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ACTINOBACTERIAL DIVERSITY DETERMINATION IN MALAYSIAN BRIS (BEACH RIDGES INTERSPERSED WITH SWALES) SOIL BY CULTURE-DEPENDENT APPROACH

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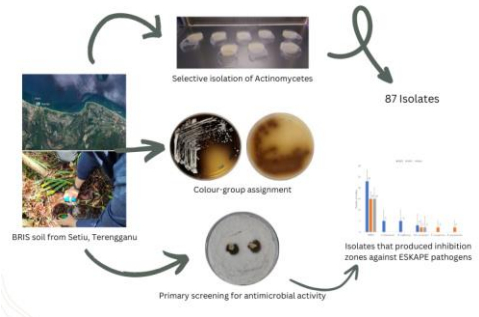
Graphical abstract



Abstract

Globally, the rapid emergence of antibiotic-resistant bacteria poses a major threat to public health, threatening the effectiveness of antibiotics. Addressing the crisis of antimicrobial resistance requires urgency in new drug discovery and the effort in finding novel compounds is dramatically evolving. However, one of the main obstacles is the rediscovery of known compounds from natural resources. To overcome this challenge, exploring underexploited location for new resource of actinomycetes, such as Beach Ridges Interspersed with Swales (BRIS) soil in Malaysia, was selected. Soil samples from Jambu Series and Rudua Series in Setiu, Terengganu were collected and analysed. A total of 87 actinomycetes strains were isolated on yeast malt agar (ISP2), Actinomyces agar and Zobell marine agar, with the highest number from the Jambu series using serial dilution and spread plate method. Antimicrobial screening revealed that 31 isolates exhibited activity against ESKAPE pathogens, with 28 and 12 isolates showing activity against Gram-positive and Gram-negative bacteria, respectively. These isolates showed strong inhibiting response towards one or more ESKAPE pathogens. This study highlights the potential of BRIS soil as an underexploited habitat for discovering actinobacterial communities which could lead to the discovery of a new potential antimicrobial producer to serve as novel drug leads.

Keywords: BRIS soil, actinomycetes, actinobacteria, microbial diversity, culture-dependent

Abstrak

Kemunculan pesat bakteria rintang antibiotik merupakan ancaman besar kepada sektor kesihatan di Malaysia. Pelbagai usaha telah dijalankan dan masih berkembang dengan pesat tetapi penemuan semula kompaun menjadi salah satu rintangan utama dalam menangani krisis ini. Bagi mengatasi cabaran ini, penerokaan lokasi yang kurang dieksploitasi sebagai sumber baru actinomycetes seperti tanah BRIS di Malaysia dipilih untuk projek ini. Sampel tanah dari Siri Jambu dan Siri Rudua di Setiu, Terengganu telah diambil dan dianalisis. Sebanyak 87 actinomycetes telah dipencilkan pada agar malt yis (ISP2), agar Actinomyces dan agar marin Zobell, dengan bilangan tertinggi dari Siri Jambu menggunakan kaedah *serial dilution* dan calitan plat. Melalui saringan antimikrob, 31 pencilan menunjukkan aktiviti terhadap patogen ESKAPE dimana 28 pencilan menunjukkan aktivtiti terhadap bakteria Gram-positif dan 12 pencilan menunjukkan aktiviti terhadap bakteria Gram-negatif. Melalui dapatan ini, pencilan ini menunjukkan tindak balas penghambatan yang kuat terhadap satu atau lebih patogen ESKAPE. Oleh itu, kajian ini menekankan potensi tanah BRIS sebagai habitat yang kurang diterokai untuk menemui komuniti Actinobacteria yang berpotensi untuk menjadi sumber kepada penemuan penghasil antimikrob baru.

Kata kunci: Tanah BRIS, actinomycetes, actinobacteria, kepelbagaian mikrob

1.0 INTRODUCTION

As a result of the rapid emergence of drug resistance in the majority of pathogenic microorganisms, the search for bioactive compounds has grown even more essential. These bioactive compounds are being sought for their potential applications in agriculture, medicine, and various industrial sectors, despite the numerous antibiotics that have been discovered over many decades [1, 2]. Out of 23,000 documented bioactive secondary metabolites originating from microorganisms, more than 10,000 are attributed to actinomycetes. Actinomycetes are Gram-positive bacteria with high guanine-cytosine (G+C) content which belong to the phylum Actinobacteria. Actinomycetes are ubiquitously present in various natural habitats, including aquatic and terrestrial environments. They play a fundamental role as integral constituents of the native soil microflora, forming a substantial component of the soil microbial community [3, 4, 5]. Previous studies have reported that Actinomycetes are primarily found in soil constituting more than 30% of the whole microbial community and through metagenomic analyses performed by Idris *et al.* (2017), 34% increase in families of actinobacterial was detected against data that are validly published [6]. The diversity of actinomycetes has been extensively determined by using culture-dependent approach which has led to the discovery of many novel strains [7].

Actinomycetes produce secondary metabolites that contribute to the production of antibiotics. About 80% of antibacterial products are derived from the genus *Streptomyces* and rare actinomycetes are reliable source of novel antibiotics [8, 9, 10, 11]. Several studies have revealed that the problem faced in the rediscovery of known compounds can be overcome by performing extensive research focusing on the rare actinomycetes in underexploited and extreme habitats that carry the potential to harbour unique actinobacterial strains with the potential of producing novel compounds [8, 12]. This is because the diversity and activity of the microbial community are heavily influenced by the specific abiotic conditions such as pH and soil moisture, prevailing in these habitats [13].

Peninsular Malaysia is known for its remarkable biodiversity and has approximately 500 unique soil series variations which are diversified into five major soil groups; Sedentary Soils, Reworked Soils, Riverine Alluvial Soils, Marine Alluvial Soils and Organic Soils. Among these, Beach Ridges Interspersed with Swales (BRIS) soils are part of the Marine Alluvial Soils [14]. BRIS soils are predominantly developed in the East Coast Peninsular Malaysia across the coastal area of Pahang – Terengganu - Kelantan. BRIS soils are known as problematic lowlands for farming or planting edible crops due to their sandy texture, resulting in limited fertility, very low water holding and nutrient capacity, very dry in drought season and prone to flood during monsoon season. They remain underexploited due to their lack of potential in supporting agricultural activities [15, 16, 17]. The first discussion and analysis conducted to isolate and characterise actinomycetes with antimicrobial activities from BRIS soil was performed by Majhool (2020) where 65% out of 155 actinomycetes isolates showed antimicrobial activity against one or more of the test organisms [18]. In this research, soil samples from two BRIS soil series were examined to identify actinomycetes with antimicrobial properties against ESKAPE pathogens.

2.0 METHODOLOGY

2.1 Collection of BRIS Soil Samples

Two BRIS soil series namely Jambu and Rudua (Figure 1) that are classified in USDA Soil Taxonomy as Spodosols (Jambu series) and Entisol (Rudua series) were selected for this study. In collaboration with DOA Terengganu, sampling sites were determined based on the soil series, location and coordinates (Table 1). At each sampling point, a spiral auger was used to dig the hole in the sampling area to reach down to 100 cm depth and samples were taken from four different depth ranges (0-20 cm, 20-50 cm, 50-70 cm, and 70-100 cm) in which soil samples from each depth ranges are labelled as in Table 2. The soil samples were placed into a sterile Falcon tube and an additional 1 kg of soil samples from each depth were transferred into a sterile plastic bag. Following transport to the laboratory at Universiti Malaysia Terengganu (UMT), the soil samples in Falcon tubes were stored in ice. Then, the soil samples were stored at 4°C at Universiti Pendidikan Sultan Idris (UPSI) for further analysis. The BRIS soil samples (1 kg) were sent to DOA for soil physicochemical analysis.

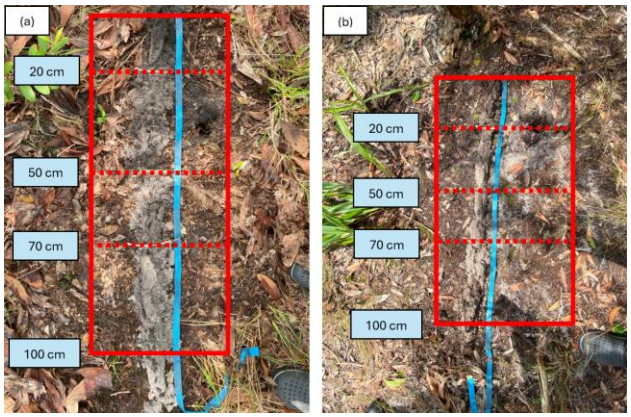


Figure 1 (a) Jambu and (b) Rudua BRIS soil series collected using a spiral auger at the sampling locations

Table 1 The coordinates of the chosen sampling locations based on BRIS soil series

Series	Point	Coordinate
Jambu	A	5°32'32.6358" 102°53'12.3072"
	B	5°32'32.0604" 102°53'12.4722"
Rudua	A	5°29'25.7238" 102°59'19.9572"
	B	5°29'24.5472" 102°59'19.9572"

Table 2 The in-situ soil parameters for each sampling location represent Jambu Series and Rudua Series

Series	Sample	Depth range (cm)
Jambu		
	jambu-a	0-20
	jambu-b	20-50
	jambu-c	50-70
	jambu-d	70-100
Rudua		
	rudua-a	0-20
	rudua-b	20-50
	rudua-c	50-70
	rudua-d	70-100

2.2 Selective Isolation of Actinomycetes from BRIS Soil Samples

Standard serial dilution and spread plate techniques were used to isolate actinomycetes from the BRIS soil samples. One gram of each soil sample was mixed with 9 mL of 25% strength sterile Ringer's solution and vortexed for homogenization followed by 1 mL from the stock solution was aliquoted into another sterile universal bottle filled with sterile Ringer's solution. The steps were repeated for the 10^{-2}, 10^{-3}, and 10^{-4} dilution factors. Afterward, 0.1 mL of 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated on selective isolation plates; yeast malt agar ISP2 [19], Actinomyces agar [20] and Zobell marine agar [21] supplemented with cycloheximide and nalidixic acid using sterile spreader in duplicate prior to incubation at 28°C for 14 days. Colony-forming units (CFU) were determined after the incubation period and representative Actinomycetes colonies were subcultured onto ISP2 medium and incubated at 28°C for 14 days. The glycerol stocks for each isolate were prepared in duplicate. The working culture was placed at -20°C and the other one was kept at -80°C for long-term preservation.

2.3 Colour-group Assignment

Eighty-seven actinomycetes isolates were chosen from the pure culture to be inoculated on ISP3 and ISP6 before incubation at 28°C for 21 days and 4 days, respectively [19]. They are chosen based on their colony morphology where actinomycetes produced leathery colonies, powdery surfaces with curved, concave, or smooth elevation and colour ranges between greenish white or grey while some also had a hard texture and hard to scrape off the plates [18]. The morphology observation and colour-group assignment were done according to The National Bureau of Standards (NBS) Colour Name Chart [22] based on its aerial spore mass, substrate mycelial, diffusible pigment, and melanin production.

2.4 Antimicrobial Activity of Actinomycetes Isolates

Standard agar plug assay was used to screen the actinomycetes isolates against six ESKAPE pathogens; Methicillin-resistance *Staphylococcus aureus* (MRSA) (ATCC43300), *Enterococcus raffinosus* (ATCC49464), *Klebsiella pneumoniae* (ATCC13883), *Enterobacter aerogenes* (ATCC51697), *Pseudomonas aeruginosa* (ATCC27853) and *Acinetobacter baumnanii* retrieved from Fakulti Sains Kesihatan, Universiti Kebangsaan Malaysia (UKM). The ESKAPE pathogens were grown in Mueller Hinton broth at 37°C overnight (concentration of 0.5 NTU – McFarland scale). Agar core (5 mm) was made with a sterile cork borer from the actinomycetes isolates plates and placed on Mueller Hinton agar with tested organisms before incubation at 37°C overnight. The inhibition of bacteria was measured by a clear zone that indicates the confluent growth of the test organisms after 24 hours of incubation [23].

3.0 RESULTS AND DISCUSSION

3.1 Selective Isolation of Actinomycetes

A total of eight soil samples from both locations with depths ranging from 0-100 cm were studied and evaluated based on their physico-chemical properties. Eighty-seven actinomycetes strains were successfully isolated, of which the total number of isolates in the Jambu Series and Rudua series were 55 and 32 isolates, respectively as shown in Table 3. The highest number of actinomycetes were isolated from the Jambu series which comprised about 63% of the total actinomycetes isolates. The temperature in all soil samples ranged from 29.5℃ to 32.5℃, which fits into the category of the optimal growing conditions for most actinomycetes, which is between 20℃ and 40℃ while neutral pH (6 to 8) is the best condition for actinomycetes growth [24, 25]. This contradicts with the result obtained where the soil pH ranges between 4.3 to 4.6 making the actinomycetes isolated from the BRIS soil samples acid-tolerant [26].

Table 3 The physicochemical properties and number of isolated Actinomycetes colonies from Jambu Series and Rudua Series

Sample	pH	Temp (℃)	Organic Carbon (%)	Organic Matter (%)	Number of Isolates
jambu-a	4.3	32.5	6.04	13.84	44
jambu-b	4.4	30.5	2.55	5.85	10
jambu-c	4.5	29.5	1.57	3.59	0
jambu-d	4.6	29.5	0.77	1.77	1
					55
rudua-a	4.3	30.5	26.49	60.75	23
rudua-b	4.4	29.5	7.81	17.9	5
rudua-c	4.5	29.5	6.21	14.24	3
rudua-d	4.4	29.5	9.07	20.79	1
					32

It is also found that the number of actinomycetes isolates declines with the increase in depth for both BRIS soil series samples. Based on previous studies, actinomycetes populations are inversely proportional to the depth of the soil which may be due to a strong physicochemical gradient which leads to reduced carbon availability and soil nutrients [27, 28]. This can be proven by the decrease in organic carbon (OC) and organic matter (OM) of BRIS soil samples with depth since, actinomycetes has a massive influence towards soil nutrients availability and organic matter content that improves soil microbial community [29, 25]. The ability of microorganisms to persist in problematic and harsh environments can often be attributed to their environmental adaptation and their capability to form resilient structures like spores [4, 30].

The highest number of actinobacterial colonies (CFUs) count was recorded on Actinomyces agar followed by ISP2 and Zobell marine agar (Table 4). However, a study by Majhool (2020) showed that the highest number of colonies were detected in ISP2 and the lowest in Actinomyces agar [18]. This contradictory result may suggest that rare actinomycetes could be cultivated in this study since Actinomyces agar targets the cultivation of rare or uncommon actinomycetes [20].

Table 4 Number of bacterial colonies (CFUs per gram dry weight soil) growing on selective isolation media after incubation at 28℃ for 14 days

Sample	ISP2	Zobell marine agar	Actinomyces agar
	Number (CFU/g)	Number (CFU/g)	Number (CFU/g)
jambu-a	2.9×10^4	2.1×10^4	3.1×10^4
jambu-b	2.1×10^4	5.0×10^3	2.7×10^4
jambu-c	3.0×10^4	1.0×10^4	1.6×10^4
jambu-d	2.0×10^4	5.0×10^3	2.5×10^4
rudua-a	4.1×10^4	4.0×10^3	6.4×10^4
rudua-b	4.0×10^3	2.0×10^3	9.0×10^3
rudua-c	2.2×10^4	3.4×10^4	3.0×10^4
rudua-d	2.5×10^4	3.0×10^4	5.0×10^4

3.2 Colour-group Assignment

Determination of actinobacterial diversity based on culture morphology revealed that the 87 actinomycetes isolates were assigned into 40 groups according to their aerial spore mass, substrate mycelial, and diffusible pigment on ISP3 and the production of melanin pigment on ISP6. Fifty-five isolates from Jambu soil samples were grouped into 14 multi-membered and 4 single-membered groups while 32 isolates obtained from Rudua soil samples were grouped into 7 multi-membered and 4 single-membered groups. Result showed that 12 colour-groups were common between Jambu and Rudua

soil samples with morphological characteristics as listed in Table 5.

Table 5 Multi-membered colour groups consist of actinomycetes isolates obtained from Jambu and Rudua soil samples *(ASM – aerial spore mass colour, SM – substrate mycelial colour, DP – colour of diffusible pigment, (xx) – designated number code for each colour)*

Colour group	Cultural characteristics			Number of Isolates	
	ASM	SM	DP	Jambu	Rudua
19	Light yellow (86)	Strong yellow (84)	-	2	1
20	Deep yellow (85)	Vivid yellow (82)	-	1	3
21	Deep yellow (85)	Strong yellow (84)	-	1	3
22	Deep orange yellow (69)	Vivid yellow (82)	-	1	1
24	Yellowish white (92)	Strong yellowish brown (74)	-	2	2
26	Yellowish gray (93)	Deep orange yellow (69)	-	1	1
27	Moderate orange yellow (71)	Brilliant orange yellow (67)	-	1	2
29	Brilliant yellow (83)	Vivid yellow (82)	-	1	1
32	Yellowish white (92)	Strong yellow (84)	-	3	1
33	Strong yellow (84)	Brilliant yellow (83)	-	2	1
34	Yellowish white (92)	Deep yellowish brown (75)	-	2	1
39	Yellowish white (92)	Deep orange (51)	-	1	1

Colour-grouping assignment of these isolates retrieved from BRIS soil samples indicates a high degree of actinobacterial diversity [31] since, different morphological characteristics represent different taxonomic group [32] that are present in the BRIS soil samples which is in line with previous studies conducted by [6, 18].

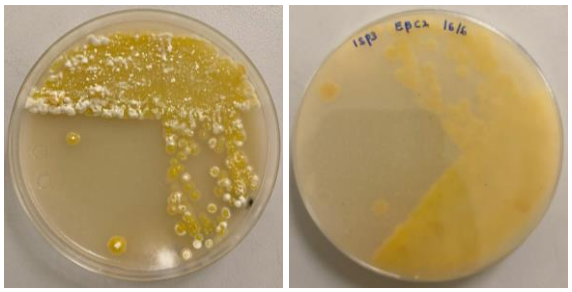


Figure 2 Appearance of actinomycetes colonies from the BRIS soil samples on ISP3 after incubation at 28℃ for 21 days

The largest multi-membered colour-group is dominated by isolates that showed morphological characteristics consisting of aerial spore mass colour of deep yellow (85) and substrate mycelial colour of vivid yellow (82) and strong yellow (84) (Figure 2). However, further analysis is needed using molecular approach to determine the genus of each member in this group.

3.3 Primary Screening of Actinomycetes Isolates

Thirty-one out of eighty-seven isolated actinomycetes (35%) showed inhibition activity against at least one or more of the tested ESKAPE pathogens with the inhibition zone ranging from 10 mm to 21 mm on both ISP2 and ISP3 media. Among these 31 isolates, 24 isolates (7%) and 7 isolates (23%) are from Rudua series and Jambu series, respectively. The result also revealed that 28 isolates and 12 isolates showed antimicrobial activity against Gram-positive and Gram-negative bacteria, respectively. This pattern highlights the varying sensitivity of Gram-positive and Gram-negative bacteria to the metabolites produced by these isolates. This is also because Gram-negative bacteria have a thin peptidoglycan cell wall and surrounded by an outer membrane containing lipopolysaccharide that serves as better protection against antibiotics compared to Gram-positive bacteria [33, 34].

ISP2 and ISP3 media showed significant differences in the number of isolates that can produce inhibition zones (Figure 3). Twenty-three isolates from ISP2 medium showed activity against Methicillin-resistance *Staphylococcus aureus* (MRSA) (ATCC43300), five isolates against *Acinetobacter baumnanii*, five isolates against *Enterococcus raffinosus* (ATCC49464) and three isolates showed activity against *Enterobacter aerogenes* (ATCC51697) while on ISP3, fifteen isolates showed activity against MRSA (ATCC43300), two isolates showed activity against *Enterobacter aerogenes* (ATCC51697), two isolates showed activity against *Pseudomonas aeruginosa* (ATCC27853) and two isolates against *Klebsiella pneumoniae* (ATCC13883). The results revealed that the effectiveness of the isolates against ESKAPE pathogens was influenced by the type of growth media used.

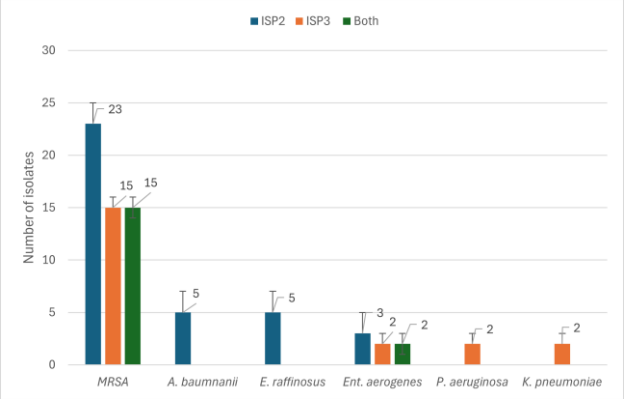


Figure 3 Number of isolates that produced inhibition zones against tested ESKAPE pathogens grown on ISP2 and ISP3 media following incubation at 37°C overnight

For instance, twenty-two isolates exhibited inhibitory effects when cultivated on ISP3 but did not show the same effect when cultured on ISP2. This discrepancy can be attributed to the distinct nutrient compositions of these media, which affected the inhibitory capabilities of the actinomycetes isolates [35, 36]. Isolate BH111 only demonstrated inhibition against one or more ESKAPE pathogens when cultured in ISP2, indicating that this medium provided the necessary nutrients for its inhibitory activity. In contrast, BH117 displayed inhibitory potential when grown in both ISP2 and ISP3. Prior research had indicated that among nineteen isolates, thirteen of them were effective against *Staphylococcus aureus,* five isolates against *Klebsiella pneumoniae* and none showed inhibitory activity against *Pseudomonas aeruginosa* [34]. In another study, it was suggested that actinomycetes isolates had a pronounced inhibitory effect against MRSA [37]. This pattern highlights the varying sensitivity of Gram-positive and Gram-negative bacteria to the metabolites produced by these isolates.

According to David and Stout (1971), the zone of inhibition can be classified into four intensities based on its diameter; >20 mm (very strong), 10-20 mm (strong), 5-10 mm (medium) and <5 mm (no response) [38]. Notable inhibitory results were observed for specific isolates against pathogens: BH33 exhibited the highest activity against *Enterobacter aerogenes* (21 mm), BH127 against *Pseudomonas aeruginosa* (13 mm), BH134 and BH111 against *Enterococcus raffinosus* (18 mm), BH140 against *Klebsiella pneumoniae* (11 mm), BH88 against Methicillin-resistance *Staphylococcus aureus* (21 mm), and BH110 showed the highest activity against *Acetinobacter baumnanii* (18 mm). These isolates showed inhibition zone ranges of 11-21 mm which indicates that these isolates have a strong inhibiting response [39] towards one or more ESKAPE pathogens.

4.0 CONCLUSION

Our findings show that, Malaysian Peninsular BRIS soil exhibits potential for harbouring diversity of actinobacterial community due to its various morphological characteristics which represent variety of taxonomic group. It also provides habitat for rare actinomycetes due to its ability to cultivate high CFUs on Actinomyces agar. These isolates also produce antimicrobial activity. The actinomycetes isolates that showed significant antimicrobial activity against ESKAPE pathogens could undergo further analysis and be considered as potential candidates for gaining deeper insights into discovering novel antimicrobial producers that may serve as new drug leads. It is advisable to consider the physicochemical characteristics of the BRIS soil in subsequent studies to ensure an accurate understanding of the actinobacterial community in Malaysian Peninsular BRIS soil.

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Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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