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Diversity of *Micromonospora* in Malaysian mangrove rhizosphere soil

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ABSTRACT Seventy putative micromonosporae were assigned to 12 single and multi- membered colour groups based on their colony colour produced on modified micromonospora (MMS) agar medium. The strains produced well-developed substrate mycelium of approximately 0.2 – 0.4 μm in diameter and had *meso*-DAP in the cell wall hydrolysates. Single spores having an average diameter of 0.4 to 1.0 μm were produced on substrate mycelium. A range of diffusible pigments produced by 29% of the isolates on the MMS agar plates included: brown, 13%; yellow, 10% and purple, blue, and black at 1% each. The results of the cell wall analysis, cultural and morphological characteristics of the selected strains tentatively indicated that they belonged to the genus *Micromonospora* Orskov 1923.

ABSTRAK Tujuh puluh pencilan micromonoporae dari sampel tanah rizosfera payah telah diagihan kepada dua belas kumpulan warna berdasar warna koloni di atas media micromonospora terubahsuai (MMT). Pencilan menghasilkan miselium substrat 0.2-0.4μm diameter dan kandungan asid amino didalam hidrolisat dinding sel adalah meso-DAP. Spora tunggal 0.4 – 1.0μm dihasilkan oleh miselium substrat. 29% pencilan menghasilkan pelbagai pigmen yang meresap kedalam plat agar MMS termasuk: perang, 13%; kuning, 10%; ungu, 1%; biru, 1% dan hitam, 1%. Kajian ini menandakan bahawa pencilan terpilih adalah secara tentatif, dari genus *Micromonospora* Orskov1923.

(Micromonospora, actinomycete, colony colour, spore ornamentation, marine mangrove rhizosphere)

INTRODUCTION

Actinomycetes, which contains more than 50 genera are a phylogenetically defined, metabolically active group of Gram-positive bacteria and prolific producers of bioactive compounds. The rare actinomycete, *Micromonospora* which was first proposed by Orskov [1] is mainly characterised by (i) absence of aerial mycelium, (ii) well developed substrate mycelium of about 0.5 μm in diameter; (iii) presence of single spores on substrate mycelium; (iv) Gram-positive; (v) cell wall contains *meso*-diaminopimelic acid; (vi) xylose and arabinose are dominant sugars in cell wall hydrolysates and (vii) the mol% of G+C of the DNA is 71-73 [2]. The colonies are initially pale yellow or light orange, becoming orange, red, brown, blue-green or purple [2].

Proper and accurate classification and identification of actinomycetes are of biotechnological and medical importance. Knowledge of the cultural properties of actinomycetes is essential for their classification. *Micromonospora* species can be identified up to genus level on the basis of colony and spore morphology [2]. However, to assess the diversity, both morphological and chemical characteristics are regarded as very important for distinguishing the isolates to the species level [2, 3 and 4]. The observations of spore and sporophore shape of cultures by electron microscopy have been used for characterisation of micromonosporae, too [2]. The objective of this study was to determine the cultural and morphological characteristics of the putative micromonosporae isolated from coastal mangrove rhizosphere habitats.

MATERIALS AND METHODS

Selection of Micromonospora strains

Presumptive micromonosporae colonies on starch-casein-nitrate [5], raffinose-histidine (RH) [6] and humic acid-vitamin (HV) [7, 8] agar isolation plates were observed under a 40× stereo-binocular microscope (Nikon SMZ-10).

About 100 colonies were picked and transferred to modified micromonospora medium (MMS) [9], International Streptomyces Project (ISP) medium no. 2 (ISP2) and ISP4 [10] and Bennet's (BM) agar plates [11]. All the plates were incubated at 28 ± 2°C for 2 weeks. After incubation, the isolates of micromonosporae were checked for purity. From the 100 strains, 70 presumptive micromonosporae were selected on the basis of their morphological characteristics, such as substrate mycelium colour, soluble pigments, if any, and spore mass colour on MMS agar plates.

Determination of cell wall diaminopimelic acid (DAP) isomer

The procedure of Lechevalier and Lechevalier [4] was followed for the determination of whole cell diaminopimelic acid (DAP) isomer of selected micromonosporae isolates.

Colour grouping of selected micromonosporae

The selected 70 presumptive micromonosporae were inoculated on to MMS agar medium to determine the colour groups. Duplicate plates were set up for each of the selected strain. The plates were then incubated at 28 ± 2°C for 3 weeks. After incubation, colony colour was determined using Methuen Handbook of Colour [12]. All the selected isolates were preserved in 30% glycerol (w/v in distilled water) at –20°C.

Morphological and chemical characteristics of selected micromonosporae

The selected isolates were characterised and identified based on a range of cultural and morphological tests. The characteristics were: (i) the absence or presence of aerial mycelium, (ii) the structure of substrate mycelium; (iii) spore shape and (iv) the cell-wall diaminopimelic acid (DAP). The colony morphology was observed on MMS and ISP4 agar medium after 3 weeks of incubation at 28 ± 2°C by eye and using a 40× stereo-binocular microscope (Nikon SMZ-10).

Scanning Electron Microscopic observation

Spore morphology of the representative isolates from each colour group was then studied by Scanning Electron Microscope (Philips, 515) [13]. As the main aim of SEM studies was know the morphology of the spores the quality of the micrographs were not emphasized here.

RESULTS AND DISCUSSION

Growth and cultural characteristics of selected micromonosporae

Aerial mycelium was absent except for the isolate M56 which produced short, scanty, whitish or greyish aerial mycelium on MMS and ISP4 agar plate after 21 days of incubation at 28 ± 2°C. Generally, species of the genus *Micromonospora* do not produce aerial mycelium but scanty whitish or grayish aerial mycelium was produced in some cultures [2]. Abundant growth of substrate mycelium; colour and spore mass were observed. 92% of the selected 70 isolates, grew well on the modified micromonospora medium (MMS) and 91% on ISP4 agar plates at 28 ± 2°C for 3 weeks. Abundant production of dark coloured spore mass of the strains were observed on the MMS agar plates compared to ISP4 agar plates.

Mucoid, waxy to dry colonies range in colours from pale orange, orange, brown, dark brown to black, red-brown, olive green, purple and blue were observed on MMS and ISP4 agar plates. In general, micromonosporae had been reported to become yellow, orange, red, brown, black, blue, green and purple colonies with the production of mature spores and sometimes the colonies become mucoid, waxy and viscid [2]. However, based on substrate mycelium pigment, it was not possible to relate selected strains with other valid *Micromonospora* strains.

Of the 70 isolates 29% of the strains produced a range of diffusible pigments on the MMS agar plates. The diffusible pigments produced included: brown, 13%; yellow, 10%; purple, 1%; blue, 1% and black, 1%. It was reported that the substrate mycelium of some *Micromonospora* spp. produce yellow-orange-red mycelial pigments which appear to offer diagnostic value in strain or species recognition [14, 2]. There are two characteristic mycelial pigments, which are known to be pH indicators: the maroon-purple pigments of *M. echinospora* subsp. *echinospora*, *M. echinospora* subsp. *ferruginea* and *M. purpurea* and blue-green pigment of *M. coerulea*. In this study, strains M8 and M15 produced dark blue and purple red diffusible pigments respectively on MMS agar plates after 3 weeks of incubation at 28 ± 2°C. However, these findings were not enough to relate with the *M. echinospora* subsp. *echinospora*, *M. echinospora*

subsp. *ferruginea* and *M. purpurea* and *M. coerulea*.

Colour grouping of the selected micromonosporae

The colour groups of 70 isolates were recorded as brown > orange brown > dark brown > orange > dark orange brown > pale brown orange > dark blackish brown > purplish brown, dark red brown; dark green yellow; pale orange and bluish black respective to 29% > 20% > 16% > 13% > 7% > 6% > 3% > 1% (Table 1). The 70 presumptive micromonosporae isolates grown on MMS agar plates at 28 ± 2°C for 3 weeks were assigned to 12 multi and single membered colour groups (Table 1). The largest colour group with initially orange colonies, which later turned to brown on MMS agar plates had 20 isolates (29%). Thus, it seemed that mangrove rhizosphere habitat might be a good source for diverse micromonosporae isolates. Hatano [15] had reported that the mangrove rhizosphere

Table 1. Colour groups of selected micromonosporae strains on MMS agar medium after 3 weeks of incubation at 28 ± 2°C *

No. of colour groups	Colony colour*	Code number of the Isolates	Total no. of isolates	% of total isolates
1	Brown	M4, M6, M10, M13, M17, M20, M22, M29, M30, M42, M50, M51, M58, M60, M62, M64, M65, M67, M68, M74	20	29%
2	Orange to brown	M1, M5, M9, M12, M24, M26, M27, M28, M32, M36, M39, M54, M66, M71	14	20%
3	Orange	M16, M38, M40, M55, M57, M61, M69, M70, M72, M73, M75	11	16%
4	Dark brown	M2, M7, M14, M23, M44, M47, M52, M59, M63	9	13%
5	Dark orange brown	M18, M19, M25, M31, M43	5	7%
6	Pale brown orange	M33, M34, M35, M53	4	6%
7	Dark blackish brown	M11, M21	2	3%
8	Purplish brown	M15	1	1%
9	Dark red brown	M56	1	1%
10	Dark green yellow	M37	1	1%
11	Pale orange	M3	1	1%
12	Bluish black	M8	1	1%

* The MMS agar plates were examined by eye and spore mass and substrate mycelium colour, colour of diffusible pigments, if any, were recorded based on Methuen Handbook of Colour [12].

habitat was a good source of diverse actinomycetes including micromonosporae.

Cultural and morphological characteristics of selected strains from each colour group

The cultural and spore morphology characteristics of representative strains from each colour group are given in Figure 1 and Table 2. All the isolates selected had well-developed substrate mycelium about 0.2 – 0.4 µm in diameter on MMS agar (Table 2). Aerial mycelium was not formed except for the isolate M56, which produced scanty whitish aerial mycelium after 3 weeks of incubation at 28 ± 2°C on MMS and ISP4 agar plates. Spores with an average diameter of about 0.4 to 1.0 µm were borne singly on the substrate mycelium (Figure 2 a - l). The spore surface of the strains appeared smooth (Figure 2 a, c, e, f, g, j, k and l) to blunt warty to rough (Figure 2 b, d, h and i).

Characteristics of "type" strain of each colour group

Strain M17

The strain M17 is a representative isolate from the brown colour group (Tables 1 and 2). The strain M17 produced brown, round, flat, smooth and waxy colonies on MMS agar medium after 3 weeks of incubation at 28 ± 2°C (Figure 1 a). The sporulation was abundant in mature colonies and oval (~ 0.5 – 0.7 µm in diameter), smooth and sessile spores were observed on substrate mycelium (~ 0.4 µm in diameter) (Figure 2 a). So far, the morphology of this strain does not match with other valid micromonosporae strains.

Table 2. Cultural and morphological characteristics of the isolates selected from each colour group

Selected isolates	Colour groups	Colony morphology	Spore morphology and size (µm)	Mycelium size (µm)
M17	1. Brown	Round; small; flat; smooth and waxy	Oval, smooth; Sessile and About (~) 0.5- 0.7	About (~) 0.4
M39	2. Orange brown	Irregular, rough; flat and dry	Oval, rough; sessile and ~ 0.4 – 0.7	~ 0.2 – 0.3
M38	3. Orange	Raised, folded; round and waxy	Spherical; smooth; sessile and ~ 0.8	~ 0.4
M7	4. Dark brown	Raised, folded; round to irregular and waxy	Elongate, rough; sessile and ~ 0.5	~ 0.3
M19	5. Dark orange brown	Round, flat; smooth and dry	Oval; smooth; sessile and long sporophore and ~ 0.8 – 1.0	~ 0.3
M33	6. Pale brown orange	Raised, round; smooth and dry	Oval, rough; long sporophore and ~ 0.8	~ 0.4
M21	7. Dark blackish brown	Raised, folded; round and dry	Spherical, rough; sessile and ~ 0.9	~ 0.4
M15	8. Purplish brown	Raised, round; smooth and waxy	Spherical, smooth; sessile and ~ 0.7	~ 0.2
M56	9. Dark red brown	Flat; rough; round and dry	Oval, rough; sessile and short sporophore and ~ 0.8 um	~ 0.2
M37	10. Dark green yellow	Flat, folded; irregular and waxy	Oval, smooth; long sporophore and ~ 0.7	~ 0.3
M3	11. Pale orange	Raised, folded; irregular and waxy	Oval, smooth; sessile and ~ 0.9	~ 0.4
M8	12. Bluish black	Round, flat; folded and dry	Oval, smooth; sessile and short sporophore and ~ 0.6	~ 0.3

Strain M39

The strain M39 from the orange to brown group produced orange-brown folded colonies and reddish-brown diffisible pigments on MMS agar medium after 3 weeks of incubation at 28 ± 2°C (Figure 1 b and Table 1). The sporulation was abundant in mature colonies. Spores were mostly oval (about 0.7 µm in diameter), rough walled and had blunt spines, sessile or long sporophore produced on the substrate mycelium (Figure 2 b).

Strain M38

Cultural characteristics of the strain M38 from the orange colour group are given in Figure 1 c and Table 2. Bright orange vegetative mycelia and spherical smooth spores were formed on the MMS agar medium (Figure 2 c). This colour remained even after 3 weeks and did not change to dark brown or brownish black, although the mycelial pigment of known *Micromonospora* spp. changes from orange to dark brown or brownish black on maturation or spore production. However, results from other studies indicated that *M. echinospora* subsp. *armeniaca* subsp. nov. produced bright orange vegetative mycelia and that colour remained orange even after 4 weeks of incubation and did not change to dark brown or brownish black [16]. It was also observed that this strain produced spherical spores on substrate mycelium [16]. Strain M38 was similar to *M. inositola*, which produced bright orange colonies, did not change to any dark colour and produced spherical to oval spores (0.8 um to 1.0 um), which were borne on short sporophores [2].

Strain M7

The strain M7 is a representative isolate from the dark brown colour group formed dark brown, raised, folded, round to irregular and waxy colonies and pale yellow diffusible pigment on MMS agar plate (Figure 1 d and Table 2). Elongate, rough and sessile spores (~ 0.5 µm in diameter) were borne on substrate mycelium (~ 0.3 µm in diameter) (Figure 2 d). So far, the morphology of this strain did not match any other described valid micromonosporae strains.

Strain M19

The strain M19 is a representative isolate from dark orange brown colour group (Figure 1 e and Table 2). Oval, smooth spores were observed (~ 0.8 – 1.0 µm in diameter) which were sessile or long sporophore of substrate mycelium (~ 0.3 µm in diameter) (Figure 2 e). The morphology of this strain did not match any other valid described micromonosporae strains.

Strain M33

Cultural characteristics of the strain M33 are shown in Figure 1 f and Table 2. This strain formed raised round pale brown orange colonies on MMS agar plate and produced brownish black diffusible pigments after 3 weeks of incubation at 28 ± 2°C. Spores were spherical to slightly oval (about 0.8 µm in diameter) in shape and hyphae were observed to be about 0.4 µm thick (Figure 2 f). Studies have shown that *M. purpureochromogenes* produced dark-brown to black agar diffusible pigment [17]. *Micromonospora purpureochromogenes* was also observed to produce single or clusters of spores, which were 0.8-1.2 µm in diameter [2]. Jensen [18] characterised *M. fusca* as producing a dark brown to black diffusible pigment, which he believed, differed from the pigments produced by chromogenic streptomycetes. On the other hand, a dark brown to black diffusible pigment is also produced by many streptomycetes [19].

Strain M21

The strain M21 from the dark blackish brown colour group produced dark brown to black colonies on the MMS agar plate (Figure 1 g). The colonies of the strain M21 produced brownish diffusible pigments on maturation. Spores spherical, about 0.9 µm in diameter appeared rough walled under scanning electron microscope (Figure 2 g). Kawamoto [2] reported that *M. echinospora* produce dark-brown to black colonies and spiny clusters of spores on agar plate.

Strain M15

The colonies of the strain M15 from the purplish brown colour group were smooth, round and purplish-brown on the MMS agar plate (Figure 1 h) and produced purplish diffusible pigments. Spores are spherical, smooth, sessile and ~ 0.7 µm diameter (Figure 2 h). It has been observed that maroon to purple mycelial pigments in *M. echinospora* subsp. *echinospora* [20] were soluble in acid alcohol and was pH-sensitive, being red in the acid range and blue-green and precipitated in the basic range [2]. Spore layers when present were purplish black, waxy to dry, not moist or viscid. Aerial mycelium was absent but occasionally a very short purplish gray bloom devoid of spores occurred on some colonies. However, the strain M15 did not produce any

aerial mycelium on the MMS or ISP4 agar medium. Other studies had shown that *M. echinospora* subsp. *ferruginea* produced maroon to purple mycelial pigments [20, 2].

Strain M56

Mycelial colonies of the strain M56 from the dark red brown were grayish red to reddish brown when grown on the MMS agar medium (Figure 1 i). The spore layer was black and waxy to dry rather than moist or viscoid. It formed a rudimentary, whitish, non-sporulating aerial mycelium. Microscopic observation showed that the hyphae of the substrate mycelium were well developed, straight to wavy and branched. Spores were formed singly on the substrate mycelium, usually as sessile or short sporophores (Figure 2 i). Electron microscopy showed that the mature spores were oval with a diameter of about 0.8 μm with a rough spiny surface (Figure 2 i). Studies have shown that *M. griseorubida* sp. nov. produced grayish red to reddish brown mycelial pigments on different agar media and these pigments behave as pH indicators, being yellow orange in the acid range and grayish red in the basic range. Further, colonies of the *M. griseorubida* produced scanty aerial mycelium [21].

Strain M37

The cultural characteristics of the strain M37 from the dark green yellow are illustrated in Figure 1 j and Table 2. Growth was good on MMS agar and colour of the colonies were light yellow to brown, which turned to olive or dark green when spores were produced. The substrate mycelium was well developed, branched, septate and approximately 0.3 μm in diameter. The spores were borne singly on long sporophores from the substrate mycelium. The mature spores were approximately 0.7 μm in diameter and oval in shape (Figure 2 j). According to the known *Micromonospora* species described in the Approved Lists of Bacterial Names [22], the strain M37 closely resembled *M. olivastrerospora* sp. nov. [23]. *Micromonospora olivasterospora* sp. nov. was characterised by its green black waxy spore layer during the formation of spores. The mature spores borne on short sporophores were sessile, spherical to oval, approximately 1.0 μm in diameter and had a rough surface when observed under phase contrast microscopy [2]. However, the spores of strain M37 were smooth and appeared on long sporophores (Figure 2 j).

Strain M3

The strain M3, a representative isolate from pale orange colour group produced oval, smooth and sessile spores (~ 0.9 μm in diameter) with pale yellow diffusible pigment (Figures 1 k and 2 k). So far, the morphology of this strain M3 did not match any valid micromonosporae strains.

Strain M8

The strain M8 from the bluish black colour group produced dark blue to greenish blue-black colonies on the MMS agar plate and dark blue diffusible pigments were produced at maturation (Figure 1 l). The spores were spherical to oval, about 0.6 μm in diameter, smooth-walled when observed under scanning electron microscopy. The spores were borne on short or long lateral sporophores (Figure 2 l). Similarly, *M. coerulea* produced blue green or bluish green black colonies with spherical smooth spores borne on short or long sporophores. It was reported a blue green mycelial pigment was produced by *M. coerulea* [18] and was water-soluble and pH sensitive [2].

Results from the current investigation showed that the average diameter of the spore ranged from about 0.4 to 1.0 μm, which is within the average size of the some previously described micromonosporae isolates (0.7 to 1.5 μm) [2]. The diameter of the well-developed mycelium ranged from about 0.2 to 0.4, which is also the average size of the some species of the genus *Micromonospora* [2]. However, based on the above findings the selected strains from each colour group had both close similarities and dissimilarities with respect to substrate mycelium pigmentation and spore morphology when compared with the valid *Micromonospora* species described previously. However, without further chemical and molecular investigation it was not possible to relate these selected strains to the valid species in Bergey's Manual of Systematic Bacteriology.

Several factors such as media components, temperature and pH might influence the morphological and cultural variation of the selected strains with the valid species of the genus *Micromonospora*. However, all the selected isolates from each colour group shared a number of cultural properties, which were consistent with their inclusion in the genus *Micromonospora* [2]. They all formed extensively branched substrate mycelium, single

spores on substrate mycelium; had no aerial hyphae except for scanty aerial hyphae of the strain M56. Based on these data, the selected strains may be considered as putative strains of the genus *Micromonospora* Orskov 1923.

CONCLUSIONS

From this investigation, the following conclusions were made with respect to cultural and morphological characteristics of selected putative micromonosporae from coastal mangrove rhizosphere habitats. The selected strains were members of the genus *Micromonospora*. However, colony colour and spore morphology were not sufficient for the accurate identification of the species and their novelty in the mangrove rhizosphere region. Further taxonomic characterisation, especially molecular systematic data are needed for identification of the species.

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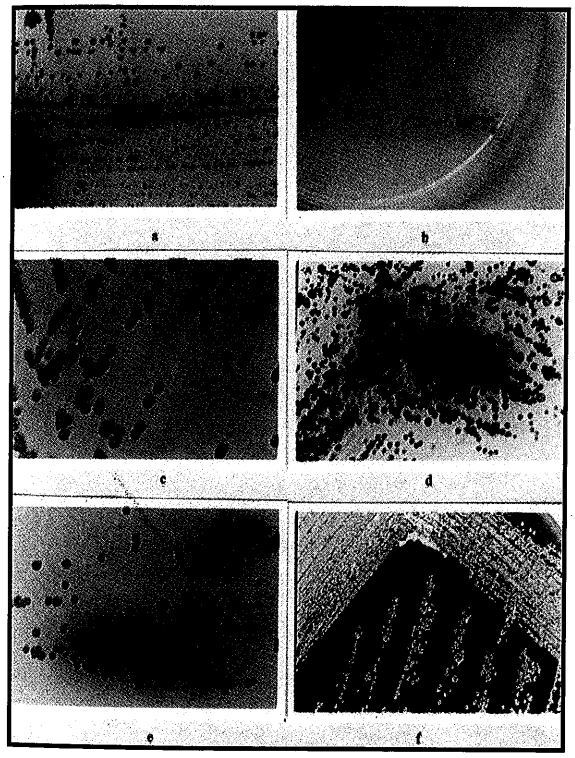
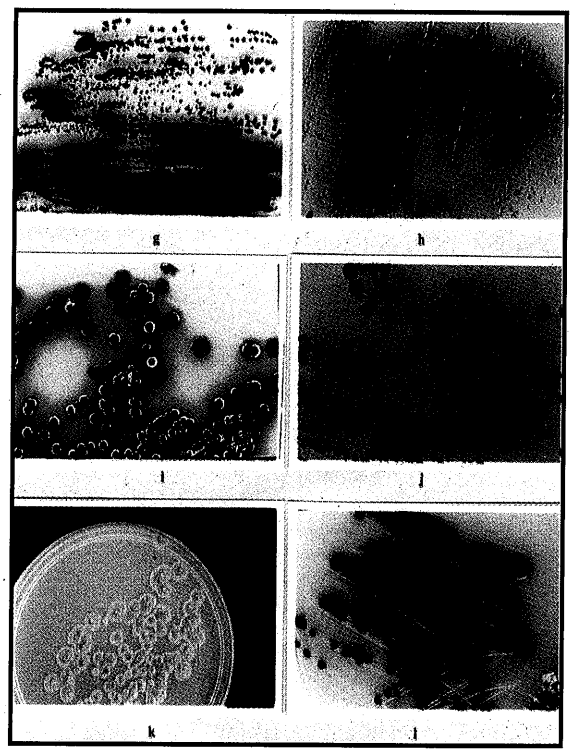
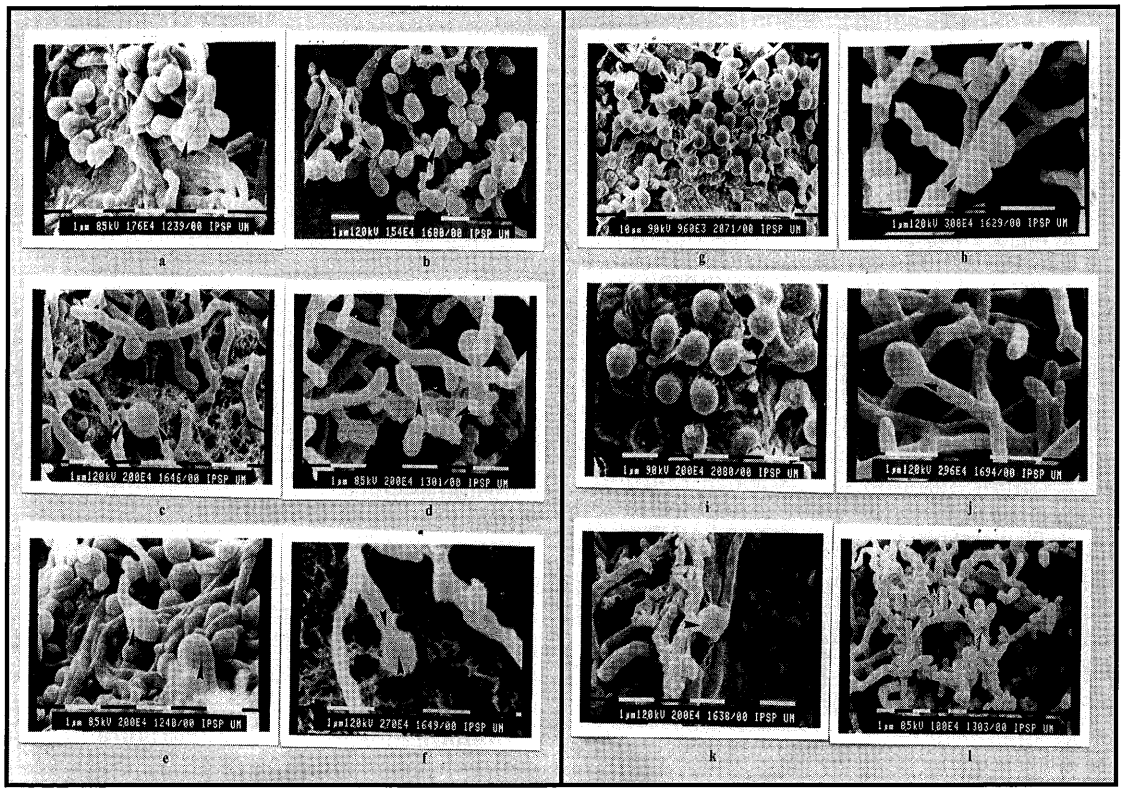


(a –f)



(g – l)

Figure 1. Colony morphology of selected *Micromonospora* isolates on MMS agar medium after 3 weeks of incubation at 28± 2°C. (a) brown colonies of the strain M17; **(b)** orange brown colonies of the strain M39; **(c)** orange colonies of the strain M38; **(d)** dark brown colonies of the strain M7; **(e)** dark orange brown colonies of the strain M19; **(f)** pale brown orange colonies of the strain M33; **(g)** dark blackish brown colonies of the strain M21; **(h)** purplish brown colonies of the strain M15; **(i)** dark red brown colonies of the strain M56; **(j)** dark green yellow colonies of the strain M37; **(k)** pale orange brown colonies of the strain M3 and **(l)** bluish black colonies of the strain M8.



(a - f) **(g – l)**

Figure 2. Spora morphology of selected *Micromonospora* isolates on MMS agar medium after 3 weeks of incubation at 28± 2°C. Arrows indicate the sporophore and spore shape **(a)** oval and sessile smooth spores of brown colonies of the strain M17; **(b)** elongated to oval rough walled sessile monospores of orange brown colonies of the strain M39; **(c)** spherical to oval and sessile smooth spore of orange colonies of the strain M38; **(d)** elongated rough walled sessile monospores of dark brown colonies of the strain M7; **(e)** oval smooth walled monospores (sessile or on short sporophores) of dark orange brown colonies of the strain M19; **(f)** spherical to oval smooth walled monospores on long sporophores of pale brown colonies of the strain M33; **(g)** spherical and sessile smooth spores of dark blackish brown colonies of the strain M21; **(h)** spherical rough sessile walled monospores of purplish brown colonies of the strain M15; **(i)** oval rough walled monospores on substrate mycelium with short sporophores of dark red brown colonies of the strain M56; **(j)** oval smooth spores on long sporophores of dark green yellow colonies of the strain M37; **(k)** spherical to oval smooth sessile monosphores of pale orange colonies of the strain M3 and **(l)** oval smooth walled sessile monospores of bluish black colonies of the strain M8. Arrows indicate the sporophore and spore shape.

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