

ACTINOMYCETES ISOLATED FROM SOIL SAMPLES FROM THE CROCKER RANGE SABAH

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ABSTRACT

A diversity of actinomycetes was isolated from various sites of top soils throughout the Crocker Range in Sabah. The soils were mainly collected during the expedition (15–25 October 1999) together with 2 soil samples collected on 28 November 1999 under Rafflesia keithii in the Rafflesia Reserve Forest, Gunung Mas. A total of 78 strains of actinomycetes, probably mostly Streptomyces, were obtained from different sites. Amongst these strains 20 have been aerobically grown in shaking liquid cultures. Acetone extracts of these cultures were screened for MAPK Kinase and MAP Kinase Phosphatase in a yeast system in the preliminary screening of novel cancer drugs. This screening system is based on the fact that the MAP kinase pathway is homologues from yeast to human. However, no such inhibitors were found. A few strains with pigmentation were collected from specific locations.

INTRODUCTION

Actinomycetes are gram positive bacteria frequently filamentous and sporulating with DNA rich in G+C from 57–75%. Some of their secondary metabolites have employed as useful microbial compounds (Prescott, Harley & Klein, 1993). Examples include streptomycin from *Streptomyces griseus* for treatment of tuberculosis caused by *Mycobacterium tuberculosis* and the immunosuppress drug, tacrolimus (FK506) produced by *S. tsukubaensis*. Actinomycetes of about 100 genera exist in soils (Yokota, 1997). In their natural habitat, such as forests, the actinomycetes interact in various ways with the higher plants.

The fallen tress, barks and flowers first provide nutrients both to the microbes and plants through microbial degradation of carbohydrates, lipids and proteins to sugars, fatty acids, glycerol and amino acids and ultimately to mineralisation. Besides providing these nutrients, plant secondary metabolites (such as dipterocarp resins) that are generally toxic to microorganisms, will need to be degraded or detoxified by certain microbes. These degraders (microbes) are selectively pressured and ultimately evolve to produce novel secondary metabolites of their possibly to counteract the toxic plant secondary metabolites (Park et al, 1999 and Ho et al, 2000).

In this study, soil samples were collected in different habitats in the Crocker Range National Park to investigate the diversity of actinomycetes. Actinomycetes were then isolated on selective medium humic-acid + modified B vitamins and extracts were screened for biological activities of the secondary metabolites.

MATERIALS AND METHODS

Soil samples: Soil samples were collected by sterile method from various locations visited throughout this scientific expedition to Crocker Range Park (Figure 1), from an area of mist forest (1400—1500m from sea level), submontane rain forest (Mahua), hill forest (uphill of Mensalog River, Ulu Senangang) to cultivated areas (of introduced *Theobroma cacao* and *Tectonia grandis*). Soil samples were air-dried under room temperature for about 30 days before isolation (Table 1). A second set (Table 2) used air-dried soils stored at room temperature over a long period (9-11 months).

Isolation of actinomycetes: 0.5g of soil samples was suspended in 9.5ml of sterile distilled water and was 1000-fold diluted. 0.1ml of the dilutions was spread on humic acid + modified B vitamins agar (HV) medium, pH 7.2, supplemented with cycloheximide. The plates were incubated at 28^oC for 2 weeks.

Classification of actinomycetes: Isolated strains were transferred from HV medium onto oatmeal agar medium, pH 7.2 and incubated at 28^oC for 14 days. Colouration of aerial mycelium (on the surface of agar), substrate mycelium (underside of plate) and diffusible pigment were observed.

Extraction of secondary metabolites: Submerged fermentation of purified cultures were carried out in liquid medium of 2% mannitol, 2% peptone and 1% glucose, pH 7.2, for 5 days at 28^oC, 220rpm. Resultant broths were added with equal volume of acetone to extract secondary metabolites (final concentration of extract in 50% acetone).

Screening: The acetone extracts were tested for inhibitory activity against MAPK kinase and MAP kinase phosphatase inhibitors into yeast strains *Saccharomyces cerevisiae* MKK1^{P386} and *S. cerevisiae* MKK1^{P386}—MSG5 respectively.

RESULTS AND DISCUSSION

A total of 78 isolates of actinomycetes were isolated from 22 soil samples (Table 1 & 2) while 16 other soil samples without any isolate (Table 3). Some strains were isolated from soil under *Theobroma cacao*, *Rhododendron* sp. and particularly *Rafflesia kethii* (Figure 2) and *R. pricei* (Figure 3). Most of the isolates were presumed to be of the genera *Streptomyces* as they showed good sporulation with compact, chalk-like dry colonies of different colours. A few pigmented strains, unique to individual sites were observed.

Table 1. Strains isolated from air-dried soils collected in Crocker Range Sabah - isolated within one month after collection (Lo & Ho, 2001)

Type of forest	Site	Collection	Soil from under	Location	Laboratory
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		Date	tree/ plant		number of isolates
Submontane rain forest, Mahua Crocker Range	SA-38	15/10/99	<i>Rafflesia pricei</i> young living bud (I)	Submontane forest Mahua,	L-51, C-2
	SA-39	15/10/99	<i>R. pricei</i> young living bud (II)	Same as above	L-52
	SA.43	15/10/99	<i>R. pricei</i> young living bud (III)	Same as above	L-46,C-3
	SA-44	15/10/99	Blossom <i>R. pricei</i>	Same as above	L-25
	SA45	15/10/99	Bamboo	Same as above	C-4
Secondary hill forest, Crocker Range	SA-47	16/10/99	<i>Lithocarpus turbinatus</i>	Secondary hill forest, above Crocker Range Park Headquarters, Keningau, 1000m from sea level	L-54, C-1
River side forest, Mahua	SA-59	17/10/99	<i>Duabanga moluccana</i>	Left side of Mahua River, primary forest, Mahua	W-1
	SA-60	17/10/99	<i>Dendroxnidae oblanculata</i>	Same as SA-59	L-34, L-56
Agricultural area in village ground, Tikolod, Tambunan	SA-58	16/10/99	<i>Lithocarpus leptogyne</i> (Korth) Soepadmo	Beside Tikolod river, Tikolod, Tambunan	L-55
Hii forest, Ulu Senagang, Crocker Range	SA-64	18/10/99	<i>Parashorea tomentella</i>	Steep slope 60°, logged forest, entrance to park, Ulu Senagang	L-55
	SA-65	18/10/99	<i>Parashorea malaanonan</i> (sapling)	Near SA-64	L-39, L-41, L-43, L-44, L-45, C-6, C-7, C-8, C-9, C10.
	SA-62	18/10/99	<i>Artocarpus odoratissimus</i>	Cultivation area, uphill of Mensalog River, Ulu Senagang	L-35, L-38, L-57, L-58, L-59
Cultivated areas, Melalap, Crocker Range	SA-66	18/10/99	<i>Tectonia grandis</i>	Near Sg. Melulut, on the way to park, Melalap	L-22, L-27, L-30, L-11, L-12
	SA-67	18/10/99	<i>Theobroma cacao</i>	Near Sg. Melulut, on the way to park, Melalap	L-28, L-29, L-31, L-60, L-61
Rafflesia Reserve Forest Gunung Mas	SA80	28/11/99	<i>Rafflesia keithii</i>	Rafflesia Reserve Plot 8 Gunung Mas, Tambunan	E-1, E-2, E-3, E-4, E-5, E-7, E-8, E-11

- All of the isolates were recovered from humic acid + B vitamins* agar plates (pH 7.2) which

had been incubated for 14-30 days at 28⁰C.

*B vitamins: thiamine-HCl, pyridoxin-HCl and inositol.

- The isolates from E1-E11 were recovered from humic acid + B vitamins** agar plates (pH7.2) which had been incubated for 14-30 days at 28⁰C.

*B vitamins: thiamine-HCl, pyridoxin-HCl, riboflavin, niacin, inositol, Ca-pantothenate & p-aminobenzoic acid.

- Isolated by 'L'-Lo, C.W; 'C'- Cheah, H-Y; 'W'-Wong, N.K. 'E'- Lai, N.S. Eric

Table 2: Strains isolated from air-dried soils collected in Crocker Range, Sabah — after long storage, 9 to 11 months, at room temperature (Lo&Ho,2001)

Type of forest	Site	Collection Date	Soil from under tree/plant	Location	Laboratory Isolates number of
Submontane rain forest, Mahua, Crocker Range	SA-35	15/10/99	<i>Canarium denticulatum</i>	Trail to Mahua Waterfall, near base camp	L-71, L-72
	SA-36	15/10/99	<i>Duabanga moluccana</i>	Near SA-35, on the right side of Mahua River	L-73, L-74, L-75, L-76, L-77
	SA_75	22/10/99	<i>Duabanga moluccana</i>	Trail to Mahua Waterfall, near base camp	L-111
	SA-60	17/10/99	<i>Dendroxnidae oblanculata</i>	Left side of Mahua River, near Mahua Base Camp	L-89, L-90, L-91, L-92, L-93, L-94
	SA-37	15/10/99	<i>Rafflesia pricei</i> (dead)	1 st colony of Rafflesia site, Mahua	L-78, L-79
	SA-59	17/10/99	<i>Duabanga moluccana</i>	Same as SA-60	L-113
Mist forest, near Telecom Tower, beside road Keningau to Ulu Kimanis Crocker Range	SA-51	16/10/99	<i>Rhododendron suaveolens</i> Sleumer	1400-1500m from sea level, Mist Forest, 4-5km from HQ, near Telecom Tower	L-80,L-104. L-105, L-106
	SA-53	16/10/99	<i>Rhododendron crassifolium</i> Stapf	Same as SA-51	L-81, L-82, L-83, L-84

- All of the isolates were recovered from humic acid + B vitamins** agar plates (pH 7.2) which had been incubated for 14-30 days at 28⁰C. Isolated by Lo.C.W.

** B vitamins: thiamine-HCl, pyridoxin-HCl, riboflavin, niacin, inositol, Capantothenate, p-aminobenzoic acid and biotin

Table 3: List of soils without isolate of actinomycetes on humic acid + B vitamins agar plates

Type of forest	Site	Collection date	Soil from under tree/plant	Location
Submontane rainforest, Mahua	SA-40	15/10/99	<i>Rafflesia pricei</i> young living bud	Submontane forest, Mahua
	SA-41	15/10/99	<i>R. pricei</i> (dead)	Same as above
	SA-61	17/10/99	<i>Epipogium reselum</i>	Collected by Dr. Axel Poulsen, 1516
	SA-74	21/10/99	<i>Marchantia acaulis</i>	Trail to Mahua Waterfall, near base camp
Secondary hill forest, Crocker Range	SA-48	16/10/99	<i>Ficus</i> sp	3 metres from SA-47, Secondary hill forest, near C.R.P Headquarter, Keningau
	SA-54	16/10/99	<i>Lithocarpus</i> sp.	1100m from sea level, Secondary forest, on the way to HQ
Mist Forest, Crocker Range	SA-49	16/10/99	<i>Cytandra gibbsiae</i>	1400-1500m from sea level, Mist Forest, 4-5 km from HQ, near Telecom Tower
	SA-50	16/10/99	<i>Clethra</i> sp.	Same as above
	SA-52	16/10/99	<i>Rhododendron crassifolium</i> Stapf	Same as above
	SA-70	19/10/99	<i>Rhododendron x planecostatium</i>	On the right side of road towards Crocker Range, Mist Forest, Ulu Kimanis
	SA-73	19/10/99	<i>Rhododendron orbiculatum</i>	1200m from sea level, Mist forest, road to Ulu Kimanis secondary camp
Degraded land/Disturbed forest	SA-55	16/10/99	<i>Schitachyum</i> sp	Open field, Sg. Pampang, Keningau
	SA-56	16/10/99	<i>Melastoma malabathricum</i>	Degraded land, edge of road near the hill land with hill paddy and bamboo trees
	SA-57	16/10/99	<i>Lithocarpus elegans</i> (Blume) Hatus. Ex Soepadmo	Beside the road, 800m beside stream of Sg. Tikolod, Tikolod check point
	SA-68	19/10/99	<i>Plorarium</i> sp.	On the way to Ulu Kimanis, degraded forest
Hill Forest, Ulu Senagang, Crocker Range Park	SA-63	18/10/99	<i>Saraca</i> sp.	Across the Mensalog River towards the park, ulu Senagang

All the isolates were grouped into 3 colour groups (white series, grey series and brown series) based on the colour of aerial mycelium on oatmeal agar, after 14 days incubation at 28⁰C (Figure 4). Majority of the strains were of the grey series, followed by white series and brown the least

(Table 4). The grey series include pale grey, light grey, medium grey and dark grey; white colour group includes yellowish white, milky white and orange white while brown colour group includes greyish orange, brownish orange and greyish brown. As description of colour is quite subjective, a colour chart from Nippon 9000(1997) was used for standardization.

A few strains varied according to sites are as follow: L-28 exhibited red pigmentation all over the agar medium, while L-27 exhibited orange colour extracellular pigment. Both strains were isolated from soil samples obtained under *Theobroma cacao* and *Tectonia grandis* respectively (Table 5). In the MAP kinase screening, 20 extracts were screened but none was found to be inhibitory.

Table 4: Colour group pf isolated actinomycetes as determined in oatmeal medium

Colour of aerial mycelium			
Grey	White	Brown	Total
35	31	12	78

Table 5: Strains with unique sporulation and pigmentation

Strain	Uniqueness	Sampling site
L-46	Good sporulation on NA and OA medium. No pigmentation	<i>Rafflesia</i> forest, Crocker Range
L-28	Red pigmentation on OA medium	Cocoa plantation, Crocker Range
L-27 & L-31	Yellow pigmentation on OA medium	Secondary forest/cultivated area, Crocker Range
L-78	Greenish yellow pigmentation on OA	<i>Rafflesia pricei</i> , Mahua

OA-oatmeal agar; NA- nutrient agar

The search for novel metabolites especially from actinomycetes requires a large number of isolates (over thousands) in order to discover a novel compound of pharmaceutical interest. The search will be more promising if diverse actinomycetes are sampled and screened. For this reason, soils were specifically collected under identified trees. This is based on the hypothesis that actinomycetes diversity may be influenced by the diversity of plant species as these bacteria grow profusely in the humus and leaf litter layer. Furthermore, different plants produce different type of secondary metabolites and some of these chemical compounds are toxic to soil microorganisms including actinomycetes. However, adaptation has in turn lead the actinomycetes to produce their own secondary metabolites.

Although the collection sites have mainly been limited to fairly disturbed forests in the fringes of Crocker Range, yet they possess many actinomycetes in the leaf-litter humus layer. The conservation of this park will ensure the survival of these commercially important industrial microbes of biotechnological and pharmaceutical importance together with striking *Rafflesia*, orchids and *Rhododendron*.

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