

***Rhodovulum mangrovi* sp. nov., a phototrophic alphaproteobacterium isolated from a sediment sample of Coringa mangrove forest, India**

Nupur¹, T.N.R. Srinivas², S. Takaichi³ and P. Anil Kumar^{1*}

¹Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology, Sector 39A, Chandigarh – 160 036, INDIA

²CSIR-National Institute of Oceanography, Regional Centre, 176, Lawsons Bay Colony, Visakhapatnam-530017, INDIA

³Nippon Medical School, Department of Biology, Kosugi-cho, Nakahara, Kawasaki 211-0063, Japan

Address for correspondence* Dr. P. Anil Kumar

Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (CSIR), Sector 39A, Chandigarh – 160 036, INDIA; Email: apinnaka@imtech.res.in; Telephone: 00-91-172-6665170

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AK41^T is HG529993.

A novel Gram-staining negative purple non-sulfur bacterium, AK41^T, was isolated from a sediment sample collected from Coringa mangrove forest, Andhra Pradesh, India. A red-brownish coloured culture was obtained on modified Pfennig medium after enrichment with 2% NaCl and 0.3% pyruvate under 2000 lux of illumination. Individual cell is ovoid-rod shaped and non motile. Bacteriochlorophyll *a* and carotenoids of the spheroidene series were present as photosynthetic pigments. Strain AK41^T was halophilic and grew photoheterotrophically with a number of organic compounds as carbon source and electron donor. It was unable to grow photoautotrophically. It did not utilize sulfide and thiosulfate as electron donors. The fatty acids were found to be dominated by C_{16:0} and C_{18:1 ω7c}. Strain AK41^T contains phosphatidylglycerol, phosphatidylethanolamine, amino lipid and four unknown lipids as polar lipids. Q-10 is the predominant respiratory quinone. The DNA G+C content of the strain AK41^T was found to be 68.9 mol%. The 16S rRNA gene sequence analysis indicated that strain AK41^T was a member of the genus *Rhodovulum* and closely related to *Rhodovulum sulfidophilum* with 96.0% similarity and with other species of the genus *Rhodovulum* similarity is between 95.8 to 93.9%. Phylogenetic analyses indicated that the strain AK41^T clustered with *Rhodovulum marinum*, *Rhodovulum kholense*, *Rhodovulum sulfidophilum*, and *Rhodovulum visakhapatnamense* with phylogenetic similarity of 95.9 to 96.2%. Based on data from the current polyphasic study, strain AK41^T is proposed as a novel species of the genus *Rhodovulum*, for which the name *Rhodovulum mangrovi* sp. nov. is proposed. The type strain of *Rhodovulum mangrovi* is AK41^T (= MTCC 11825^T = JCM 19220^T).

Key words: *Rhodovulum mangrovi*, Coringa mangrove forest, 16S rRNA gene based phylogeny, chemotaxonomy, *Alphaproteobacteria*

Mangrove ecosystems are one of the most active and bio-diverse wetlands on our earth. However, these characteristic coastal tropical habitats are among the major endangered ecosystems on our globe and may be deteriorating more swiftly than inland ecosystems. By partial endeavour, 48 novel microbes belonging to a number of species and higher taxa (*Actinobacteria*, *Firmicutes* and *Proteobacteria*) were isolated from various mangrove ecosystems described in the last few years (Srinivas *et al.*, 2013). Out of these a number of anoxygenic phototrophic bacteria were isolated from different mangrove ecosystems includes *Marichromatium indicum*, *Thiophageococcus mangrovi*, *Rhodobacter aestuarii* and *Rhodovulum kholense* (Anil Kumar *et al.*, 2008a, b; Arunasri *et al.*, 2005; Ramana *et al.*, 2009).

The genus *Rhodovulum* was proposed by Hiraishi & Ueda (1994), with the type species *Rhodovulum sulfidophilum* along with other species *Rhodovulum adriaticum*, and *Rhodovulum euryhalinum* (Hiraishi & Ueda 1994). Phylogenetically, the genus belongs to the family *Rhodobacteraceae* within the class *Alphaproteobacteria*. The genus *Rhodovulum* comprises Gram-negative, ovoid to rod-shaped cells, 0.3 to 0.9 μm wide, and 0.9 to 2.0 μm long, motile by means of a single polar flagellum or nonmotile, dividing by binary fission. Growth occurs under photoautotrophic/photoheterotrophic (anaerobically under light with sulfide or thiosulfate or organic carbon as the electron donor)/chemoheterotrophic (aerobically under dark) conditions. Cells grown phototrophically form vesicular intracytoplasmic membranes along with bacteriochlorophyll *a* (esterified with phytol) and carotenoids of the spheroidene series and appear yellow-green to yellow-brown, while aerobically grown cultures are pink to red. Mesophilic and halophilic and require 0.5 to 7.5% (w/v NaCl) for optimal growth. Preferred growth mode is photoorganotrophy with various organic compounds. Most preferred carbon sources are intermediates of the citric acid cycle, lower fatty acids, lactate, pyruvate, and some sugars. Some strains grow at high sulfide concentrations (2 mM or more). Sulfide is finally oxidized to sulfate. Comprises ubiquinone 10 as the major quinone, C_{18:1} as the major fatty acid, and the G+C content of the genomic DNA ranges from 58 to 69 mol% (Hiraishi & Ueda 1994; Kompantseva *et al.*, 2010; Srinivas *et al.*, 2007a). Presently the genus comprises fifteen species with validly published names isolated from diverse habitats like sea water (Hiraishi & Ueda, 1995; Kompantseva, 1985; Neutzling *et al.*, 1984; Srinivas *et al.*, 2006; Srinivas *et al.*, 2007b), coloured ponds (Chakravarthy *et al.*, 2009; Lakshmi *et al.*, 2011; Srinivas *et al.*, 2012), aquaculture pond (Srinivas *et al.*, 2007a), coastal sediments (Straub *et al.*, 1999), mangrove forest (Anil Kumar *et al.*, 2008a), and soda lakes (Kompantseva *et al.*, 2010; Kompantseva *et al.*, 2012). In the course of bacterial diversity studies of mangrove samples, a bacterial strain, AK41^T, was isolated from a mangrove sediment sample collected near Coringa, Andhra Pradesh, India. Phylogenetic analysis

based on 16S rRNA gene sequences revealed that strain AK41^T was closely related to the genus *Rhodovulum* of the family *Rhodobacteraceae*. In this study, a polyphasic approach, including genotypic, phenotypic and chemotaxonomic characterizations, was used to determine the taxonomic position of strain AK41^T.

The strain AK41^T was isolated from the sediment sample collected from Coringa mangrove forest, India. The temperature and pH of the sediment sample was 30 °C and 7.5, respectively. Anoxygenic phototrophic bacterium AK41^T was isolated using photolithoautotrophic enrichments of the sample and subsequently purification was performed as previously described (Anil Kumar *et al.*, 2008) in modified Pfennig medium (Pfennig & Trüper 1992) supplemented with NaCl (2 % w/v), pyruvate (0.3 % w/v), sodium thiosulfate (2 mM) and ammonium chloride (0.12 % w/v). Modified Pfennig medium was used throughout the study unless mentioned otherwise. Preservation was done at -80°C in modified Pfennig broth with 20% glycerol.

Colony morphology was studied after 72 hr growth of the strain on modified Pfennig medium at 30°C under 2000 lux illumination anaerobically. The isolated pure colony was checked for cell morphology and motility by using phase contrast microscopy (Olympus, USA) at 1000X magnification. Gram reaction was determined by using the HIMEDIA (Mumbai, India) as described by manufacturer's protocol. Cell size, morphology (negative staining) and intracytoplasmic structures (sections) were viewed through a transmission electron microscope (Jeol JEM 2100) at operating voltage of 200 kV as previously described (Ramana *et al.*, 2006). Sudan black (Smibert & Krieg, 1981) or Nile blue staining (Ostle & Holt, 1982) were used to confirm the presence of Polyhydroxyalkanoates. Physiological and biochemical characteristics were determined as previously described (Anil Kumar *et al.*, 2008a; Srinivas *et al.*, 2006).

Strain AK41^T was grown on modified Pfennig medium with 2% NaCl at 30 °C under 2000 lux illumination anaerobically for 3 days to prepare fatty acid methyl esters (classical methods) and were analysed using Sherlock Microbial Identification System (MIDI-6890 with database TSBA50) by the protocol described by the manufacturer. Freeze-dried cells following growth were analysed for polar lipids and quinones. Cells were extracted for the polar lipid analysis (Bligh & Dyer, 1959) and analyzed by 2D thin-layer chromatography followed by spraying with appropriate detection reagents (5 % ethanolic molybdatophosphoric acid, molybdenum blue, ninhydrin and Molisch reagents) (Komagata & Suzuki, 1987). Extracted isoprenoid quinones (Collins *et al.*, 1977) analyzed by high performance liquid chromatography (Groth *et al.*, 1997). *In vivo* absorption spectra were measured

with a Spectronic Genesys 2 spectrophotometer in sucrose solution (Trüper & Pfennig, 1981). Absorption spectra of pigments extracted with acetone were also recorded.

DNA G+C content and 16S rRNA gene sequence analysis were determined as previously described in Srinivas *et al.* (2013). The resultant sequence of the 16S rRNA gene (1428 bp) was subjected to EzBioCloud (Kim *et al.*, 2012) to identify the nearest taxa. To reveal the exact phylogenetic position of strain in the domain *Bacteria*, the 16S rRNA gene sequence was aligned against an ARB dataset using the ARB program package (Ludwig *et al.*, 2004). 16S rRNA gene sequences of the closely related members of the genus *Rhodovulum* were downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov>) and aligned using the CLUSTAL_W program within MEGA5 package (Tamura *et al.*, 2011). Phylogenetic trees were constructed using tree-making algorithms, the neighbor joining and maximum likelihood methods using the MEGA5 package (Tamura *et al.*, 2011) and the resultant tree topologies were evaluated based on 1000 resamplings.

Colonies were circular, smooth, brown, shiny, opaque and flat with entire margin on modified Pfennig medium. Transmission electron microscopy revealed the cells were ovoid to rod shaped with cell diameter of 0.5-1.0 μm (Fig. 1), non-motile and multiplied by binary fission. Electron microphotographs of ultrathin sections of the cells revealed vesicular internal membranes (Supplementary Fig. S4). The Sudan black staining confirmed the presence of lipophilic granules but not Polyhydroxyalkanoates as the Nile blue stained cells did not fluorescence at an excitation wavelength of 460 nm.

Strain AK41^T grows photoorganoheterotrophically (optimum light intensity is 2000 lux) and chemoorganoheterotrophically (aerobically in the dark). Strain can grow at the atmospheric level of oxygen. Photolithoautotrophic growth [anaerobic, light (2000 lux), Na₂S, Na₂S₂O₃ (0.5 mM), NaHCO₃ (0.1%, w/v)], chemolithoautotrophic growth [aerobic, dark, thiosulfate (0.5 mM), NaHCO₃ (0.1%, w/v)] and fermentative growth (anaerobic, dark with glucose/pyruvate (0.3%, w/v)) were not detected. Salt (NaCl) was not required for growth, but tolerates up to 12% (w/v, NaCl), the optimum NaCl concentration being 1–9% (w/v). The pH range for growth of strain AK41^T was 5.0–9.0 with the optimum at 7.0–8.0. The temperature range was from 25 to 38 °C and the optimum was at 28 °C. No growth factors are required for growth. The colour of photosynthetically grown cell suspensions was brown. The whole-cell absorption spectrum (Supplementary Fig. S2a) of strain AK41^T in sucrose solution gave maxima at 379, 462, 592, 803, 855 nm, confirming the presence of bacteriochlorophyll *a*. The absorption spectrum of acetone extracted pigments (Supplementary Fig. S2b) gave maxima at 456, 486 nm, indicating the presence of the carotenoids. The carotenoid composition of strain

AK41^T, as determined by usnig C₁₈-HPLC analysis, was neurosporene (3%), demethylspheroidene (43%), spheroidene (49%), OH-spheroidene (1%), spheroidenone (4%). They were identified based on absorption spectra in the C₁₈-HPLC eluent of methanol, the retention times on the C₁₈-HPLC, and the relative molecular masses of major three carotenoids (Takaichi & Shimada 1992). Other physiological characteristics are given in the species description and Table 1.

The cellular fatty acid composition of strain AK41^T showed a profile of seven fatty acids of C_{10:0}3OH (2.1), C_{16:0} (21.2), C_{17:1} ω8c (2.0), C_{18:0} (4.3), C_{18:1} ω7c (68.3), C_{12:0} aldehyde/iso-C_{16:1}I/C_{14:0}3OH (1.0), and C_{16:1} ω7c/iso-C_{15:0}2OH (1.1). Unsaturated fatty acids constituted 70.3 to 71.4%. The major polar lipids consisted of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), one unidentified aminolipid (AL) and four unidentified lipids (L) (Supplementary Fig. S3). Q-10 is the predominant respiratory quinone. The polar lipids and quinones are in line with the genus *Rhodovulum* and nearest genus *Rhodobacter* (Imhoff, 1984; 1991). The DNA base composition of strain AK41^T was 68.9 mol% G+C (*Tm*).

For phylogenetic assessment of strain AK41^T, 1428 bp of 16S rRNA gene sequence was amplified and sequenced. The sequence was compared with the database of type strains with validly published prokaryotic names [Available online <http://eztaxon-e.ezbiocloud.net/> (Kim *et al.*, 2012)], which revealed its closeness to *Rhodovulum* genus of the *Alphaproteobacteria*. Based on pair-wise sequence similarity, strain AK41^T was closest to *Rhodovulum sulfidophilum* with 96.0% similarity and to other members of the genus *Rhodovulum* with 95.8 to 93.9% similarities. Phylogenetic tree based on neighbour joining method showed the clustering of strain AK41^T with *Rhodovulum marinum*, *Rhodovulum kholense*, *Rhodovulum sulfidophilum*, and *Rhodovulum visakhapatnamense* with a phylogenetic similarity of 95.9 to 96.2% (Fig. 2). In maximum likelihood phylogenetic tree also strain AK41^T clustered with *Rhodovulum marinum* and together clustered with *Rhodovulum kholense*, *Rhodovulum sulfidophilum*, and *Rhodovulum visakhapatnamense* (Supplementary Fig. S1).

Based on the phylogenetic analysis a comparison was made between the characteristics of strain AK41^T with the type strains of the genus *Rhodovulum* (*Rhodovulum sulfidophilum* DSM1374^T, *Rhodovulum kholense* JA297^T, *Rhodovulum marinum* JA128^T and *Rhodovulum visakhapatnamense* JA181^T), of the family *Rhodobacteraceae* (Table 1) and a number of differences were observed. For instance, strain AK41^T differed from the type strains with respect to cell size, cell shape, motility, colour of cell suspension, NaCl range and optimum, temperature growth range and optimum, pH growth range and optimum, photoautotrophic growth, vitamin requirement, carbon substrate utilization, nitrogen sources, and G + C content (Table 1). Thus, the cumulative differences that

strain AK41^T exhibits with the above closely related type strains unambiguously supports the creation of a new species of the genus *Rhodovulum* for which the name *Rhodovulum mangrovi* sp. nov. is proposed.

Description of *Rhodovulum mangrovi* sp. nov.

Rhodovulum mangrovi (man.gro'vi. N.L. gen.n. mangrovi, of or belonging to mangrove).

Cells are Gram-stain-negative, ovoid to rod-shaped, 0.5–1.0 µm diameter, non-motile and division by binary fission. Growth occurs under anaerobic conditions in the light (photoorganoheterotrophy) or under aerobic conditions in the dark (chemoorganoheterotrophy). Internal photosynthetic membranes are of vesicular type and also contains lipophilic granules as storage material. The colour of phototrophic cultures is brown, while aerobic cultures are pink. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 379, 462, 592, 803, 855 nm. The absorption spectrum in acetone extracted pigments gives absorption maximum at 456 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series. The type strain is mesophilic (range 25–38 °C, optimum 28 °C), can grow both acidic and alkaline conditions (pH range 5.0–9.0, optimum 7.0–8.0) and does not required NaCl for growth but can tolerate concentrations of NaCl up to 12% w/v with optimum growth between 1-9% NaCl. Photoorganoheterotrophy with various organic compounds is the preferred growth mode. Substrates that are utilized as carbon source/electron donor under photoheterotrophic condition include acetate, butyrate, crotonate, fumarate, glucose, glycerol, propionate, pyruvate, succinate, valerate and yeast extract. Casamino acids, citrate, ethanol, fructose, glutamate, glycolate, malate, methanol, sucrose and trehalose are not utilized under photoheterotrophic condition. Photoautotrophic and chemoautotrophic growth is not possible in the presence of sulfide and thiosulfate as electron donor and NaHCO₃ as carbon source. Fermentative growth is not possible under anaerobic dark conditions. Ammonium chloride and yeast extract are utilized as nitrogen source but not nitrate, nitrite, glutamine and glutamate. Thiosulfate is utilized as sulfur source under photoheterotrophic condition but not sulfide and sulfur. Ferrous iron cannot act as electron donor. There is no vitamin requirement. The dominant fatty acids are C_{16:0}, C_{18:1 ω7c}. The polar lipids consist of diphosphatidylglycerol, phosphatidylethanolamine, one unidentified aminolipid and four unidentified lipids. Q-10 is the predominant respiratory quinone. DNA G+C base composition is 68.9 mol% (*Tm*).

The type strain AK41^T (= MTCC 11825^T = JCM 19220^T) was isolated from a sediment sample collected from Coringa mangrove forest, Andhra Pradesh, India. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AK41^T is HG529993.

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Figure Legends:

Fig. 1. Electron micrograph of negatively stained cells of strain AK41^T showing lipophilic granules.

Bar, 0.2 μm.

Fig. 2. Neighbor-joining tree based on 16S rRNA gene sequences representing the phylogenetic relationships between the strain AK41^T and the related type strains obtained from the database of type strains with validly published prokaryotic names at EzTaxon-e identification service. Numbers at the nodes are bootstrap values >70%. *Rhodospirillum sulfurexigens* JA143^T (AM710622) was taken as the out group organism. The scale bar corresponds to the expected number of changes per nucleotide position.

Supplementary Fig. S1. Maximum likelihood tree based on 16S rRNA gene sequences representing the phylogenetic relationships between the strain AK41^T and the related type strains obtained from the database of type strains with validly published prokaryotic names at EzTaxon-e identification service. *Rhodospirillum sulfurexigens* JA143^T (AM710622) was taken as the out group organism. The scale bar corresponds to the expected number of changes per nucleotide position.

Supplementary Fig. S2. Whole-cell absorption spectrum (a) of strain AK41^T and absorption spectrum (b) of acetone extracted pigments.

Supplementary Fig. S3. The polar lipid profile of strain AK41^T. The polar lipids were identified as follows: phosphatidylglycerol (PG), phosphatidylethanolamine (PE), unidentified aminolipid (AL), unidentified lipid (L).

Supplementary Fig. S4. Electron micrograph of an ultrathin section of cells of strain AK41^T showing the vesicular nature of intracytoplasmic membranes.

Table 1 Phenotypic features that distinguish strain AK41^T with the closely related type strains of *Rhodovulum* species

Taxa: 1, stain AK41^T; 2, *Rdv. sulfidophilum* DSM1374^T; 3, *Rdv. kholense* JA297^T; 4, *Rdv. marinum* JA128^T; 5, *Rdv. visakhapatnamense* JA181^T. All the strains grow photoheterotrophically and chemoheterotrophically, aerobic, anaerobic, Gram-stain-negative. All strains utilise D-glucose, fumarate, pyruvate, succinate and yeast extract but not citrate, glycolate and methanol. All strains require NH₄Cl as nitrogen source. Data is from the present study. C, chains; O, ovoid; R, rod-shaped; B, brown; YB, yellowish brown; RB, reddish brown; b, biotin; n, nicotinic acid; paba, *p*-aminobenzoic acid; t, thiamine; +, positive; -, negative

Characteristic	1	2	3	4	5
Cell diameter (µm)	0.5-1.0	0.6-1.0	0.5-1.0	0.5-0.6	0.9-1.2
Cell shape	O to R	O to R	O to R	O to R, C	O to R
Motility	-	+	+	-	-
Colour of cell suspension	B	RB	YB	YB	YB
NaCl range (%)	0-12	0-10	0.05-6	0.05-8	0-10
NaCl optimum (%)	1-9	1-6	1-4	0.5-3	1-6
pH range	5.0-9.0	5.0-9.0	6.0-8.0	5.5-7.5	4.0-9.0
pH optimum	7.0-8.0	6.5-7.5	6.0-7.0	6.0-6.8	6.0-8.0
Temperature range (°C)	25-38	25-40	20-35	25-35	20-35
Temperature optimum (°C)	28	25	30	30	30
Photoautotrophy	-	-	+	-	+
Vitamin requirement	None	b, n, paba, t	b, n, t	t	b, n, paba, t
DNA G+C content (mol%)	68.9	66.5	63	62	61.2
<i>Carbon/electron donors</i>					
Sulfide	-	+	+	-	-
Thiosulfate	+	+	+	-	+
Sulfur	-	+	-	-	-
Ferrous iron	-	+	-	-	-
Propionate	+	+	-	-	+
Butyrate	+	+	+	-	+
Valerate	+	+	+	-	+
Ethanol	-	-	-	+	-
Malate	-	+	+	-	+
Glutamate	-	+	+	-	+
Fructose	-	-	+	+	+
Glycerol	+	+	-	+	+
Citrate	-	-	-	-	-
Methanol	-	-	-	-	-
Malate	-	+	+	-	+
Crotonate	+	+	-	-	-
Acetate	+	+	-	-	-
Casamino acids	-	-	+	+	+
Sucrose	-	+	-	-	-
Glycolate	-	-	-	-	-
<i>Nitrogen Sources</i>					
Nitrate	-	-	-	+	-
Nitrite	-	-	-	-	-
Glutamine	-	-	+	+	+
Glutamate	-	+	+	+	+

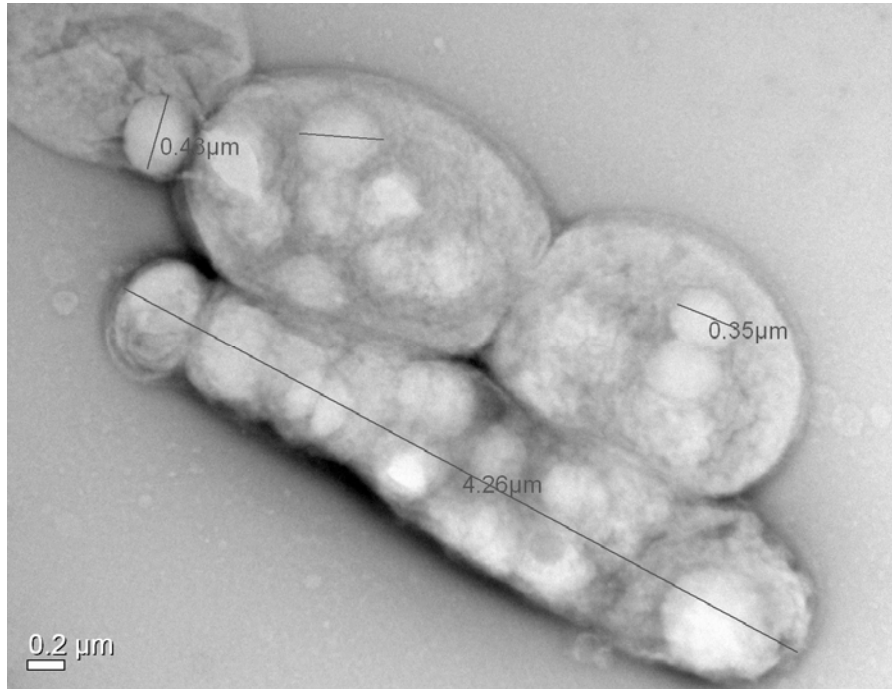


Fig. 1

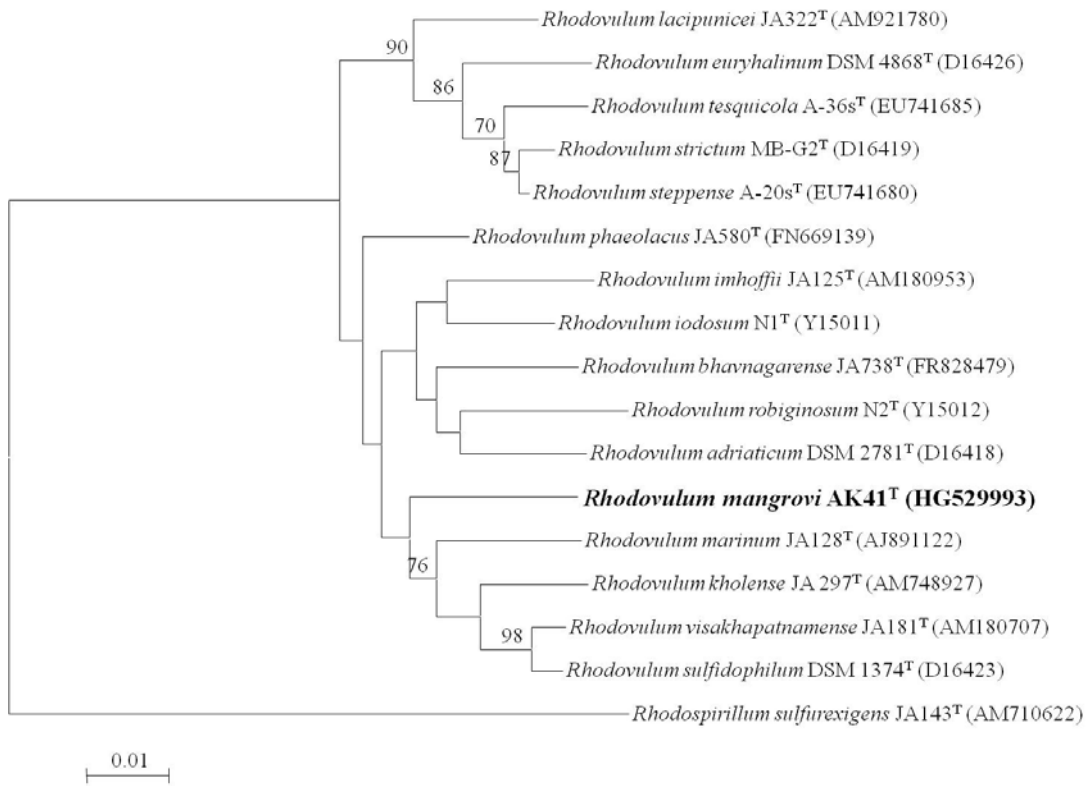
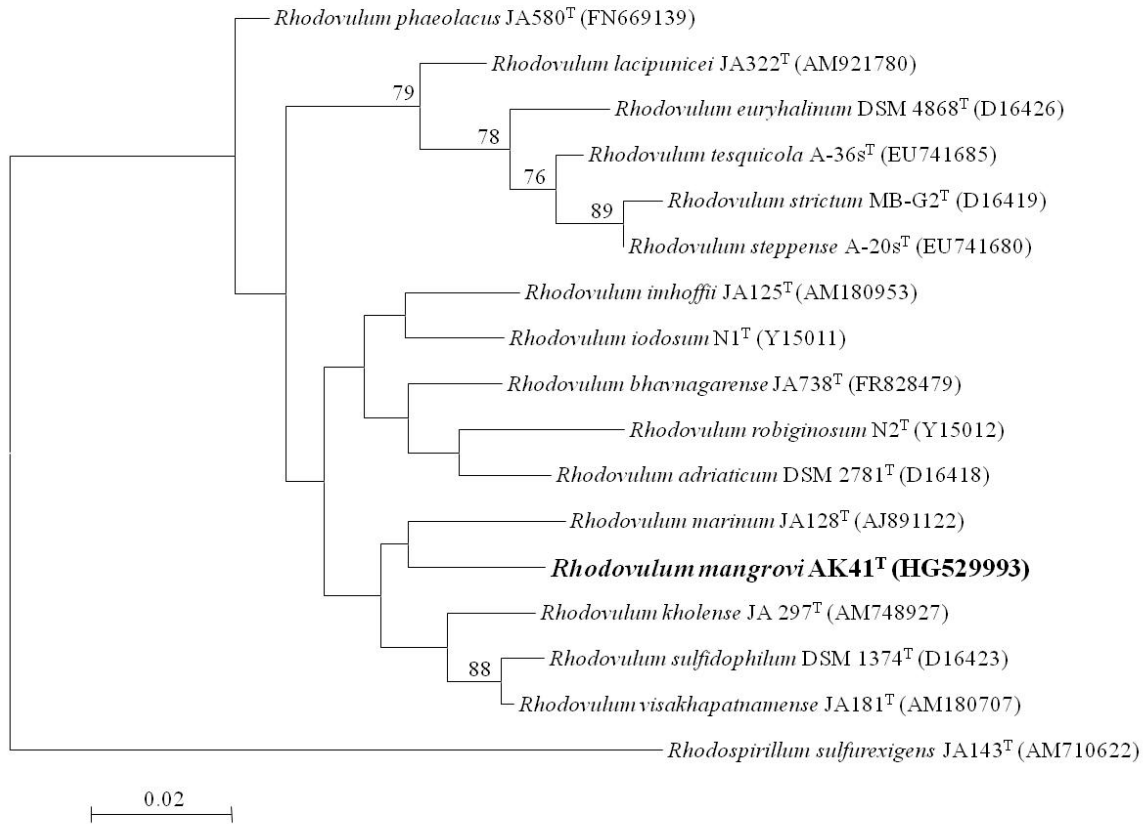
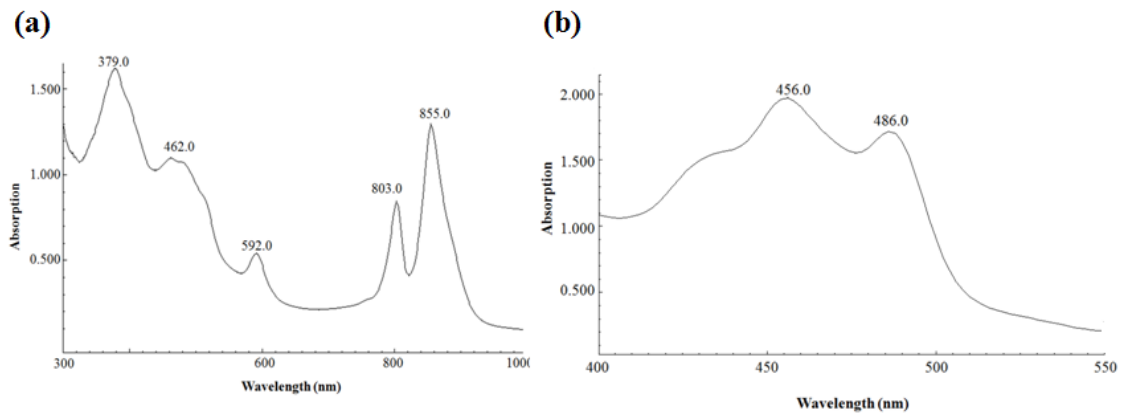


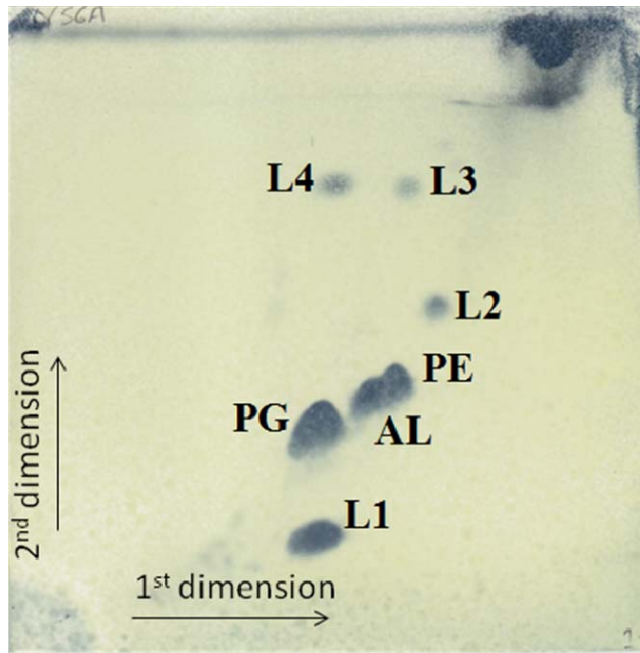
Fig. 2



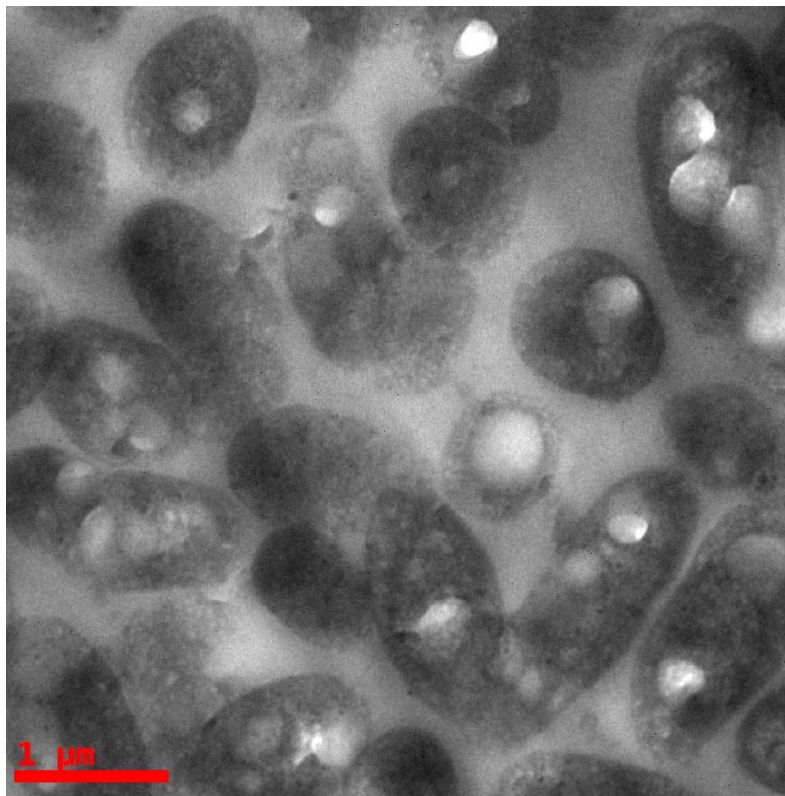
Supplementary Fig. S1



Supplementary Fig. S2



Supplementary Fig. S3



Supplementary Fig. S4