In primary screening, all selected isolates were streaked as a single straight line at the centre of nutrient agar plates. These plates were incubated at 30°C for 6 days and the experiment was done in duplicates. On the 7th day of incubation, test bacteria were inoculated by a single streak that was perpendicular to the actinomycete growth streaked at single straight line at the centre of the plate followed by incubation at 37°C for 24 hours. Inhibition zones (the distance between the edge of the test bacterial growth and the actinomycete colony) formed were measured and recorded in millimetre (Oskay, 2009b).

2.7.Secondary screening by disc and well-diffusion assays

The selected actinomycetes with antibacterial activity were subjected to secondary screening by disc and well diffusion assays. All the four test bacteria were inoculated into 10 ml nutrient broth and incubated at 37°C for 2-6 hours. Turbidity was standardized to 0.5 McFarland standards using sterile physiological saline, corresponding to the absorbance 0.08-0.13 at 625 nm. All active actinomycete isolates were inoculated into the flasks containing 50 ml SCB and were incubated in an orbital shaker of 200 rpm at 30°C for 6 days. At the end of incubation, the broth cultures were centrifuged at 10000 rpm for 10 min. Supernatant collected aseptically was checked for antibacterial production. Precisely, the sterile filter paper discs were placed on fresh test bacterial lawn culture on nutrient agar medium and loaded with 20 µl of actinomycete supernatant for disc-diffusion assay.

In well-diffusion assay, wells of approximately 6 mm in diameter and 5 mm in depth were made aseptically on nutrient agar plate containing fresh test bacterial lawn culture using cork-borer and each well was loaded with 100 µl of actinomycete supernatant. The test was duplicated for each actinomycete isolate. All plates were incubated at 37°C for 24 hours and were examined for inhibition zones produced as a result of antibacterial activity. Diameter of inhibition zones was measured and expressed in millimetre. Ampicillin (20 µg) and empty disc and/or well without any antibiotic or supernatant of actinomycetes were treated as positive and negative control respectively.

2.8.Antibacterial activity of crude antibiotic compounds

The isolates were inoculated into flasks containing 100 ml of SCB and incubated at 30°C in a shaker at 200 rpm for 6 days. The broth culture was filtered using Whatman No. 1 filter paper (11 µm) followed by 0.45 µm membrane filters. Organic solvent methanol was added to the filtrate in the ratio of 1:1 (v/v). The mixture was shaken vigorously for 1 hour and subsequently evaporated to dryness at 80-90°C in water bath. The residual crude extract obtained was dissolved in 2 ml of 0.2 M phosphate buffer (pH 7.0) by vortexing and preserved at 4°C. The antibacterial activity of the crude extract was determined by well-diffusion assay using 100 µl of crude extract. After 24 hours of incubation at 37°C, the diameter of inhibition zones was measured as stated previously.

2.9.Characterization of actinomycetes possessing antibiotic activity

Colour of mycelium, spores as well as pigmentation of the actinomycete isolates were determined following the method described by Shirling and Gottlieb (1966) on oatmeal agar (ISP3 medium), whereas the structure of mycelium was observed using light microscope at 1000x magnification employing oil immersion (Lumenera, Canada) with five days old actinomycete cultures. The cultures were also stained using Gram's staining and acid-fast staining methods and observations were made.

Biochemical tests that were carried out to characterize selected actinomycetes included hydrolysis of starch, casein, urea, lipid and gelatine and tryptophan utilization. Also, utilization of tryptophan and citrate, sugar fermentation, methyl red test and catalase production test were carried out.

3. Results & Discussion

3.1. Effect of calcium carbonate on isolation and enumeration of actinomycetes

Soil actinomycetes are distributed in both virgin and cultivated soils and over the past few years, actinomycetes have been isolated from various sources; marine sediments (Sahu et al., 2005), rhizosphere region of soil (Ramakrishnan et al., 2009), desert soil and fallen leaves (Tokohashi and Ōmura, 2003). In rhizosphere region, plants supply nutrient to actinomycetes via secretions of carbohydrates, amino acids, organic acids and other exudates as well as by sloughing root epidermal cells, whereas the actinomycetes play a major role as biocontrol agents via their antagonistic activity. At different point of soils profile, the highest number of terrestrial origin actinomycetes was isolated at a depth of 11-15 cm, where the average pH ranges from neutral to slight alkaline (Davies and Williams, 1970). Out of 25 CaCO3 treated soil samples subjected for isolation of actinomycetes, 18 soil samples produced higher number of actinomycetes population. The maximum and minimum number of actinomycetes produced from CaCO3 treated soil samples were 6.33×107 cfu/g and 1.0×105 cfu/g respectively. However, the soil samples that were not treated with CaCO3 produced a maximum of 6.7×105 cfu/g and a minimum of 0.3×105 cfu/g respectively (Fig. 1). The effect of CaCO3 treatment in stimulating isolation of soil actinomycetes was significant in rhizosphere soil samples collected from rhizosphere region of lemon tree, LM1 and ladyfinger, LF5 where, the actinomycete population had increased to the tune of 100-fold as compared with untreated soils collected from the same crops and other crops. CaCO3 is commonly employed to increase the number of actinomycete population from air-dried soils (El-Nakeeb et al., 1963; Oskay, 2009a). However, the precise mechanism of CaCO3 effect is

not well-studied. The increase in actinomycete population due to the addition of CaCO3 might be attributed as increase in pH, stimulation of formation aerial mycelium by Ca2+, which was observed in some actinomycetes viz., Streptomyces ambofaciens and Streptomyces alboniger (Natsume et al., 1989; Qin et al., 2009). Also, Ca2+ ion was found to be an essential component for germination of certain Streptomyces species such as S. streptomycini (Ensign, 1978). Based on the culture morphology, a total number of 300 actinomycetes that were morphologically different from each other had been isolated from both CaCO3 treated and untreated soil samples. However, only 280 actinomycetes were successfully recovered following the 1st subculture, whereas the remaining 20 actinomycetes were discarded as no growth was observed on subculturing. The 280 actinomycete isolates were subcultured for the 2nd time to ensure viability. Subsequently, these 280 actinomycetes isolates were subcultured again for the 3rd time to select the fast-growing strains. A total of 50 actinomycetes were found to be fast growers following the 3rd subculture.

3.2.Primary screening for antibiotic producing actinomycetes

The experimental results (Fig. 2) indicated that 9 out of 50 actinomycetes were found to possess antibacterial activity against test bacteria. The actinomycete isolate, MG1A was found to exhibit antagonistic activity against both Gram-positive and Gram-negative bacteria while LM1A and LF4A were inhibiting only Gram-positive test bacteria. However, other isolates including BJ3A, BJ3B, BJ3C, GV3A, RA2A and SS2A were found to inhibit certain bacterial species. Inability of the antibacterial compound to inhibit both Gram-positive and Gram-negative bacteria could be due to variation in the cell wall permeability of bacteria to different drugs (Struelens, 2003). B. subtilis was observed to be the most susceptible bacteria among the four test bacteria employed in this study to actinomycete LM1A whereas the actinomycete MG1A exhibited the strong inhibitory effect on the growth of Salmonella sp., Serratia sp. and S. aureus. It was noticed that the Gram-positive bacteria were more susceptible to antibacterial activity of actinomycetes than Gram-negative bacteria. Ability of Gram-negative bacteria to resist antibacterial agent is attributed to cell wall structure of bacteria. The Gram-negative bacteria possess an outer membrane of lipopolysaccharide, protein and phospholipids, which is attached to thin layer of peptidoglycan. The phospholipids of outer membrane and lipopolysaccharide form a lipid bilayer that serve as barrier for antibacterial drugs (Struelens, 2003). However, some antibacterial compounds could still be effective against Gram-negative bacteria. Hydrophobic antibiotics may penetrate Gram-negative bacteria cell wall by direct solubilisation through lipid layer of outer membrane, whereas hydrophilic antibiotics may enter the cell via water-filled membrane protein porins (Struelens, 2003).

Fig. 1. Effect of $CaCO_3$ treatment of soil samples on the enumeration of actinomycetes.

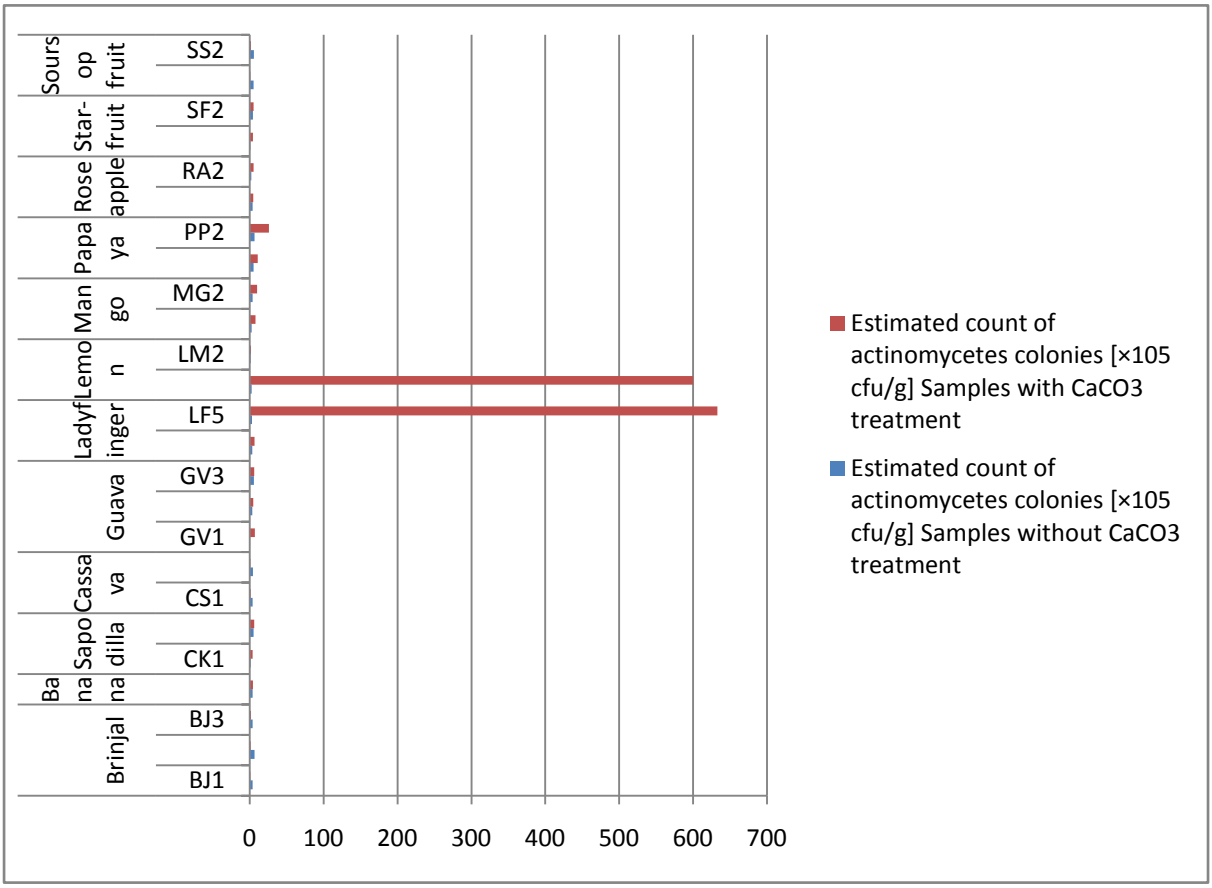
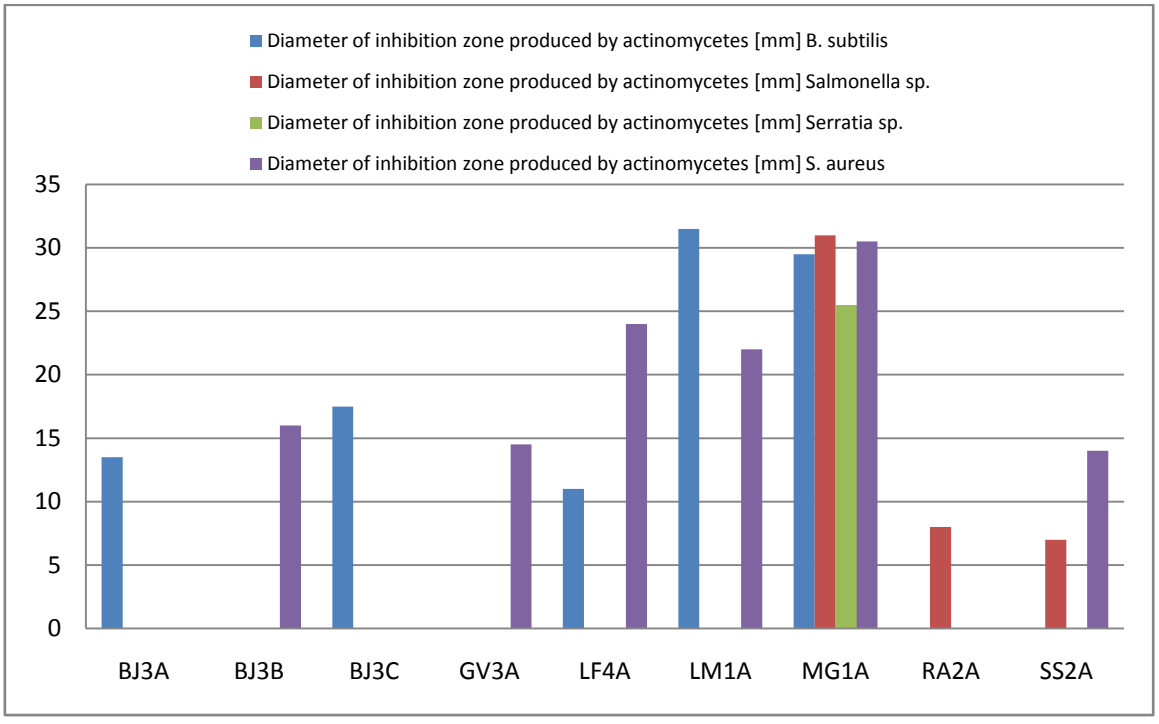


Fig. 2. Primary screening of selected actinomycetes for antibacterial activity by cross-streak method



3.3.Secondary screening for antagonistic activity by disc and well-diffusion assays

A total of 9 active actinomycetes, namely BJ3A, BJ3B, BJ3C, GV3A, LF4A, LM1A, MG1A, RA2A and SS2A screened from primary screening were subjected to secondary screening. Of nine actinomycetes tested for antagonistic activity against test bacteria, two actinomycetes viz., LM1A and MG1A were observed to inhibit test bacteria when tested using disc and well-diffusion assays. Among the two actinomycetes, LM1A and MG1A that were antagonistic against test bacteria, MG1A was superior to LM1A in controlling the bacterial growth. Apart from the LM1A and MG1A, the other actinomycete cultures such as BJ3C and SS2A had also shown the inhibitory effect on *B.subtilis* and *S.aureus* respectively (Table 1). By comparing the potential of supernatant of actinomycetes with 20 µg of ampicillin, the positive control, ampicillin (20 µg) was capable of inhibiting all test bacteria. *S. aureus* was observed to be the most susceptible to ampicillin, followed by *Salmonella* sp., *Serratia* sp and *B. subtilis*. The difference of antagonistic activity produced by ampicillin in disc and well-diffusion assays were observed to be the largest in *B. subtilis* plate, which was calculated as 3 mm whereas the smallest variation was given as 1 mm as observed in *Salmonella* plates. As observed from the result table, ampicillin produced a larger inhibition zones in disc-diffusion assay than in well-diffusion assay when the drug was tested against *B. subtilis*, *Serratia* sp. and *S. aureus*. However, a smaller inhibition zone was produced by ampicillin when the drug was tested against *Salmonella* sp. in disc-diffusion assay than in well-diffusion assay. Loss of antibacterial activity by several actinomycetes *viz*. BJ3A, BJ3B, GV3A, LF4A and RA2A was observed in secondary screening. The reason might be due to the culturing of actinomycetes in SCB in secondary screening whereas nutrient agar was used in primary screening. According to Shomura et al. (1979) and Pickup et al. (1993), the loss of antibiotic activity of actinomycetes was believed to be related with the fragmentation of vegetative mycelia in submerged cultures. Also, the inhibitory activity of actinomycetes against test bacteria in primary screening could be due to the combined effect of bacteriocin and other intracellular metabolites produced during the assay period. However, in secondary screening, the spent culture media of actinomycetes might not contain the metabolites released from actinomycetes (Lertcanawanichakul and Sawangnop, 2008). In addition, medium composition of SCB and nutrient agar vary which might have affected the biosynthesis of antibiotics by hampering the growth, metabolic activity and expression of antibiotic-coded gene (Doull and Vining, 1990; Sánchez et al., 2010).

3.4.Antibacterial activity of crude antibiotic compounds

The colour of all the crude extracts obtained from nine actinomycete cultures were maroon and soluble in 0.2 M phosphate buffer, except for the compound isolated from LF4A, which was observed as white sticky clumps and insoluble in buffer. This was believed to be one of the reasons for the absence of antagonistic activity produced by the bioactive compound extracted from LF4A (Table 2). It was also noticed that the compounds extracted from the actinomycetes MG1A and SS2A had not shown any inhibitory effect against the test bacteria whereas these cultures had expressed antibacterial activity in secondary screening. The loss of activity might be due to the extraction of compounds by evaporation at high temperature, which could have possibly damaged the heat-labile antibiotic compounds. Moreover, evaporation might have led to loss of analytes following partial evaporation or adsorption to the equipment. Furthermore, traces of acids and bases present in the extract might have

degraded acid or basic-labile components when the solvent volume approached to dryness during evaporation (Fletouris, 2007). The bioactive compounds extracted from the isolates BJ3B, BJ3C and LM1A were capable of inhibiting test bacteria. The crude compound extracted from BJ3C was active against *B. subtilis* as observed in secondary screening but with the production of larger inhibition zone than in secondary screening. By contrast, the actinomycete BJ3B which had not shown any inhibitory effect against any of the test bacteria during secondary screening had expressed antagonistic activity against three of the four test bacteria tested in this experiment. The crude extract of actinomycete culture LM1A was found to be active against all the four test bacteria whereas this particular culture had inhibited only the Gram-positive bacteria when tested under secondary screening.

Table 1.Secondary screening of selected actinomycetes by disc and well-diffusion assays using cell-free supernatant

Drug/ Actinomycetes	Diameter of inhibition zones produced by supernatant of actinomycetes by diffusion methods [mm]							
	Disc-diffusion assay				Well-diffusion assay			
	B. subtilis	***Salmonella* sp.**	***Serratia* sp.**	***S. aureus***	***B. subtilis***	***Salmonella* sp.**	***Serratia* sp.**	***S.aureus***
Ampicillin	30.0	49.0	47.0	66.5	27.0	50.0	45.5	64.5
BJ3A	- [a]	-	-	-	-	-	-	-
BJ3B	-	-	-	-	-	-	-	-
BJ3C	-	-	-	-	8.0	-	-	-
GV3A	-	-	-	-	-	-	-	-
LF4A	-	-	-	-	-	-	-	-
LM1A	16.0	-	-	10.0	20.5	-	-	17.0
MG1A	6.0	-	8.0	14.0	14.0	13.0	13.0	19.0
RA2A	-	-	-	-	-	-	-	-
SS2A	-	-	-	-	-	-	-	17.0

[a] Indicated that the test bacterium was not inhibited by cell-free supernatant of actinomycetes, characterized by the absence of inhibition zone

Table 2.Antibacterial activities of crude antibiotic compounds extracted from actinomycetes isolated from orchard soil.

Actinomycetes	Diameter of inhibition zone produced by crude extracts [mm]			
	B. subtilis	***Salmonella* sp.**	***Serratia* sp.**	***S. aureus***
BJ3A	- [b]	-	-	-
BJ3B	-	10.0	8.5	8.0
BJ3C	24.0	-	-	-
GV3A	-	-	-	-
LF4A	-	-	-	-
LM1A	9.0	10.0	9.0	25.5
MG1A	-	-	-	-
RA2A	-	-	-	-
SS2A	-	-	-	-

[b] Indicated that the test bacterium was not inhibited by the crude compounds.

3.5.Characterization of promising actinomycetes

The actinomycete cultures LM1A and MG1A were selected for characterization based on the antibacterial activity and spectrum broadness. Both actinomycete cultures produced aerial and substrate mycelia coupled with heavy spore formation on oatmeal agar. The colour of aerial mycelia was found to vary along with incubation time (Table 3). Both were capable of producing pigments on oatmeal agar and the colour of pigment was found to vary with incubation time for LM1A. Both actinomycetes LM1A and MG1A were Gram-positive but acid-fast negative bacteria due to lack of mycolic acid in the cell wall (Table 4). The mycelia of both isolates were straight to flexible and MG1A was observed to have fragmentation of mycelium into rod-shaped elements. The isolate LM1A was able to hydrolyse starch and lipid via production of amylase and lipase, whereas MG1A was capable of hydrolysing casein by caseinase activity. Both actinomycetes were able to hydrolyse urea by urease production and were not sugar fermenter. LM1A was able to utilize citrate and MG1A was capable of degrading H_2O_2 via catalase action. Both actinomycetes were presumably characterized as *Streptomyces* sp.

Table 3. Morphological characteristics of the actinomycetes LM1A and MG1A on oatmeal agar

Morphological characteristics	Colour changes of actinomycetes colonies					
	LM1A			MG1A		
	7 days	14 days	21 days	7 days	14 days	21 days
Aerial mycelium	Ash	Whitish grey	Yellowish grey	Pink	Pinkish grey	Pinkish grey
Substrate mycelium	Yellowish orange	Yellowish orange	Yellowish orange	White	White	White
Pigmentation	Yellowish orange	Yellowish orange	Yellowish orange	Light grey	Light brown	Brown

Table 4. The morphology of mycelium, staining and biochemical characteristics of LM1A and MG1A.

Characteristics	Reaction of actinomycetes	
	LM1A	MG1A
Differential staining		
Gram reaction	+ [c]	+
Acid-fast reaction	- [d]	-
Mycelium		
Morphology	Straight to flexible	Straight to flexible
Fragmentation	-	+
Musty odor production	+	+
Hydrolysis tests		
Starch	+	-
Casein	-	+
Urea	+	+
Lipid	+	-
Gelatin	-	-
Tryptophan	-	-

Sugar fermentation test		
Slant	Unchanged	Red
Butt	Unchanged	Red
Hydrogen sulfide production	-	-
Gas production	-	-
Methyl red test	-	-
Citrate utilization test	+	-
Catalase test	-	+

[c] Indicated a positive result and [d] indicated a negative result

4. Conclusion

In this study, $CaCO_3$ treatment on rhizosphere soil samples was capable of encouraging isolation of actinomycetes. This shows that the rhizosphere region of farming soil is a rich source of clinically important microorganisms. Since both macroscopic and microscopic morphology coupled with few biochemical tests could merely provide a presumptive genus or species identification, both prominent actinomycetes LM1A and MG1A should be further identified up to species level by molecular 16S rRNA assay and cell wall component analysis.

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References

Davies, F.L., and Williams, S.T. (1970). Studies on the ecology of actinomycetes in soil: I. The occurrence and distribution of actinomycetes in a pine forest soil. Soil Biology and Biochemistry. 2, 227-238.

Doull, J.L., and Vining, L.C. (1990). Physiology of antibiotic production in actinomycetes and some underlying control mechanisms. Biotechnology Advances. 8, 141-158.

El-Nakeeb, M.A., and Lechvalier, H.A. (1963). Selective isolation of aerobic actinomycetes. Applied Microbiology. 11, 75-77.

Ensign, J.C. (1978). Formation, properties and germination of actinomycetes spores. Annual Review of Microbiology. 32, 185-219.

Fguira, L.F., Fotso, S., Ameur-Mehdi, R.B., Mellouli, L., and Laatsch, H. (2005). Purification and structure elucidation of antifungal and antibacterial activities of newly isolated *Streptomyces* sp. strain US80. Research in Microbiology. 156, 341-347.

Fletouris, D.J. (2007). Clean up and fractionation methods. In: Pico Y (ed) Food toxicants analysis: techniques, strategies and development. Elsevier Ltd. Printed in Great Britain. pp. 306.

Küster, E., and Williams, S.T. (1964). Selection of media of aerobic actinomycetes. Nature. 202, 928-929.

Lee, S.H. (2008). Isolation, characterization and identification of actinomycetes from agriculture soils at Semongok, Sarawak. African Journal of Biotechnology 7(20), 3697-3702.

Lertcanawanichakul, M. and Sawangnop, S. (2008). A comparison of two methods used for measuring the antagonistic activity of *Bacillus* species. Walailak Journal of Science and Technology 5(2), 161-171.

Mellouli, L., Ameur-Mehdi, R.B., Samiha, S., Mansour, S., and Samir, B. (2003). Isolation, purification and partial characterization of antibacterial activities produced by a newly isolated *Streptomyces* sp. US24 strain. Research in Microbiology. 154, 345-352.

Moellering, R.C. (2010). NDM-1- A cause of worldwide concern. New England Journal of Medicine 363(25), 2377-2379.

Natsume, M., Yasui, K., and Marumo, S. (1989). Calcium ions regulates aerial mycelium formation in actinomycetes. Journal of Antibiotics 42(3), 440-447.

Oskay, M., Tamer, A.U. and Azeri, C. (2004). Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. African Journal of Biotechnology 3(9), 441-446.

Oskay, M. (2009a). Comparison of *Streptomyces* diversity between agricultural and non-agricultural soils by using various culture media. Scientific Research and Essay 4(10), 997-1005.

Oskay, M. (2009b). Antifungal and antibacterial compounds from *Streptomyces* strains. African Journal of Biotechnology 8(13), 3007-3017.

Pickup, K.M., Nolan, R.D., and Bsuhell, M.E. (1993). A method for increasing the success rate of duplicating antibiotic activity in agar and liquid cultures of *Streptomyces* isolates in new antibiotic screens. Journal of Fermentation and Bioengineering 76(2), 89-93.

Pisano, M.A., Sommer, M.J., and Brancaccio, L. (1989). Isolation of bioactive actinomycetes from marine sediments using rifampicin. Applied Microbiology and Biotechnology. 31, 609-612.

Qin, S., Li, J., Chen, H.H., Zhao, G.Z., Zhu, W.Y., Jiang, C.L., Xu, L.H., and Li., W.J. (2009). Isolation, diversity and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China. Applied and Environmental Microbiology 75(19), 6176-6186.

Ramakrishnan, J., Shunmugasundaram, M., and Narayanan, M. (2009). *Streptomyces* sp. SCBT isolated from rhizosphere soil of medicinal plants is antagonistic to pathogenic bacteria. Iranian Journal of Biotechnology 7(2), 75-81.

Sahu, M.K., Kumar, K.S., and Kannan, L. (2005). Isolation of actinomycetes from various samples of the Vellar Estuary, southeast coast of India. Pollution and Research. 24, 45-48.

Sánchez, S., Chávez, A., Forero, A., Garcia-Huante, Y., Romero, A., Sánchez, M., Rocha, D., Sánchez, B., Avalos, M., Guzmán-Trampe, S., Rodríguez-Sanoja, R., Langley, E., and Ruiz, B. (2010). Carbon source regulation of antibiotic production. Journal of Antibiotics. 63, 442-459.

Shirling, E.B., Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. International Journal of Systematic Bacteriology 16(3), 313-340.

Shomura, T., Yoshida, J., Amano, S., Kojima, M., Inouye, S., and Niida, T. (1979). Studies on Actinomycetales producing antibiotics only on agar culture: screening, taxonomy and

morphology- productivity relationship of *Streptomyces halstedii*, strain SF- 1993. Journal of Antibiotics 32(5), 427- 435.

Singh, L.S., Baruah, I., and Bora, T.C. (2006). Actinomycetes of Loktak habitat: isolation and screening for antimicrobial activities. Biotechnology 5(2), 217-221.

Struelens, M.C. (2003). The problem of resistance. In: Finch RG (ed) Antibiotic and chemotherapy: anti- infective agents and their use in therapy, 8th Ed. Elsevier Ltd. Printed in Great Britain. pp. 28.

Thakur, D., Yadav, A., Gogoi, B.K., and Bora, T.C. (2007). Isolation and screening of *Streptomyces* in soil of protected forest areas from the states of Assam and Tripura, India, for antimicrobial metabolites. Journal of Medical Mycology. 17, 242-249.

Tokohashi, Y., and Ōmura, S. (2003). Isolation of new actinomycetes strains for the screening of new bioactive compounds. The Journal of General and Applied Microbiology. 49, 141-154.

Unno, Y., Shinano, T., Wasaki, J., and Osaki, M., (2004). Microspatial distribution of bacteria in the rhizosphere. In: Werner D (ed) Biological resources and migration. Springer Ltd. Printed in The United States. pp. 157.

Waksman, S.A. (1959). The Actinomycetes Volume 1. Lippincott Williams and Wilkins. Printed in The United States. pp. 53, 113, 116, 117, 126, 134, 146.

Williams, S.T., Goodfellow, M., Alderson, G., Wellington, E.M.H., Sneath, P.H.A., and Sackin, M.J. (1983). Numerical classification of *Streptomyces* and related genera. Journal of General Microbiology. 129, 1743-1813.