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Characterization and antimicrobial activities of two *Streptomyces* isolates from soil in the periphery of Universiti Putra Malaysia

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Abstract. This study was to assess the identification and antimicrobial activities of two actinomycete isolates. The two isolates designated as B8 and C2, were isolated from a patch of soil in the peripheral area of Universiti Putra Malaysia by streaking on starch casein agar after standard serial dilution procedures. Their antimicrobial activities were first evaluated against eight clinical laboratory strains namely *Bacillus* sp., *Enterococcus* sp., *Escherichia coli*, *Klebsiella* sp., *Pseudomonas* sp., *Salmonella* sp., *Staphylococcus aureus*, and *Staphylococcus epidermidis* by perpendicular streak method on Mueller Hinton and Tryptic Soy agar. In both media, a broad-spectrum antibacterial activity was observed for both isolates, with B8 against all the test bacteria and C2 against five of them (*Bacillus* sp., *E. coli*, *Pseudomonas* sp., *S. aureus* and *S. epidermidis*). Re-assessment against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 strains by similar method showed antibacterial activities by isolate B8 against both ATTC strains while C2 only against *S. aureus* ATCC 25923. *Streptomyces griseus* ATCC 10137 was included in the later experiment and showed antibacterial activity against both ATCC strains. Subsequently, the two isolates were identified by PCR/sequencing techniques and phylogenetic analysis to be *Streptomyces* species (>93% homology based on *16S rRNA* and *rpoB* genes). Characterization on cultural characteristic and viable count at different temperatures (37ºC and 28ºC), on different microbiological media (AIA, ISP-2, MHA, NA, PDA and TSA), were performed. More morphological features were observed on ISP-2 for both isolates. A higher growth yield was also observed at 28ºC in all media but in comparing that between the two isolates, isolate B8 outnumbered C2 at all experimental conditions. The observed variation in cultural traits and growth yield indicate unique properties between the two antibiotic-producing isolates.

INTRODUCTION

Infectious diseases account for 13.3 billion deaths worldwide, which constitute for about 25% of all deaths (Cassell & Mekalanos, 2001). The chemotherapeutic treatments of infectious diseases are also getting limited due to the emergence of antibiotic resistant pathogens, which have been widely and continuously reported. For example, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), extended spectrum β-lactamases (ESBL)-producing *Escherichia coli*, and multi-drug resistant *Mycobacterium tuberculosis* (MDR-MTB) are of a major concern nowadays (Selvameenal *et al.*, 2009). In consequence, search for new antibiotics has been a major attempt throughout the world. Environmental

microorganisms have been the most feasible source for new antimicrobial compounds due to their high abundance and remarkable diversity in nature. Numerous antimicrobial agents of these kinds have been isolated such as streptomycin, aminoglycoside, anthracyclines, chloramphenicol, β-lactams, macrolides and tetracyclines by the members of actinomycetes, in particular the *Streptomyces* species (Goodfellow *et al*., 1988). *Streptomycetes* are fungus-like filamentous gram-positive bacteria which are responsible for the musty odor of soil. They have been the major source of such antimicrobial compounds and more than 70% of all known antibiotics in the market (Ozgur *et al*., 2008). Due to the biodiversity of *Streptomycetes* in both aquatic and terrestrial locales, the variability in antibacterial compounds is also expected. Thus searching for new territory that has yet to be explored for new *Streptomycetes* may lead to the discovery of new bioactive compounds with antibacterial properties (Mincer *et al*., 2002).

Meanwhile, the cultivation methods that can be considered as a standard for cultivating *Streptomyces* species are still elusive. This is due to the complexity of the members of the actinomycete group that different isolates from diverse habitats and locations may require different nutritional needs and growth conditions (Rho & Lee, 1994; Jensen *et al*., 2005). Thus, the selection of media and incubation temperatures in a study may not be able to accommodate growth of the different isolates in other studies. In view of the important roles of *Streptomycetes* as the antibiotic producing organisms, we have been isolating and characterizing the isolates from soil samples at the Universiti Putra Malaysia and nearby areas (Zin *et al*., 2010). In this present study, basic conventional and molecular approaches for the identification of two actinomycete-resembling isolates with a broad spectrum-activity against various bacterial pathogens were conducted. In addition to morphological and antibacterial characteristics, the growth yield on several common laboratory media as well as those commercially formulated for actinomycetes at different incubation temperatures were quantitatively evaluated for a definite understanding on the growth pattern and preferences of these isolates.

MATERIALS AND METHODS

Isolation and phenotypic characterization

The two actinomycete isolates were from a patch of soil taken at a small forested area at the periphery of Universiti Putra Malaysia. Isolation was done by standard serial dilution and spread plate methods using Starch Casein Agar (SCA) as the primary isolation agar with incubation temperature at 28ºC for 7 to 14 days (Zin *et al*., 2010). Actinomycete colonies on the plates were tentatively identified as colored, dried, and rough with irregular or regular margin (Williams & Cross, 1971). Based on the morphological characteristics and musty odor, cultures were selected and further purified by several subculturing on ISP-2 (International *Streptomyces* Project) agar medium (Difco, USA). Pure isolates were studied for their basic colony appearance, Gram-stained, and tested for catalase and oxidase activities (Wu, 1995). Further morphological characterizations on aerial mass, substrate mycelium, pigment and spore production of matured cultures on Actinomycete Isolation Agar (AIA) (Difco), ISP-2, Mueller Hinton Agar (MHA) (Merck, Germany), Nutrient Agar (NA) (Merck), Potato Dextrose Agar (PDA) (Merck) and Tyrptic Soy Agar (TSA) (Merck) were done as described by Shirling & Gottlieb (1966). Stock cultures were preserved in Actinomyces broth (BBL, USA) with 15% glycerol (v/v) at -20ºC.

Antimicrobial screening

The antimicrobial activity of the two isolates was first evaluated against eight clinical laboratory strains; *Bacillus* sp., *Enterococcus* sp., *E. coli*, *Klebsiella* sp., *Pseudomonas* sp., *Salmonella* sp., *S. aureus* and *Staphylococcus epidermidis* by perpendicular streak method on MHA and TSA at 30ºC (Duraipandiyan *et al*., 2010). Re-assessment of the antibacterial activity of the two isolates was done by similar method against pathogenic *E. coli* ATCC 25922 and *S. aureus*

ATCC 25923 strains. *Streptomyces griseus* ATCC 10137 was also included in the later experiment as a positive control.

Molecular identification by *16S rRNA* and *rpoB* gene sequence homology

The two isolates were subjected to molecular identification by polymerase chain reaction (PCR)/sequencing technique targeting *16S rRNA* and *rpoB* genes. For each isolate, a loopful of culture containing mycelium and spores was scraped from colonies on ISP-2 agar medium and suspended in Tris-EDTA (TE) buffer. Genomic DNA of the isolates was extracted using the GF-1 bacterial DNA extraction kit (Vivantis Tech., Malaysia). The DNA was semi-quantified on a 0.8% agarose gel in 1XTris-borate-EDTA (TBE) and visualized under ultraviolet (UV). Concentration and quality of DNA extract was checked by using the biophotometer (Eppendorf, Germany). The *16S rRNA* gene was detected and amplified using a set of published primers targeting almost the full length of the gene: 27F (5'-AGT TTG ATC CTG GCT CAG-3') and 1492R (5'-ACG GCT ACC TTG TTA CGA CTT-3') (Duraipandiyan *et al*., 2010). The reaction was carried out in a 50 µl reaction volume consisting the following reagents: 10 µl of 5Xi-PCR RED Master Mix (i-DNA Biotech., Singapore), 0.2 µl of forward primer, 0.2 µl of reverse primer, 38.6 µl of water and 1 µl of template DNA, with thermal cycling conditions as follows: 94ºC, 52ºC and 72ºC for 1 min each for 30 cycles followed by another incubation at 72ºC for 10 min (Duraipandiyan *et al*., 2010). For *rpoB* gene, the primers were SRPOF1 (5'-TCG ACC ACT TCG GCA ACC GC-3') and SRPOR1 (5'-TCG ATC GGG CAC ATG CGG CC-3') (Kim *et al*., 2004). The reaction was carried out similarly as described earlier with thermal cycling conditions as follows: 30 cycles at 95ºC for 30 s, 60ºC for 30 s and 72ºC for 45 s. The reaction was then held at 72ºC for 5 min (Mun *et al*., 2007). PCR amplification was detected by agarose gel electrophoresis and UV visualization. PCR product was purified using the GF-1 PCR clean-up kit (Vivantis Tech.), checked for purity, and sequenced using the same primers for PCR (First Base Lab., Malaysia). The generated sequences were searched for sequence homology, using the BLAST software available at the National Center of Biotechnology Information (http://www.ncbi-nlm-nih.gov). The determined 16S rDNA nucleotide sequence of the two isolates were subjected to phylogenetic analysis in comparison to those of other related species as shown by the BLAST searches. The later sequences, including some of the *Micromonospora* species to serve as the out-group, were retrieved from the GenBank sequence databases. A distance matrix tree was then built by UPGMA clustering method with Needle-Wunsch algorithm and 100X bootstrap analysis, using the Bionumerics software version 6.1 (Applied Maths, Belgium).

Viable cell count

The two isolates were grown on ISP-2 agar medium at 28ºC. The spores produced were scraped and transferred into normal saline in sterile test tubes. The density of the spore suspension was checked at OD_{600} of 0.10 – 0.15 (~0.5 McFarland standard). A 10-fold serial dilution of the spore suspension was carried out and 0.1 ml of each dilution was spread onto different media; AIA, ISP-2, MHA, NA, PDA and TSA. Plating was done in two sets for 7 days-aerobic incubation at 37ºC and 28ºC respectively. All experiments were done in triplicate and a mean value was obtained. Kruskal Wallis and Mann Whitey tests were used for statistical analysis with $P \leq 0.05$ as the significant level.

RESULTS

The two isolates, labeled as B8 and C2 were colored, dried, rough, and irregular in margin with a strong musty smell on the agar plate. Basic microbiological description on the colony appearance showed generally raised and opaque colonies, with filamentous form and rugose surface. Microscopic observation showed rod shaped-branching filaments with a gram positive reaction to further indicate the characteristics of actinomycetes. In the biochemical characterizations, catalase and oxidase tests showed that the two isolates were positive for the enzymes. The summary

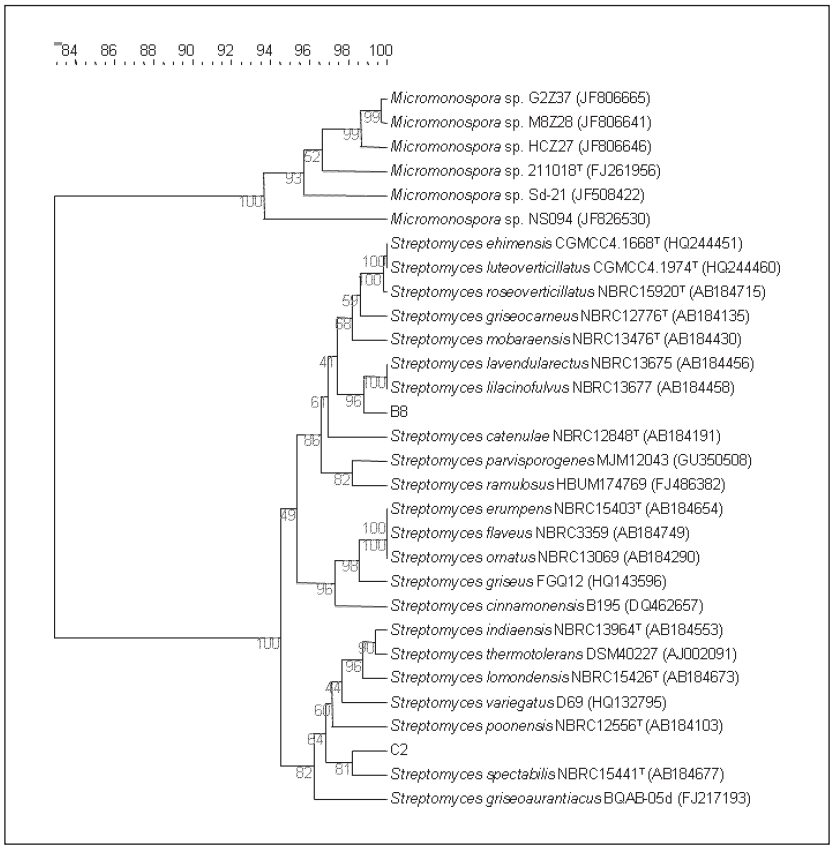

Figure 1. Phylogenetic dendogram of 16S rDNA distance matrix-based analysis showing the position of isolates B8 and C2 and their phylogenetic neighbors (GenBank accession numbers in parentheses). The above scale indicates the pairwise similarity scores (%) calculated from a total of 30 aligned sequences comprising *Micromonospora* species. The later serve as the out-group to root the tree. Numbers at the branch nodes indicate the bootstrap values (%)

of the studied phenotypic properties of the isolates was listed in Table 1.

Further morphological characterizations of the isolates on the different media were summarized in Table 2. Overall, a color homogeneity (pale yellow) was observed for both aerial mass and substrate mycelium of isolate B8 on all media except ISP-2 that showed a different color (dark brown) for substrate mycelium. On the other hand, a variety of color was observed for isolate C2 on the different media with a color contrast between aerial mass and substrate mycelium (orangish and dark brown) also on ISP-2. Soluble pigment was observed for both isolates on ISP-2, MHA and TSA whereas that on PDA was observed only for isolate C2. While the presence of spore mass was obviously visible only on ISP-2 for both isolates, and each was of different color.

In the antimicrobial screening by perpendicular streak method (Table 1), isolate B8 showed an inhibition zone against the entire test bacteria while isolate C2 against five of them (*Bacillus* sp., *E. coli*, *Pseudomonas* sp., *S. aureus* and *S. epidermidis*) on both MHA and TSA. Re-assessment against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 strains also showed a similar result on MHA and TSA whereby isolate B8 had antibacterial effect against both ATCC strains while isolate C2 only against *S. aureus* ATCC 25923. *S. griseus* ATCC 10137 demonstrated inhibitory effect against both ATCC strains on MHA and TSA.

Table 1. Phenotypic properties of isolates B8 and C2

Test	Result	
	B8	C2
Colony appearance		
Optical character	Opaque	Opaque
Elevation	Raised	Raised
Form	Filamentous	Filamentous
Surface	Rugose	Rugose
Cell characteristic		
Gram's reaction	+	+
Shape	Filament	Filament
Biochemical test		
Catalase	+	+
Oxidase	+	+
Antibacterial activity		
Bacillus sp.	+	+
Enterococcus sp.	+	–
E. coli	+	+
Klebsiella sp.	+	–
Pseudomonas sp.	+	+
Salmonella sp.	+	–
S. aureus	+	+
S. epidermidis	+	+
E. coli ATCC 25922	+	–
S. aureus ATCC 25923	+	+

'+' = positive reaction, '–' = negative reaction

Table 2. Cultural characteristics of isolates B8 and C2 on different media

Medium	Aerial mass		Substrate mycelium		Soluble Pigment		Spore	
	B8	C2	B8	C2	B8	C2	B8	C2
AIA	Pale yellow	Pale yellow	Pale yellow	Pale yellow	–	–	–	–
ISP-2	Pale yellow	Orangish brown	Dark brown	Dark brown	+	+	+ (White)	+ (Orange)
MHA	Pale yellow	Pale yellow	Pale yellow	Pale yellow	+	+	–	–
NA	Pale yellow	Pale yellow	Pale yellow	Pale yellow	–	–	–	–
PDA	Pale yellow	Pinkish orange	Pale yellow	Pinkish orange	–	+	–	–
TSA	Pale yellow	White	Pale yellow	White	+	+	–	–

'+' = present, '–' = not present

The presumptively identified actinomycete-isolates B8 and C2 were successfully identified as *Streptomyces* species (>93%) following sequence homology search of the generated *16S rRNA* and *rpoB* gene sequences in the GenBank. Based on 16S rDNA nucleotide sequences, BLAST results showed isolate B8 to have the highest similarity (99%) to *Streptomyces lavendularectus* NBRC 13675 (AB184456) and *Streptomyces lilacinofulvus* NBRC 13677 (AB184458), while isolate C2 has the highest similarity (99%) to *Streptomyces spectabilis* NBRC 15441^T (AB184677). The phylogenetic tree further shows the two isolates to be in a very close contiguity with their respective highest matches of *Streptomyces* species as in the BLAST analysis, with each in a distinct evolutionary cluster. For the out-group, all *Micromonospora* species were collectively separated from the *Streptomyces* clades as expected. The 16S rDNA nucleotide sequences of isolates B8 and C2 including those for *rpoB* gene have been submitted into the GenBank database with accession no. JN116247, JN116248, JN116249 and JN116250 respectively.

Viable count on the different media showed that growth of isolate B8 was about 10-fold higher than that of isolate C2 (10^6 vs. 10^5cfu/ml) at all experimental conditions. Nevertheless, for both isolates, the growth yield was higher at 28°C of incubation temperature than at 37°C (Tables 3 and 4). In comparing the growth yield on various media for the respective isolates, viable count differed significantly at both temperatures. For isolate B8, the order of media that generated from low to high growth yield was as follows: PDA<NA<TSA<MHA<AIA<ISP-2 at 37°C while that at 28°C was NA<TSA<MHA<AIA<ISP-2<PDA (Table 3). Based on the order of growth yield, a consistent pattern of media preference was observed (underlined) at both temperatures except for PDA that harbored the highest growth at 28°C but turned out to be the lowest at 37°C. A different media preference was nevertheless observed for isolate C2 as follows: at 37°C: PDA<MHA<ISP-2<NA<TSA<AIA, and at 28°C: PDA<MHA<AIA<NA<TSA<ISP-2 (Table 4). Still, media preference of isolate C2 at the two temperatures was mostly consistent (underlined) except for AIA and ISP-2 whereby the later was preferred at 28°C but not at 37°C, whereas AIA was preferred at 37°C but not at 28°C. As a whole, ISP-2 was frequently the best media to harbour higher growth except for isolate C2 at 37°C. PDA was the lowest to harbour growth except at 28°C for isolate B8, while the effects of other media were variable.

Table 3. Viable cell count (cfu/ml) of isolate B8 on various microbiological media at different temperatures

Temperature/ Medium	37°C (mean ± standard deviation)	28°C (mean ± standard deviation)	P-value[1*]
AIA	$2.47 \pm 0.035 \times 10^6$	$4.77 \pm 0.058 \times 10^6$	0.046
ISP-2	$2.57 \pm 0.036 \times 10^6$	$5.13 \pm 0.058 \times 10^6$	0.046
MHA	$2.04 \pm 0.021 \times 10^6$	$4.53 \pm 0.252 \times 10^6$	0.050
NA	$1.78 \pm 0.042 \times 10^6$	$3.50 \pm 0.265 \times 10^6$	0.050
PDA	$1.20 \pm 0.105 \times 10^6$	$6.17 \pm 0.569 \times 10^6$	0.050
TSA	$1.88 \pm 0.067 \times 10^6$	$4.27 \pm 0.155 \times 10^6$	0.046
P-value[2*]	**0.005**	**0.007**	

[1]Comparison of growth at different temperatures on respective media
[2]Comparison of growth on different media at respective temperatures
[*]Significant values in bold

Table 4. Viable cell count (cfu/ml) of isolate C2 on various microbiological media at different temperatures

Temperature/ Medium	37°C (mean ± standard deviation)	28°C (mean ± standard deviation)	P-value[1*]
AIA	$6.70 \pm 0.173 \times 10^5$	$6.77 \pm 0.153 \times 10^5$	0.637
ISP-2	$6.07 \pm 0.252 \times 10^5$	$7.50 \pm 0.458 \times 10^5$	0.050
MHA	$5.93 \pm 0.306 \times 10^5$	$6.00 \pm 0.200 \times 10^5$	0.822
NA	$6.40 \pm 0.300 \times 10^5$	$6.80 \pm 0.173 \times 10^5$	0.105
PDA	$3.60 \pm 0.400 \times 10^5$	$5.50 \pm 0.458 \times 10^5$	0.050
TSA	$6.60 \pm 0.200 \times 10^5$	$7.33 \pm 0.503 \times 10^5$	0.077
P-value[2*]	**0.017**	**0.012**	

[1]Comparison of growth at different temperatures on respective media
[2]Comparison of growth on different media at respective temperatures
[*]Significant values in bold

DISCUSSION

Actinomycetes are a diverse group of microorganisms that identification approach by biochemical tests may be tedious and troublesome. Due to the specificity of molecular technique, it may substitute robust conventional identification by giving a faster and reliable result. In this study, some basic conventional identification tests for *Streptomycetes* such as Gram-stain, odor and morphological examination followed by molecular detection using PCR/sequencing technique targeting *16S rRNA* and *rpoB* genes were performed. The former gene has been long used and shown to be effective in identification and evolutionary tracing of various microorganisms including *Streptomyces* strains but the later has also been extensively studied as an alternative target for species identification due to its greater polymorphism to render it more discriminatory than the former gene (Khamis *et al*., 2004; Adékambi *et al*., 2008; Ki *et al*., 2009). Until a consensus is reached to treat *rpoB* gene as so, it was used in this study as an additional validation at genus level only. The BLAST analysis and generated 16S rDNA based-phylogram suggested that isolates B8 and C2 are *Streptomyces* species with potential different lineage of evolution. Considering that the isolates were from a patch of soil that there could be a potential accumulation and dissemination of clones, some of their phenotypic characters which are dissimilar in term of colour and growth capacity on the different media may support the phylogenetic inference that they are different isolates.

Streptomyces strains have been known for their valuable potential as sources for antimicrobial agents. But their diversity obscures the selection of feasible media for a sustainable growth of various unknown isolates. To maximize the isolation and recovery of various actinomycetes from environmental samples on agar media, many studies have been employing various sources of carbon, nitrogen and mineral in media preparation (Narayana & Vijayalakshmi, 2008; Valanarasu *et al*., 2009), while one study used the soil itself as part of the media component (Hamaki *et al*., 2005). It is not known whether these specially formulated media could be equally effective to provide growth for the *Streptomyces* strains from habitat and location of different origins as these media were designed to suit the local environments in the respective studies. A major shortcoming is that these media are not commercially available to be readily utilized by different laboratories throughout the world. International *Streptomyces* Project (ISP) has come up with media ISP-1, -2, and -4 to select stable properties of *Streptomyces* strains for characterization purposes

(Shirling & Gottlieb, 1966). In this study, ISP-2 agar medium was used as the maintenance media following primary isolation on SCA. Pure culture was obtained after several subculturings and growth characteristics were then evaluated on various common commercial microbiological media; MHA, NA, PDA and TSA. The AIA and ISP-2 media are commercially formulated for *Streptomyces* and growth on these media was compared with those on the other common media for potential differences. Malaysia is a tropical country which is very warm during the day-time and often humid, while it is fairly cool during the night. Thus the incubation temperatures used in this study were 37°C and 28°C to represent the local surrounding temperature. The later temperature was widely used in many other studies but fewer reports elucidated the pattern of growth at 37°C in comparison to that at 28°C in a quantitative manner (Suthindhiran *et al*., 2009; Valanarasu *et al*., 2009; Kumar & Kannabiran, 2010).

The ISP-2 agar medium contains yeast and malt extract which are rich in nutrients such as nitrogen, amino acids and vitamins, and dextrose to serve as the source of carbon. But the exact amounts of such individual nutritional elements in the extract are not known. On the other hand, AIA contains defined amounts of nitrogen source (sodium caseinate) and amino acid (asparagines) with added glycerol (0.5%) as source of carbon. None of these types of nutritional sources in ISP-2 and AIA media are present in the other media used in this study but TSA contains peptone from yeast. With a standardized spore suspension (~0.5 McFarland standard) as the starting inocula, viable counts showed that growth for both isolates B8 and C2 were within10^6 and 10^5cfu/ml respectively indicating that all media were capable of supporting growth at certain extents. In term of incubation temperatures, growth yield at 28°C were always higher particularly isolates B8 that reached significant level in all media conditions. Nevertheless the two isolates showed different media preferences that changed in response to the incubation temperatures. These indicate that different isolates may have different capability in utilizing different sources of nutritional components and the capability may change at different temperatures. Frequently, ISP-2 medium sustained a good growth yield for both isolates in comparison to the other media which varied. In addition, more morphological characteristics of the isolates were exhibited on ISP-2 medium such as the color variety of aerial mass, substrate mycelium and spores, while those on other media were generally homogenous. This supports ISP-2 as a medium suitable for describing discrete morphological features of *Streptomyces*. Nevertheless, as far as the growth output is concerned regardless of incubation temperature, pigment and spore production, MHA may be favorable for isolate B8 whereas TSA for isolate C2 for the choice of media other than those specifically devised for *Streptomyces* (ISP-2 or AIA). It is difficult to comment on the impact of the individual components of the media used as in general, these are undefined media that utilize extracts from various sources whereby the specific amounts and types of element in the extract are not known. But, due to the frequent good growth and expression of varied cultural traits on ISP-2 agar media in this study, the potential sources of nutrient would be those present in the media; the yeast and malt extract, to serve as a basis for further exploitation in media formulation. Yeast and malt extract are commercially available and have been widely used as supplement in combination with various media to support growth of fastidious organisms.

Although growths of the two isolates were shown to vary on different media and temperatures, the question on the amount and quality of antibacterial metabolites produced is still unknown. Primary antibacterial screening on MHA and TSA performed in this current study showed the inhibitory activity but the ability of the antibacterial metabolites produced by the two isolates to equally diffuse in the different media may not be similar due to different media composition. Secondary screening using metabolite produced in broth culture followed by disc or well diffusion method on a standardized test media is usually required to further validate the antimicrobial activities. However, it is still

controversial as production of the antibacterial metabolites may be affected by the different growth environment on agar surface and in broth suspension (Pandey *et al.*, 2004).

On the whole, findings from this study showed variations in phenotypic characteristics, particularly the growth yield on different media at different temperatures to indicate unique features between the two local *Streptomyces* isolates with antibacterial properties. In view of the diversity of the genus, a lot more isolates with distinct characteristics are anticipated to further complicate the selection of feasible media for their cultivation. How those could relate to the production of antimicrobial metabolites is of much interest.

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