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Prescreening of bioactivities from actinomycetes isolated from forest peat soil of Sarawak

(Penyaringan awalan aktiviti biologi aktinomiset dari hutan tanah gambut Sarawak)

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Abstract

Forest soil has been long regarded as a place for the isolation of rare and beneficial microorganisms such as actinomycetes. In this study, 40 isolates of actinomycetes were screened using multi-stage Dispersion and Differential Centrifugation (DDC) method. All the 40 isolates were later tested for their ability to secrete bioactivities such as cellulase, mannanase, xylanase, lipase, protease and antimicrobial compounds. Prescreening of the 40 isolates of actinomycetes showed that 45% produced cellulase, 5% mannanase, 12.5% xylanase, 12.5% protease and 32.5% lipase. It was observed that these actinomycetes also produced antimicrobial activities against various test pathogens such as *Ralstonia solanacearum* (10%), *Pantoea stewartii* (20%) and *Bacillus subtilis* (10%). Eight of the best producers of the bioactivities were identified using 16S rRNA primers binding to the 16S rRNA sequence of the microorganisms. Seven of the actinomycetes were identified to be from the genus *Streptomyces* while one was from the genus *Kitasatosproria*. These eight potential actinomycetes need further study to fully characterize their benefits before application in any industry.

Introduction

For decades, actinomycetes have been known as excellent sources of useful natural products of high commercial value, such as antimicrobial compounds and valuable extra cellular enzymes (proteases, cellulases, xylanases, lipases, glucose-isomerase, pectinolytic enzymes) (Bhat and Bhat 1997; Jeffrey and Azrizal 2007a). This group of bacteria is still being explored for new bioactive substances with diverse chemical structure and biological activities (Watve et al. 2001; Jeffrey et al. 2007b). Actinomycetes are widely distributed in nature and they thrive in soil where they play an important role in the bioremediation, mineralization and decomposition of organic matter with the production of numerous extracellular enzymes.

In the past few decades, extensive studies had been conducted to test and evaluate the potential of actinomycetes species as a potential biological control of numerous plant pathogens. In these studies actinomycetes showed ability to reduce pathogen survival and infectivity in infested soil. Several studies that showed encouraging results demonstrated the ability of actinomycetes to act antagonistically with Pythium Root Rot of sugarcane and corn by *Pythium* spp. (Johnson 1954); root rot and wilt diseases of *Lens Culinaris*

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due to *Fusarium* and *Sclerotium* infection (Mehrotra and Caludius 1972); fungal root and seed rots by various pathogenic fungi (Yuan and Crawford 1995) and *Fusarium* wilt in banana (Cao et al. 2004).

Moncheva et al. (2002) studied the antagonistic activity of Antarctica actinomycetes against phyto-pathogenic bacteria (*Xanthomonas* spp., *Pseudomonas syringae pv. tomato* and *Clavibacter michiganensis*). Similar study was also conducted by Ndonde and Semu (2001), Barakate et al. (2000) and Oskay et al. (2004) for *Streptomyces* spp. isolated from soil samples from Tanzanian, Morocco and Turkey respectively.

Since Malaysia is known as one of the twelve world's mega biodiversity countries as it possesses vast unexplored flora and fauna as well as rich with microorganisms, attempts have been made to discover the potential of actinomycetes in controlling plant diseases (Lo et al. 2002; Jeffrey et al. 2007b). The diversity of Malaysian actinomycetes is poorly studied and information gathered remains scarce (Vikineswary et al. 1997; Ismet et al. 2002; Lo et al. 2002; Numata and Nimura 2003; Jeffrey et al. 2007ab).

Currently, there is only a handful of documentation from our fellow Malaysian researchers on the Malaysian actinomycetes strains with the potential of synthesizing useful bioactive compounds. This paper highlights the isolation and screening of bioactive compounds from actinomycetes isolated from peat soil in Malaysia.

Peat soil is an accumulation of organic matter resulting from incomplete decomposition of plant material. Koesnandar et al. (2006) described peat soil characteristics as low in pH, high in organic matter and cation exchange capacity and low in base saturation. These characteristics reduce the ability of plants to utilize the nutrients such as potassium, calcium, and magnesium. Study on the isolation of actinomycetes and their properties from peat soil would provide researchers with a scenario on the diversity of actinomycetes in these areas.

Materials and methods

Collection of soil samples

Soil samples were collected from MARDI peat land research station at Sessang, Sarawak. Soil collection was carried out using a spade (up to 10 cm depth) after removing ~3 cm of the soil surface. Samples were sieved (<6 cm diameter) and kept in sterile zip-lock polyethylene bags during transportation.

Isolation and enumeration of actinomycetes bacteria

The multi-stage Dispersion and Differential Centrifugation (DDC) method (Hopkins et al. 1991) was used for the isolation of actinomycetes bacteria with minor modifications. Commercially available Chelex 100 sodium form was used as substitution of the sodium form chelating resin as suggested by Herron and Wellington (1990). The actinomycetes dissociation was achieved in three successive stages, thus obtaining four different fractions for each individual soil sample. Supernatants (S1, S2 and S3) retrieved from the interval differential centrifugations at 500 rpm and pellet or residue (R) collected by the end of the protocol were kept at 4 ºC.

Serial dilutions (10^{-1}–10^{-3}) were done for all fractions and spread plate onto Starch Casein Agar pH 7.0, supplemented with Cycloheximide (50 µg/ml). The plates were incubated at 28 ± 2 ºC for 14 days. Total plate count were calculated from average counts of three replicates and expressed as colony forming units (cfu) per gram of dry soil.

Preliminary screening of extra-cellular enzyme and anti-microbial activity

Screening for extra-cellular hydrolysis enzyme activities (manannase, xylanase and cellulase) of actinomycetes isolates were carried out on minimal medium containing the following components (in grams per

litre): bacteriological peptone, 1; yeast extract, 1; KH_2PO_4, 0.5; $(NH_4)_2HPO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.5; agar, 15; and substrate, 1 which contained Megazyme: AZO-CM-Cellulose, AZO-Carob-Galactomannan and AZO-Xylan (oat).

Protease test was conducted using gelatin hydrolysis assay as described by Frazier (1926) while lipase activity was screened using method for determination of esterastic activity (Sierra 1957) with little modification. Tween 80 used in the esterastic assay test was replaced with Tween 20. Pure actinomycetes cultures are spot-inoculated on substrate medium. Size of clearing zone observed was recorded for the duration of 3 days.

Plate diffusion method (Bauer et al. 1966) with the modification suggested by Barakate et al. (2000) was applied in screening of anti-microbial activities. Actinomycetes strains on Starch Casein Agar (SCA) medium were removed using a sterile cork bore (5 mm in diameter) to make agar stabs and placed onto pathogenic bacteria lawn plates. Formation of clearing zone was observed and measured after 3 days of incubation. Inhibition zone was measured from diameter of clear zone as observed from the antagonistic reaction. Pathogenic bacteria strains used for screening were *Xanthomonas campestris, Ralstonia solanacearum, Bacillus subtilis* and *Pantoea stewartii*.

Isolation of genomic DNA

Genomic DNA (gDNA) was isolated from 8 selected bacterial cultures using the BACTOZOL™ Kit and according to manufacturer's protocol (Molecular Research Center).

Polymerase chain reaction (PCR) amplifications

Eight of the best producing microbes selected were identified using primers flanking their 16S rRNA region. Polymerase chain reaction (PCR) was carried out using 15.5 µl sterile distilled H_2O, 2.5 µl 10X PCR buffer, 2.0 µl 25 mM MgCl, 0.6 µl dNTPs, 0.4 µl Taq polymerase, 1.0 µl of 20 pmol of each primer Com1 and Com2 (Schwieger and Tebbe 1998) and 2.0 µl of g DNA per reaction.

PCR was performed as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s and elongation at 72 °C for 2 min and a final elongation for 10 min at 72 °C. PCR products were electrophoresed on a 1.0 % agarose gel at 80 V for 50 min and viewed using the Gel Documentation System from Biorad. PCR products were later purified using the Invitrogen gel purification kit according to manufacturer's protocol (http://www.invitrogen.com).

Sequencing of PCR products

Sequencing of the purified PCR products was performed by First Base Laboratories Sdn. Bhd., (Selangor) using the ABI PRISM® 377 DNA Sequencer (Applied Biosystems). The 16S rRNA sequences obtained were identified by comparing to sequences in the National Centre for Biotechnology Information (NCBI) genebank database using the Basic Local Alignment Search Tool (BLAST) (Altschul et. al. 1990).

Conservation of potential isolates

All the potential microbes were kept on slant as working cultures and in –80 °C glycerol stock form for conservation purposes.

Results and discussion

Isolation and enumeration of actinomycetes

A total of 40 actinomycetes with different morphology and characteristics were isolated (*Table 1*). The average colony forming unit/g (cfu/g) for the soil was 3.0 x 10^4 for the whole collection area regardless of the sample locations. The cfu/g obtained was considered to be low as compared to those collected from non-peat land forest such as Ayer Keroh Recreational Park, Melaka (2.8–5.6 x 10^6) (Jeffrey et al. 2008).

Table 1. Colour of actinomycetes colonies observed from 40 isolates

Colony colour	Isolate number	Number of isolates
Grey	TN01,TN02,TN04,TN05,TN06,TN10,TN11, TN12,TN13,TN14,TN16,TN18,TN19,TN20, TN25,TN26,TN31,TN33,TN39,TN40	20
Brown	TN03,TN09,TN17,TN21,TN22,TN23,TN30, TN32,TN34,TN35	10
White	TN07,TN08,TN15,TN24,TN27,TN28,TN29, TN36,TN37,TN38	10
	Total	40

Preliminary screening of extra-cellular enzyme and anti-microbial activity

A total of 77.5 % (31/40) of isolates exhibited one or more secondary activities while 22.5 % (9/40) did not show any activity. These actinomycetes were isolated mainly from soil beneath and surrounding different rotten fallen tree trunks and areas near roots of forest trees with dense mat of fallen leaves and other plant debris that accumulated and are being degraded all year long.

All these biomasses are plant components that contain high percentage of cellulose and hemicelluloses. The type of nutrient available and plant secondary metabolites produced may have pressured the microbes to produce their own secondary metabolites in order to thrive and survive in the ecosystem (Lo et al. 2002; Oskay et al. 2004).

Hydrolysis of substrates on minimal medium was observed as a decolourized clearing zone on the medium due to the production of extra-cellular enzymes (*Figure 1*). The diameter of the halo zone produced indicated the intensity of the extra-cellular enzyme excreted. Of 40 isolates tested, 24 (60 %) of the actinomycetes bacteria had one or more enzymatic activity on the substrates, with 5 isolates (12.5 %) producing xylanase, 18 isolates (45 %) producing cellulase, 2 isolates (5 %) producing galacto-mannanase, 13 isolates (32.5 %) producing lipase (*Figure 2*) and 5 isolates (12.5 %) producing protease (*Figure 3*). The distribution of hemicelluloses in wood fibres varies depending on the wood species. In hard wood, most of the hemicellulose is xylan, while softwood is mainly formed by glucomannan (Montiel et al. 1999).

Cellulases and xylanases encompass a collection of enzymes whose primary function is for hydrolyzing β-1, 4-glycosidic linkages in the major plant structural polysaccharides, cellulose and xylan (Gilbert and Hazlewood 1993). Actinomycetes can produce several lignocellulytic enzymes such as mannanase (Montiel et al. 1999), xylanase (López et al. 1995), cellulases (Wilson 1992), peroxide (Rob et al. 1997) and laccases (Berrocal et al. 1997).

Actinomycetes isolates that produce lignocellulosic degrading enzymes have good potential to be exploited as inoculums in composting agricultural wastes (Lacey 1997). Actinomycetes incorporation in composting of oil palm empty fruit bunches (Thambirajah et al. 1995) and green waste compost (Lacey 1997) have proven its potential with high viability throughout composting process for both mesophilic and thermophilic strains.

In the antagonism study conducted, 40 % (16/40) of the actinomycetes isolates had shown antimicrobial activity against one or more plant pathogens. Antagonistic abilities were observed against *Ralstonia solanacearum* 10 % (4/40), *Pantoea stewartii* 20 % (8/40) and *Bacillus subtilis* 10 % (4/40) (*Figure 4* and *Table 2*).

Figure 1. Actinomycetes secreting cellulase creating a clear zone around the colony

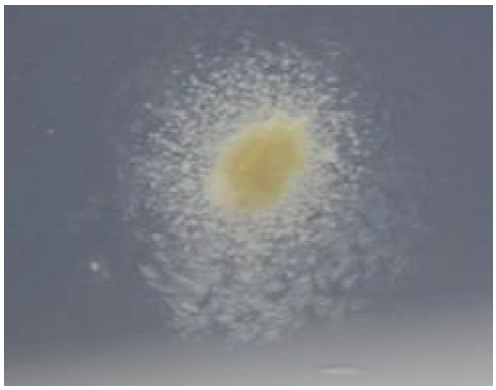

Figure 2. Actinomycetes producing lipase forming residue of particles around the microbes

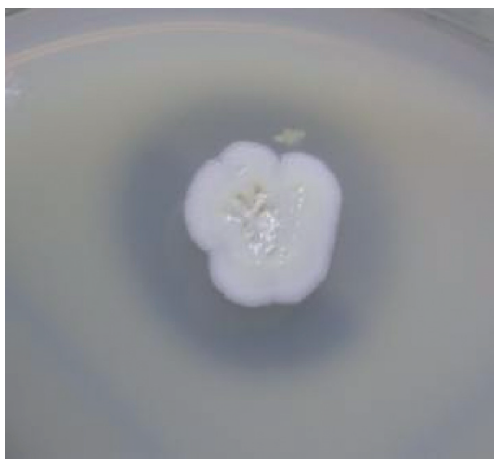

Figure 3. Actinomycetes secreting protease forming a clear zone around the microbes

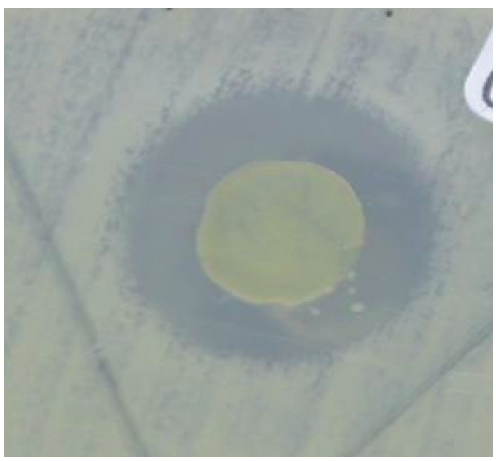

*Figure 4. Actinomycetes producing antimicrobial compounds against **Bacillus subtilis** and forming a clear zone around the organisms*

This study showed that isolates which inhibited the growth of gram-negative bacteria were also expressing antibacterial activity towards gram-positive bacteria. This phenomenon observed suggested that these actinomycetes produced antibiotic substances which had a broad spectrum of activity (Ndonde and Semu 2001; Moncheva et al. 2002). Due to the large number of morphological diverse types of actinomycetes which are antagonistic to the same pathogens tested, it has been suggested that several different antibiotics are involved (Johnson 1954: Barakate et al. 2000).

In actinomycetes, the rediscovery rate for antibiotics is as high as 99 % (Zaehner and Fiedler 1995). The sensitivity of the tested pathogenic bacteria to actinomycetes isolates suggests that these pathogens maybe simply sensitive to different antimicrobial substances and antibiotics of different types and concentrations. Also it is possible that the pathogen does not possess the antibiotic resistance as they may have not been exposed to similar antibiotics previously (Ndonde and Semu 2001).

It is possible that other useful antimicrobial compounds were also produced and not being screened (Barakate et al. 2000). Porter (1971) hypothesized that all actinomycetes possessed some antimicrobial properties which can be

assessed when cultured and studied under suitable conditions. Kokare et al. (2004) demonstrated that antimicrobial activity of actinomycetes was dependent on the media used for cultivation.

Molecular identification of actinomycetes

It was observed from the BLAST analysis of the sequences obtained from the eight isolates of the actinomycetes that seven belonged to the genus *Streptomyces*, while only one belonged to the *Kitasatosproria* genus (*Table 2*).

Conclusion

Preliminary screening of actinomycetes showed that 40 isolates produced various bioactivities such as cellulase (45%), galacto-mannanase (5%), xylanase (12.5%), protease (12.5%) and lipase (32.5%). It was observed that these actinomycetes also produced antimicrobial activities against various test pathogens; *Ralstonia solanacearum* (10%), *Pantoea stewartii* (20%) and *Bacillus subtilis* (10%). Seven out of eight best producers of bioactivities were identified as *Streptomyces* and hence we can conclude that *Streptomyces* species in the soil is the most significant. These potential actinomycetes can be further exploited for use in various industries such as agriculture, food and paper.

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Table 2. Bioactivities produced by eight potential actinomycetes

Isolate number	Identity from BLAST	Enzymatic activity (Clear zone in mm)					Antimicrobial activity (Clear zone in mm)			
		Celllulase	Mannanase	Xylanase	Protease	Lipase	*Ralstonia solanacearum*	*Xanthomonas campestris*	*Bacillus subtilis*	*Pantoae stewartii*
TN01	*Streptomyces* sp.	18	0	0	0	20	15	0	10	30
TN03	*Streptomyces* sp.	16	0	28	0	10	0	0	0	25
TN04	*Streptomyces* sp.	35	0	25	20	15	0	0	8	25
TN06	*Streptomyces* sp.	20	0	0	0	30	20	0	15	33
TN07	*Streptomyces* sp.	25	18	0	0	28	19	0	19	20
TN10	*Kitasatospora cystarginea* strain AS 4.1587	8	0	0	0	32	0	0	0	32
TN17	*Streptomyces* sp.	0	20	0	18	0	16	0	0	30
TN30	*Streptomyces aureofaciens*	29	0	24	28	0	0	0	0	28

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Abstrak

Tanah hutan sudah sekian lama dianggap menjadi tempat pemencilan mikrob seperti aktinomiset yang jarang ditemui dan berguna. Dalam kajian ini, 40 pencilan aktinomiset telah disaring dengan menggunakan teknik Pengemparan Serakan dan Kebezaan (DDC) pelbagai peringkat. Kesemua 40 pencilan kemudiannya diuji kebolehan mereka menghasilkan bioaktiviti seperti enzim selulase, mannanase, xilanase, protease, lipase dan sebatian antimikrob. Penyaringan ke atas 40 pencilan tersebut mendapati 45% menghasilkan sellulase, 5% mannanase, 12.5% xilanase, 12.5% protease dan 32.5% lipase. Aktinomiset ini juga didapati menghasilkan pelbagai aktiviti antimikrob terhadap patogen yang diuji seperti *Ralstonia solanacearum* (10%), *Pantoea stewartii* (20%) dan *Bacillus subtilis* (10%). Lapan penghasil terbaik bioaktiviti dikenal pasti melalui jujukan 16S rRNA mikroorganisma tersebut. Tujuh daripada aktinomiset ini dikenal pasti berasal daripada genus *Streptomyces* dan satu lagi daripada genus *Kitasatosporia*. Lapan aktinomiset yang berpotensi ini perlu dikaji dengan lebih rapi sebelum digunakan dalam mana-mana industri.

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