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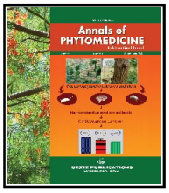

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Diversity of anoxygenic phototrophic purple bacteria from India

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Abstract

The photosynthetic purple bacteria are typical anoxygenic photosynthetic bacteria and one of the suitable microorganisms showing various biotechnological applications. In this study, we made an attempt to report the diversity of purple bacteria from various habitats of India. Among the phototrophic purple bacteria, purple non-sulfur group (PNSB) were dominated in various geographical locations. Most of the strains are isolated from water sample, soil mat, sand and an estuarine soil associated with xerophytes plant. The phototrophically grown cultures had shown distinct variation in color varying from yellow-brown, green, brown-red, to pink. Whole cell absorption spectrum of cultures revealed the presence of BCHL-a and carotenoid pigments. From the present study, total thirty isolates were isolated from enrichments and purified. Among them, fourteen isolates are tentatively identified based on their colour, microscopic observation and colony morphology are belonged to the genera, *Rhodobacter, Rhodopseudomonas, Marichromatium* and *Allochromatium*. Sixteen isolates were isolated, purified and identified through 16S rRNA gene sequencing analysis and affiliated to the genera, *Rhodobacter, Rhodovulum, Rhopseudomonas, Rhodobium, Rhodospirillum, Afifella, Marichromatium* and *Allochromatium*. *Rhodobacter* and *Rhodovulum* are frequently isolated among PNSB. Marichromatium and *Allochromatium* were the only genera belonging to purple sulfur bacteria were isolated. Phylogenetic analysis based on 16S rRNA gene sequence showed all the strains to cluster to their nearest phylogenetic neighbours and are diverse among themselves.

1. Introduction

Anoxygenic phototrophic purple bacteria are gram-negative prokaryotes which perform anoxygenic photosynthesis using more redox compounds like sulfide, elemental sulfur, thiosulfate, and sulfate without evolving oxygen as an end product. Purple sulfur bacterium (PSB) varies from purple non-sulfur bacteria (PNSB) in their metabolism and in phylogenetic relationship, but species belonging to this groups habitually occur in highly illuminated anoxic environments. PSB posses strong photoautotrophic growth mode and have the ability to exist in photoheterotrophy, limited growth in dark condition. Whereas, PNSB are photoheterotrophs by nature, limited photoautotrophy, and have various capabilities to grow in dark conditions. Occurrence of purple bacteria in extreme environments such as high sulfide concentrations, high temperature, pH, and salinity is well studied (Madigan *et al*., 2009). Members of purple bacteria belong to the alpha-*Proteobacteria*, beta-*Proteobacteria*, and gamma-*Proteobacteria*. Most of the known species from purple bacteria (>160 species) are classified into 57 genera, 12 families, and 7 orders of the *Proteobacteria* (Hallenbeck *et al*., 2017). The present communication focus on the studies of culturable anoxygenic phototrophic PSB and PNSB , their dominance and hidden diversity among themselves from various habitats.

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2. Materials and Methods

Random sampling was done which are unique with respect to their geographical positions and habitats such as sea coastal area, estuarine, marine water, beach sand, industrially polluted fresh water lakes in India. Total one hundred and forty five (145) samples were collected with sample types like sediment, water, algae, starfish, corals, soil, microbial mat, dessert sand, xerophyte plant soil. Sample pH and salinity was noted down while collection and it ranges from pH 6 - 8 and 0.8-1 %(w/v).

Enrichments, isolation and identification through 16S rRNA gene sequence analysis were performed at author's laboratory.

2.1 Enrichment

Enrichment method was employed to isolate PNSB and PSB due to their limited abundance when compared to total bacterial populations in the natural environment. Samples were subjected for enrichment of purple sulfur and non-sulfur bacteria, in a fully filled screw cap bottles (45 ml) inoculated with approximately 0.5 g or 0.5 ml of the samples. Modified bieble and pfennigs medium was used for enrichment of purple non-sulfur bacteria (PNSB) with 0.5 mM sufide concentration and a mineral medium with different combinations including high salinity more than optimum concentration 2%, high pH, and high concentration of sulfide or gradual increase from optimum to 10 mM and replacing organic substrates with malate and benzamide (2 mM) were employed and for purple sulfur bacteria (PSB) 1 mM sulfide was used in the basal medium. All the inoculated screw capped bottles were incubated under light (2,400 lux) at 28°C ± 1 under anaerobic conditions.

2.2 Isolation and purification

Phototrophically grown enrichment cultures of purple bacteria (purple sulfur and purple non-sulfur bacteria) were purified by repetitive streaking on agar slants (using respective media on which they were enriched) and maintaining strict anaerobic conditions with argon flush. Purity of the cultures was evaluated by streaking onto nutrient agar (Difco Manual, 1998; $g.l^{-1}$: peptone-5, yeast extract-3 and agar-15) plates and incubating under aerobic conditions at 28°C ± 1 (Chandel *et al*., 2021). Contamination from other chemotrophic bacteria was checked by monitoring the cultural characters like color of the culture, colony morphology and microscopic observation. Purified cultures were grown in completely filled screw cap test tubes under phototrophic conditions, as described previously (Lakshmi *et al*., 2011). A modified Biebl and Pfennig's medium (Srinivas *et al*., 2014) was used for purification and maintenance of the cultures. The medium contained ($g.l^{-1}$): $CaCl_2.2H_2O$ (0.05), $MgSO_4.7H_2O$ (0.2), yeast extract (0.3), NaCl (0.4), KH_2PO_4 (0.5), NH_4Cl (0.6), sodium pyruvate (3.0), ferric citrate (5 ml l^{-1} from a 0.1% w/v stock) and trace element solution SL 7 (1 ml. l^{-1}). SL 7 contained (mg l^{-1}): $CoCl_2.6H_2O$ (200), $MnCl_2.4H_2O$ (100), $NaMoO_4.2H_2O$ (40), HCl (25% v/v; 1 ml), $ZnCl_2$ (70), H_3BO_3 (60), $CuCl_2.H_2O$ (20) and $NiCl_2.6H_2O$ (20).

Morphological characteristics such as cell shape, division, size, aggregate formation and motility were observed under phase contrast microscope (Olympus BH-2).Whole cell absorption maxima of isolates for pigments (Meenakshi *et al*., 2018) was measured by UV-visible spectrophotometer by the sucrose method of Pfennig and Trüper (1981). 5 g of sucrose was added to broth culture, approximately 3.5 ml and mixed vigoursly on a vortexer and heat treatment can be done as per need. The absorption spectrum wave length from 300-1100 nm was measured on a Spectronic Genesys 2 spectrophotometer using sucrose as a blank in the medium.

2.3 Identification

A commercially available DNA extraction kit (Nucleopore gDNA Fungal Bacterial Mini Kit) was used to extract the genomic DNA used for 16S rRNA amplification. The 16S rRNA gene amplification and sequencing were performed as described previously (Subhash *et al*., 2013). 16S rRNA gene sequence similarities of the strains were identified by BLAST search on the EZTAXON BioCloud database (Yoon *et al*., 2017). The MUSCLE program in MEGA6 software (Tamura *et al*., 2013) was used for multiple sequence alignment and distances were calculated by using the Kimura two-parameter model (Kimura *et al*., 1980) in a pair-wise deletion manner. Neighbor-joining (NJ) method in the MEGA6 software was used to build the phylogenetic trees.

3. Results

Enrichment cultures of phototrophic purple bacteria has yielded fifty eight (58) positive for purple sulfur (35) and purple non-sulfur bacteria (23) from a photoheterotrophic medium under anaerobic conditions. Thirty (30) isolates were purified after repeated streaking on agar slants with desired medium under anaerobic conditions. Well-defined colonies were obtained with distinct color variation from yellow-brown, green, brown-red to pink. The color differences formed the basis for strain designations and numbers were allotted for individual isolates. Pure cultures were tentatively identified based on colour of cell suspension, microscopic observation of cell morphology and also based on 16S rRNA gene sequence similarity percentage. Three isolates were yellow-brown (JA826, Figure 1a), JA816 and JA967), dark brown (JA825), green, (JA814, Figure 1b), JA824) reddish brown, (JA965, JA970, JA817, JA822, JA823) brown(JA966, JA971, JA821), and pink (JA968 Figure1c) JA969). Strains which are yellow brown when exposed to air turns to reddish brown in colour. Whole cell absorption maxima (300-1100 nm) reveals the presence of bacteriochlorophyll- *a* for all purple bacterial strains. The absorption spectrum for yellow brown strains pigment extracted with acetone showed absorption maxima at 430, 450, and 489 nm indicates the presence of spheroidenone series of carotenoids. For green coloured strain, the absorption maxima in acetone was 441, 471 and 556 nm, indicating the presence of neurosporene series carotenoids. Pink coloured strains have absorption maxima at 377, 415, and 587 nm, indicates the spirilloxanthin series of carotenoids. Well isolated colonies of PSB and PNSB under anaerobic conditions had shown a variety of colony morphologies on agar slants after a week of incubation. Green colour isolates were initially small, convex, round, moist, and reached to medium sized over a period of time. Yellow brown pigmented colonies were big, round, sticky, convex. Pink pigmented colonies of isolates were small, round, convex, moist and smooth. 14 strains of PNSB were oval to rod shaped, motile and form aggregates. Cell division is by binary fission. One strain dark brown in colour is spiral shaped, highly motile, spiral chains formation and binary fission mode of cell division. Another strain pink colored is oval to mostly rod shaped, motile, cell division is by budding. 16 strains of PSB were rod shaped, big, and motile and multiplied by binary fission. Sulfur granules were deposited inside the cell of all strains. Out of 30 strains of all purple bacterial strains sixteen, were sequenced with 16S rRNA gene and the similarities were observed in EZTAXON biocloud e server (Table 1). The result hits have shown highest pair wise sequence similarities with the type strains of the species belonging to the genus *Rhodovulum* (2), *Rhodobacter* (3), *Rhodopseudomonas* (1), *Rhodospirillum* (1), *Afifella* (1), *Rhodobium* (1) of *alpha proteobacteria* and *Marichromatium* (5), *Allochromatium* (2), of *Gamma proteobacteria.*

(a) (b) (c)

Figure 1: Broth cultures of (a) *Rhodobacter* sp. JA826, (b) *Rhodovulum* sp. JA814, (c) *Afifella* sp. JA968.

Both PSB and PNSB obtained were tentatively identified based on their easily recognizable morphological attributes like cell shape, cell dimensions, motility, mode of cell division, colour of cell suspension and pigments. Among 30 pure isolates obtained, 14 were tentatively identified upto the genus level based on phenotypic characteristics

and microscopic observation. Among 14 purple bacteria, 6 PSB isolates which are reddish brown in cell suspension, long, stout rod shaped, highly motile and divided by binary fission was tentatively identified to be belonged to the genus *Marichromatium* and the other 3 strains were of reddish brown in pigmentation, short and stout rods, divided by binary fission were affiliated to the genus *Allochromatium*. PNSB isolates which are tentatively identified as *Rhodobacter* sp. (4) and *Rhodopseudomonas* sp. (1). Both of the isolates belonged to the genus are rod shaped, motile and cell suspension is yellow brown (*Rba*.) and deep red (*Rps*.) in colour. Clear visibility of sulfur deposition as granules (4 to 5) has been observed during the early growth phase.

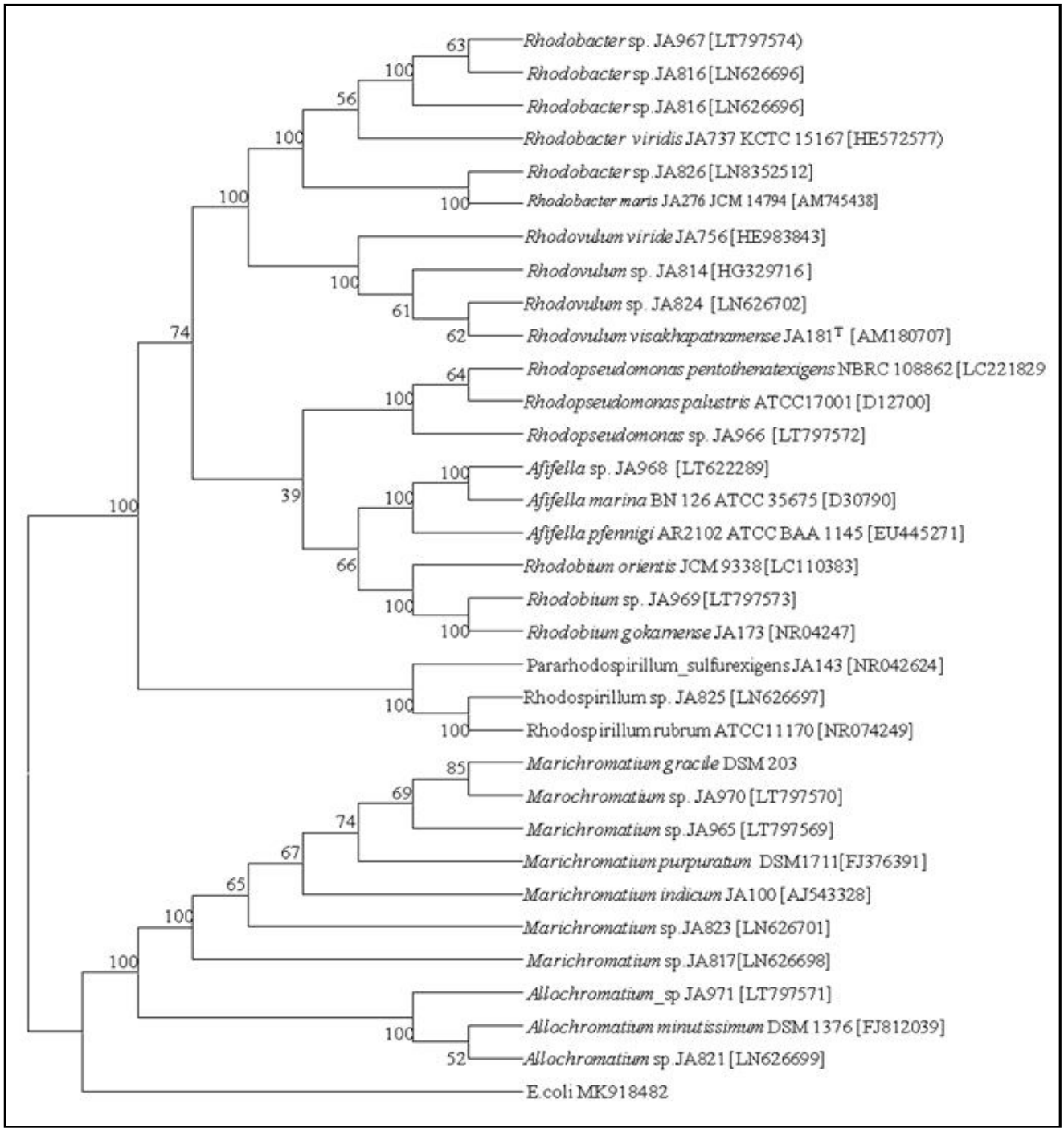

Figure 2: Phlyogenetictree computed by using MEGA6 software based on 16S rRNA gene sequences showing relationship of strains with the members of their respective genera. The GenBank accession numbers for 16S rRNA gene sequences are shown.

Out of 30 strains of all purple bacterial strains, sixteen (16) were sequenced with 16S rRNA gene and the similarities were observed in EZTAXON biocloud e server. The result hits have shown highest pair wise sequence similarities with the type strains of the species belonging to the genus *Rhodovulum* (2), *Rhodobacter* (3), *Rhodopseudomonas* (1), *Rhodospirillum* (1), *Afifella* (1), *Rhodobium* (1) of Alpha-*Proteobacteria* and *Marichromatium* (5), *Allochromatium* (2), of gamma-*Proteobacteria***.** All the 16 strains were assigned with a strain number and 16S rRNA gene sequences were deposited in Genbank/EMBL/DDBJ. The accession numbers were assigned to the respective strains.

Table 1: Isolation of phototrophic purple bacterial strains from diverse habitats of India

	Sample details				Strain details			
S.No	**Sample location**	**Sample type**	**pH**	**GPS coordinates**	**Strain No.**	**Strain colour**	**16S rRNA gene sequence similarity (%) with nearest typestrain (Ex-taxon result)**	**EMBL accession number**
1	Ramnad, Tamilnadu	Algae	5	N 9˚31’ E 79˚0’	JA814	Green	100.0% - *Rhv. viride* JA756^T	HG329716
2	Hubli, Karnataka	Sediment	7	N15˚26’ E75˚20’	JA826	Yellow-brown	98.5% - *Rba.maris* JA276^T	LN835251
3	Ramnad,Tamilnadu	Water	7	N 9˚28’ E 79˚05’	JA816	Yellow-brown	100% -*Rba.megalophilus* JA194^T	LN626696
4	Runn of kutch, Gujarat	Water	8	N22˚48’ E70˚03’	JA824	Yellow-green	99.7% - *Rhv.visakhapat namense* JA181^T	LN626702
5	Ramnad, Tamilnadu	Soil mat	5	N 9˚31’ E 79 ˚0’	JA825	Dark brown	100% - *Rhodospirillum sulfurexigens* JA143^T	LN626697
6	Kerala	Brown sediment	7	N 9˚49’ E 76˚31’	JA966	Pink	99.2% - *Rhodopseudomonas pentothenateexigens* JA575^T	LT797572
7	Orissa	lemma	7	20.25N 85.77E	JA967	Yellow-brown	100% - *Rba. Johrii* JA192^T	LT797574
8	Gujarat	Xeropyte plant soil	7	N 21˚48’ E 69˚93’	JA968	Pink	99.6%-*Afifella marina* DSM2698^T	LT622289
9	Huda beach, Gujarat	Water	8	N 21˚5’ E 72˚3	JA969	Pink	99.8%-*Rhodobium gokarnense* JA173^T	LT797573
10	Khavda dessert, Gujarat	Water	8	N22˚48’ E70˚03’	JA817	Reddish brown	99.8%-*Marichromatium indicum* JA100^T	LN626698
11	Ramnad, Tamilnadu	Sediment	8	N 9˚ 31’ E 79˚ 0	JA821	Red-brown	100%-*Allochromatium phaeobacterium* JA144^T	LN626699
12	Runn of kutch, Gujarat	Algal mat	7	N 23˚68’ E 68˚52’	JA822	Reddish brown	99.87%-*Marichromatium indicum* JA100^T	LN626700
13	Runn of kutch, Gujarat	Brown salt	8	N 22˚48’ E 70˚03’	JA823	Pale brown	100%-*Marichromatium indicum* JA100^T	LN626701
14	Gudur, AP	Sediment	7	N 14˚16’ E 79˚79’	JA971	Reddish brown	99.5% - *Allochromatium minnitissumum* DSM1376^T	LT797571
15	Runn of kutch, Gujarat	Brown mat soil	6	N 21˚62’ E 72˚28’	JA965	Brown	100%-*Marichromatium gracile* DSM203^T	LT797569
16	Orissa	Sand	7	21.49E 86.91N	JA970	Brown	100%-*Marichromatium gracile* DSM203^T	LT797570

Abbreviations: N, north; E, east. Temperature for all the samples was close to 30°C.

4. Discussion

The present study on the photosynthetic purple bacterial diversity from different habitats had revealed that the isolates of PNSB obtained from the enrichments were belonged to class alpha-*Proteobacteria* and genera of *Rhodopseudomonas*, *Rhodobium*, *Rhodospirillum*, *Afifella*, *Rhodobacter* and *Rhodovulum*. The genera *Rhodovulum* and *Rhodobacter* are found to be predominant as the conditions of the growth, oxygen requirement and medium composition in minimal concentrations of nutrients had favored them to grow and dominate over other genera in their number. Both the genera have a very flexible mode of growth and facultative nature in terms of oxygen requirements and fast growing ability. In the same

way isolates of PSB were belonged to class gamma-*Proteobacteria* and genera *Marichromatium* and *Allochromatium*. The genus *Marichromatium* is dominant among the isolates, followed by *Allochromatium*. These members need obligate requirement of anoxygenic conditions and more concentration of sulfide than required for purple non-sulfur bacterial members. The limited genera of PSB are isolated in the present study, may be due to the artificial medium and incubation conditions. Presently, only six validly published species names were reported from this genus and diversity studies are limited. Hence, isolates of this genus can be exploited for various applications as they are versatile photoheterotrophs and acts as a good probiotic in aquaculture (Cui *et al*., 2021)

5. Conclusion

Most of the isolates resulted from present study are purple non-sulfur bacterial group and very few are purple sulfur bacteria. PNSB members belonged to class alpha-*Proteobacteria* were frequently isolated from different habitats are six genera namely *Rhodopseudo monas*, *Rhodobium*, *Rhodospirillum*, *Afifella*, *Rhodobacter* and *Rhodovulum*. The genera *Rhodovulum* and *Rhodobacter* are found to be predominant and easily cultivated. PSB isolates obtained were affiliated and limited to only two genera *Marichromatium* and *Allochromatium*. Thirty (30) pure isolates had yielded from the present study, of which sixteen (16) strains were sequenced with 16S rRNA gene and phylogenetic analysis revealed the strains were affiliated to their respective genera. Fourteen (14) isolates were tentatively identified based on rapid typing. These strains can be further studied for various biotechnological (Kiruthiga et *al*., 2021), therapeutical applications (Shamna *et al*., 2021; Nath *et al*., 2021) and also assess their industrial exploitation.

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Conflicts of interest

The authors declare that no conflicts of interest relevant to this article.

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