2. DIVERSITY OF PNSB IN SHRIMP PONDS

2.1 INTRODUCTION

2.1.1 Purple photosynthetic bacteria

Anoxygenic phototrophic purple bacteria are a major group of phototrophic microorganisms that inhabit aquatic and terrestrial environments. Purple photosynthetic bacteria are gram-negative prokaryotes that convert light energy into chemical energy by the process of anoxygenic photosynthesis. Purple bacteria contain photosynthetic pigments like bacteriochlorophylls and carotenoids and can grow autotrophically with CO$_2$ as sole carbon source. Purple bacteria share with oxygenic phototrophic prokaryotes, the cyanobacteria, the ability to conserve energy by photophosphorylation. Photosynthesis in purple bacteria only occurs under anoxic (O$_2$-free) conditions. This is also true of the other classical anoxygenic phototrophs: green sulfur bacteria, green nonsulfur bacteria, and the heliobacteria (Imhoff, 1995).

Purple bacteria require anoxic conditions for phototrophic growth because pigment synthesis in these organisms is repressed by molecular oxygen (Cohen-Bazire et al., 1957). Thus, the competitive success of purple bacteria in nature requires both light and anoxic conditions. This combination is most commonly found in lakes, ponds, estuaries, and other aquatic environments where H$_2$S is present (Pfennig, 1967; 1978; 1989). Sulfide concentration, pH, light intensity and temperature, controls the abundance and diversity of purple bacteria that develop there (Madigan, 1988). In certain habitats which are particularly favorable for their development, purple bacteria have been shown to be significant primary producers (Czeczuga, 1968; Takahashi and Ichimura, 1968; Overmann et al., 1994; 1996; 1999).
Purple bacteria participate in the anoxic cycling of carbon both as primary producers (CO₂ fixation, photoautotrophy) and as light-stimulated consumers of reduced organic compounds (photo heterotrophy).

2.1.2 Habitats of purple non sulfur bacteria (PNSB)

Representatives of the purple nonsulfur bacteria (PNSB) are widely distributed in nature and are found in all kinds of stagnant water bodies, like lakes, waste water ponds, coastal lagoons, and in other aquatic habitats, and also in sediments, moist soils, and paddy fields. They live in aquatic habitats with significant amounts of soluble organic matter and low oxygen tension, but rarely form colored blooms, like those of purple sulfur bacteria. However, often they are found accompanying the purple sulfur bacteria in stratified environments. They have been found not only in freshwater, marine and hypersaline environments, and most frequently in habitats of moderate temperatures, but also in thermal springs and in cold polar habitats (Imhoff, 2006). Some purple non sulfur bacteria also occur in acidic, boggy waters and soils, which grows optimally at pH 5.5– 5.8 (Pfennig, 1969).

Ecological niches of phototrophic α-Proteobacteria are those anoxic parts of water and sediments that receive light of sufficient quantity and quality to allow phototrophic development. Purple non sulfur bacteria occasionally form dense blooms in habitats where levels of sulfide are either low or undetectable. Purple nonsulfur bacteria are usually present in only low numbers in blooms of purple sulfur bacteria, probably because of their sulfide sensitivity. Purple nonsulfur bacteria are present also in sewage (Holm and Vennes, 1970; Siefert et al., 1978) and waste water lagoons which offers excellent conditions for growth of purple nonsulfur bacteria (Jones, 1956; Cooper et al.,
Purple non sulfur bacteria are found in various marine habitats like mangroves (Vethanayagam, 1991), mud flats (Hansen and Veldkamp, 1973; Hiraishi and Ueda, 1995), salt pans (Kawasaki et al., 1993a; Sørensen et al., 2005), aquaculture ponds (Bender et al., 2004) and coral reefs (Imhoff and Trüper, 1976; Caumette et al., 2007).

In marine habitats of India, the work of Vethanayagam (1991) reports the occurrence of PNSB in Pichavaram mangrove environment present in the Bay of Bengal coast, Tamilnadu, India. A novel purple non sulfur bacterium *Rhodovulum imhoffii* sp. nov., (Srinivas et al., 2007a) has been reported from an marine aquaculture pond present in Bhimunipatnam, situated near the Bay of Bengal coast, Andhra pradesh, India. Likewise novel PNSB strains namely, *Rhodovulum marinum* sp. nov. (Srinivas et al., 2006), *Rhodovulum visakhapatnamense* sp. nov. (Srinivas et al., 2007b) and *Roseospira visakhapatnamensis* sp. nov., (Kalyan Chakravarthy et al., 2007) were isolated from marine environments of Visakhapatnam, situated near Bay Bengal coast, Andhra pradesh, India and *Roseospira goensis* sp. nov.(Kalyan Chakravarthy et al., 2007) reported from Kurka saltern, located near the Arabian sea, Goa, India.

Purple non sulfur bacterial members isolated from shrimp culture ponds have been found to be useful in shrimp ponds as potential bioremediators (Watanabe et al., 2003; Zhou et al., 2007; Panwichian et al., 2010; 2011).

### 2.1.3 Characteristic features of PNSB

Purple nonsulfur bacteria is by far the most diverse group of the phototrophic purple bacteria (Imhoff and Trüper, 1989). This diversity is reflected in greatly varying morphology, internal membrane
structure, carotenoid composition, utilization of carbon sources, and electron donors, among other features (Table 1-3). Most species are motile by flagella, gas vesicles are not formed by any of the known species. In all purple bacteria the photosynthetic pigments and the photosynthetic apparatus are located within a more or less extended system of intracytoplasmic membranes that is considered as originating from and being continuous with the cytoplasmic membrane. These intracytoplasmic membranes consist of small fingerlike intrusions, vesicles, tubules or lamellae.

The major photosynthetic pigments are bacteriochlorophyll a or b and various carotenoids of the spirilloxanthin, rhodopinal, spheroidene (alternative spirilloxanthin), or okenone series (Schmidt, 1978). Anoxygenic photosynthesis depends on the presence of a complex membrane-bound photosynthetic apparatus, which includes reaction center and light harvesting (antenna) pigmentprotein complexes. The preferred growth mode of all species is photoheterotrophic under anaerobic conditions in the light with various organic substrates. Many species also are able to grow photoautotrophically with either molecular hydrogen or sulfide as electron donor and CO₂ as the sole carbon source (Hansen and Van Gemerden, 1972).

Chemoorganoheterotrophic growth in the presence of oxygen is common among purple nonsulfur bacteria and most of the known species are facultatively chemotrophic. While some species are very sensitive to oxygen, others grow equally well under oxic conditions in the dark at the full oxygen tension of air. Also chemolithoautotrophic growth with hydrogen or reduced sulfur compounds as electron donors and oxygen as electron acceptor has been demonstrated (Madigan and Gest, 1979; Siefert and Pfennig, 1979). In the absence of external
electron acceptors, a number of purple nonsulfur bacteria can use fermentative processes for energy generation (Uffen, 1978). Most of the purple nonsulfur bacteria can use a variety of different organic carbon sources. Intermediates of the tricarboxylic acid cycle in addition to acetate and pyruvate are generally used. A number of purple nonsulfur bacteria use straight-chain saturated fatty acids with 5–18 carbon atoms (Janssen and Harfoot, 1987). Also organic acids, amino acids, alcohols and carbohydrates support growth of many of these bacteria (Imhoff, 2006).

Purple nonsulfur bacteria vary greatly in their sulfur metabolism. Most purple nonsulfur bacteria, in particular freshwater species, are inhibited by sulfide even at low concentrations. Some species, however, are quite tolerant to this toxic compound and use it as a photosynthetic electron donor (Hansen and Imhoff, 1985; Hansen and Van Gemerden, 1972). The sulfide oxidizing species of purple non sulfur bacteria deposit elemental sulfur inside the cells. The oxidation product in most species is not sulfate, only elemental sulfur is formed. But, members of *Rhodovulum sulfidophilum*, *Rhodopseudomonas palustris* and *Blastochloris sulfoviridis* oxidize sulfide to sulfate without formation of elemental sulfur (Neutzling *et al.*, 1985) and three species of the genera *Rhodovulum* spp., viz., *R. veldkampii*, *R. adriaticum* and *R. euryhalinum* oxidize sulfide to sulfate which is deposited extracellularly and they further oxidize this sulfur to sulfate (Hansen and Imhoff, 1985; Kompantseva, 1985).

In addition to sulfide, some purple nonsulfur bacteria can use thiosulfate as an electron donor (Hansen and Veldkamp, 1973). Ammonia, dinitrogen, and several organic nitrogen compounds (e.g., glutamate, aspartate or yeast extract) are the most appropriate nitrogen sources of most purple nonsulfur bacteria. Nitrate is assimilated only
by a few species and growth yields with nitrate are considerably lower than with other nitrogen sources (Göbel, 1978). The ability to fix dinitrogen is a common property of most phototrophic purple bacteria (Madigan, 1995). Hydrogen evolution in purple nonsulfur bacteria also occurs during fermentative growth under anoxic dark conditions. This hydrogen evolution is catalyzed by a reversible hydrogenase or by formate hydrogenlyase (Sasikala et al., 1993).

2.1.4 Isolation and enumeration of purple non sulfur bacteria.

Isolation of phototrophic bacteria from their habitats requires the use of specialized techniques. Selective enrichment techniques were first used by Sergei Winogradsky, and his technique is known as the Winogradsky column. In this pioneering work, the anoxygenic organisms were present as a distinct layer in the column. This paved the way for isolating purple non sulphur bacteria. Variations of this column technique for the enrichment and isolation of purple non sulphur bacteria (PNSB) was adopted by Van Niel (1944), who elaborated the physiological basis for the enrichment of purple nonsulfur bacteria and was the first to develop a defined medium which could be used for their enrichment and cultivation. For selective enrichment of purple nonsulfur bacteria, media have been used with lowered sulfate concentration to avoid production of sulfide by sulfate-reducing bacteria. An anaerobic enrichment culture using a common organic substrate, when placed in the light usually will give rise to the development of purple nonsulfur bacteria. For the isolation and cultivation of purple nonsulfur bacteria from freshwater and marine sources, the AT medium (Imhoff and Trüper, 1976; Pfennig and Truper, 1981; Imhoff, 1982) with slight modifications has been successfully employed (Vethanayagam, 1991; Zhang et al., 2002; 2003). Methods of direct isolation and enumeration of the phototrophic bacteria from natural samples employ Membrane filters
(Swoager and Lindstrom, 1971), Agar shake dilution (Biebl and Pfenning 1981). MPN method (Elbadry et al., 1999), Paraffin wax overlay of pourplate (Archana et al., 2004), and Plating on agar followed by incubation in anaerobic gas jars (Okubo et al., 2005).

2.1.5 Systematics of purple non sulfur bacteria

The first official recognition which purple bacteria received as a systematic group came with the creation of the order Thiobacteria by Migula, in 1900 (Van Niel, 1944), which included purple sulfur and purple non sulfur bacteria. However Molisch (1907) removed the purple sulfur bacteria from the Thiobacteria and considered purple non-sulfur bacteria under *Rhodobacteriales*.

Since that time, pigmentation and ability to perform anoxygenic photosynthesis were considered of primary importance for assignment of bacteria to the *Rhodobacteriales*, later called *Rhodospirillales* (Pfennig and Truper, 1971). Because the *Rhodospirillaceae* (Pfennig and Truper, 1971) do not represent a phylogenetically distinct group of bacteria, it was proposed to abandon the use of the family name. The term purple nonsulfur bacteria (PNSB) has been proposed for the physiological groups of anaerobic phototrophic *Alphaproteobacteria* and *Betaproteobacteria* that contain photosynthetic pigments and are able to perform anoxygenic photosynthesis (Imhoff et al., 1984).

Purple nonsulfur bacteria have been traditionally, classified into genera representing the rod shaped *Rhodopseudomonas* species and the spiral- shaped *Rhodospirillum* species (Pfennig and Trüper, 1974) and later into a third genus containing the half-circle to circle shaped *Rhodocyclus purpureus* (Pfennig, 1978). With the recognition of their genetic relationships and chemotaxonomic diversity, purple nonsulfur bacteria of the α and β-Proteobacteria were taxonomically separated
(Imhoff et al., 1984; Imhoff and Trüper, 1989). Later, bacteria within these groups were rearranged according to phylogeny, chemotaxonomic characteristics and eco physiological properties. The α-proteobacteria has been taxonomically subdivided into α1, α2, and α3-proteobacteria. Most of the phototrophic bacteria that belong to the α1 Proteobacteria have been previously known as *Rhodospirillum* species and are of spiral shape. At present, the only non spiral representative is *Rhodopila globiformis*. Genera included in this group are *Rhodospirillum* sp., *Phaeospirillum* sp., *Rhodospira* sp., *Roseospira* sp., *Rhodocista* sp., *Roseospirillum* sp., and also *Rhodopila* sp., *Rhodothalassium* sp., and *Rhodovibrio* sp.. Based on the 16s rDNA sequencing the spiral shaped, phototrophic α-Proteobacteria are phylogenetically quite distantly related to each other and do not warrant classification in one and the same genus (Kawasaki et al., 1993a; Imhoff et al., 1998).

These bacteria also demonstrate great phenotypic diversity. Therefore, a reclassification of the spiral-shaped phototrophic α-Proteobacteria was proposed, based on distinct phenotypic properties and 16S rDNA sequence similarities. *Rhodospirillum centenum* was transferred to a new genus as *Rhodocistacentenaria* (Kawasaki et al., 1992). Other Rhodospirillum species were transferred to the new genera *Phaeospirillum*, *Rhodovibrio*, *Roseospira* and *Rhodothalassium* (Imhoff et al., 1998). Only *R. rubrum* and *R. photometricum* were maintained as species of the genus *Rhodospirillum*. In addition, new species were described of this group: *Rhodospira trueperi* was assigned to a new genus on the basis of significant phenotypic and genotypic differences from *Rhodospirillum rubrum* and other known PNSB (Pfennig et al., 1997). For similar reasons, the new bacterium *Roseospirillum parvum* was assigned to a new genus (Glaeser and Overmann, 1999).
The characteristics of phototrophic bacteria of the group α-2 (\textit{Rhodopseudomonas}) group is the budding mode of growth and cell division and the presence of lamellar internal membranes lying parallel to the cytoplasmic membrane. Most of these phototrophic bacteria have been previously known as \textit{Rhodopseudomonas} species. Genera of this group now include \textit{Rhodopseudomonas} sp., \textit{Rhodoplanes} sp., \textit{Rhodoblastus} sp., \textit{Blastochloris} sp., \textit{Rhodomicrobium} sp., and \textit{Rhodobium} sp. (Imhoff, 2006).

After the removal of purple nonsulfur bacteria that contained vesicular internal photosynthetic membranes and those that of β-Proteobacteria from the genus \textit{Rhodopseudomonas}, only those species remained within this genus that had lamellar internal membrane structures and grew and reproduced by budding (Imhoff \textit{et al.}, 1984). The bacteria removed from \textit{Rhodopseudomonas} are now recognized as species of \textit{Rhodopila}, \textit{Rhodobacter}, \textit{Rhodovulum} and \textit{Rubrivivax}. Thereafter, what remained of the genus \textit{Rhodopseudomonas} (together with \textit{Rhodomicrobium vannielii}) still represented a heterogeneous assemblage of species (Imhoff \textit{et al.}, 1984) now recognized as genera of the α-2 Proteobacteria. Primarily due to the availability of sequence data of the 16S rDNA (Kawasaki \textit{et al.}, 1993a) and in part supported by the isolation and description of new species and additional data, the following proposals have been made. \textit{Rhodopseudomonas marina} was transferred to the new genus \textit{Rhodobium} as \textit{R. marinum} together with the new species \textit{Rhodobium orientis} (defined as the type species of this genus; Hiraishi \textit{et al.}, 1995). \textit{Rhodopseudomonas rosea} was transferred to the new genus \textit{Rhodoplanes} and designated as the type species of this genus, \textit{R. roseus} (Hiraishi and Ueda, 1994). At the same time, \textit{Rhodoplanes elegans} was described as a new species of this genus. \textit{Rhodopseudomonas viridis} and \textit{Rhodopseudomonas sulfoviridis} were assigned to the new genus \textit{Blastochloris} as \textit{B. viridis} and \textit{B. sulfoviridis}.
(Hiraishi, 1997). Quite recently, *Rhodopseudomonas acidophila* was transferred to a new genus as *Rhodoblastus acidophilus* (Imhoff, 2001). *Rhodopseudomonas blastica* was removed from this genus and transferred to *Rhodobacter blasticus* (Kawasaki *et al.*, 1993b). Its 16S rDNA sequence is most similar to and clusters with those of the *Rhodobacter* species. *Rhodopseudomonas rutila* (Akiba *et al.*, 1983) was considered as a later subjective synonym of *Rhodopseudomonas palustris* (Hiraishi *et al.*, 1992). In addition to *Rhodopseudomonas palustris*, *Rhodopseudomonas julia* (Kompantseva, 1989) and *Rhodopseudomonas cryptolactis* (Stadtwald-Demchick *et al.*, 1990) have been affiliated to this genus, though both species so far have not been validated and no 16S rDNA sequence of them is available (Imhoff, 2006).

A characteristic feature of the phototrophic α-3 Proteobacteria (*Rhodobacter* group) is the presence of carotenoids of the spheroidene series and their extraordinary metabolic versatility and flexibility. These bacteria have been previously known as *Rhodopseudomonas* species and belong to the genera *Rhodobacter* and *Rhodovulum* (Pfennig and Trüper, 1974, Imhoff *et al.*, 1984, Hiraishi and Ueda, 1994). The former are freshwater bacteria and the latter true marine bacteria. Species of both genera have distinct 16S rDNA sequences (Hiraishi and Ueda, 1994, Hiraishi and Ueda, 1995; Hiraishi *et al.*, 1996). Two new species, *Rhodovulum iodosum* and *Rhodovulum robiginosum*, have been described that use ferrous iron as photosynthetic electron donor (Straub *et al.*, 1999). *Rhodobaca borogenensis*, a new isolate from an alkaline soda lake with low salt concentration, has adapted in its salt response to this habitat (Milford *et al.*, 2000).
According to 16S rDNA sequence comparisons, the phototrophic β-Proteobacteria of the genera *Rhodoferax*, *Rubrivivax* and *Rhodocyclus* are well separated phylogenetically from their counterparts of the α-Proteobacteria and belong to different phylogenetic lines within the β- Proteobacteria (Hiraishi, 1994). Prior to the recognition of their genetic relationship on the basis of 16S rDNA sequence comparison, the phototrophic purple nonsulfur bacteria belonging to the β-Proteobacteria have been included in the Rhodospirillaceae together with the phototrophic α-Proteobacteria (Pfennig and Trüper, 1974).

In addition to their clear phylogenetic separation (Gibson *et al.*, 1979; Fox *et al.*, 1980; Hiraishi, 1994), a number of chemotaxonomic properties clearly distinguish the phototrophic β-proteobacteria from the phototrophic α-Proteobacteria: They have ubiquinone and menaquinone (or rhodoquinone) derivatives with eight isoprenoid units in the side chain (Q-8, RQ-8 and MK-8); they have a “small type” cytochrome c551 as typically found in species of the Chromatiaceae and Ectothiorhodospiraceae, but not in phototrophic α-Proteobacteria (Ambler *et al.*, 1979; Dickerson, 1980). Their lipopolysaccharides characteristically contain significant amounts of phosphate and amidelinked 3-OH-capric acid (3-OH-C-10) in their lipid-A moiety (Weckesser *et al.*, 1995) or 3-OHC- 8:0, which is the major hydroxy fatty acid of *Rhodoferax fermentans* (Hiraishi *et al.*, 1991). As a consequence, *Rhodospirillum tenue* (Pfennig, 1969) was transferred to *Rhodocyclus tenuis* (Imhoff *et al.*, 1984). Also *Rhodopseudomonas gelatinosa* was transferred to this genus as *Rhodocyclus gelatinosus* (Imhoff *et al.*, 1984), but because of its phylogenetic distance to *Rhodocyclus purpureus*, it was assigned later to a new genus as *Rubrivivax gelatinosus* (Willems *et al.*, 1991). Since then *Rhodoferax fermentans* has been described as a new genus and new species.
(Hiraishi and Kitamura, 1984; Hiraishi et al., 1991) and *Rhodoferax antarcticus* as another new species of that genus (Madigan et al., 2000).

### 2.1.6 Taxonomic disparities in purple non-sulfur bacteria

- **Rhodopseudomonas julia**, published in Mikrobiologiya by Kompantseva, (1989) was later validated in *Int. J. Syst. Bacteriol.*, 1993 (Validation List No.44). The type strain was deposited in DSMZ and ATCC as DSM 11549 and ATCC 51105 respectively. However, the 16S rRNA gene sequence of the strain DSM 11549 indicates that it is a *Rhodobium* strain and it is identical with that held in the ATCC (ATCC 51105). But, according to Kompantseva, this strain does not correspond with the original description, indicating that an incorrect strain may be in circulation.


- **Roseospirillum parvum**, published in Arch. Microbiol. by Glaeser and Overmann, 1999 though was later validated in *Int. J. Syst. Evol. Microbiol.*, 2001 (Validation List No. 80), however, as the culture was deposited only in one culture collection DSMZ (DSM 12498) at the time of publication, its validation remains pending until further deposition in other culture collection.
“Roseospira thiosulfatophila” published in *Arch. Microbiol.* by Guyoneaud *et al.*, 2002 was deposited only in ATCC (ATCC BAA-449) and not validated so far.


*Rhodoplanes cryptolactis* which was effectively published in *J. Gen. Appl. Microbiol.* by Okamura *et al.*, 2007 and deposited in two culture collection centers DSMZ and ATCC as DSM 9987 and ATCC 49414 respectively was not validated so far.

*Rhodovastum atsumiense* effectively published in *J. Gen. Appl. Microbiol.* by Okamura *et al.*, 2009a and deposited in NBRC and KCTC as NBRC 104268 and KCTC 5708 respectively was not validated so far.

According to Okamura *et al.*, 2009b, the type strain of *Rhodopseudomonas palustris* which was originally deposited in ATCC as ATCC 17001<sup>T</sup> and later deposited in other culture collections like DSMZ (as DSM 123<sup>T</sup>) and NBRC (as NBRC
100419\textsuperscript{T} are not the same. According to them, only the phenotypic properties of strain ATCC 17001\textsuperscript{T} are in accordance with the original description of *Rhodopseudomonas palustris* (Imhoff *et al.*, 2005; Van Niel, 1944) and they presume that the mislabeling of the type strain of *Rhodopseudomonas palustris* took place during its transfer from ATCC to other culture collections. Hence, according to Okamura *et al.*, 2009b, the original type strain of *Rhodopseudomonas palustris* is only ATCC 17001\textsuperscript{T}, and DSM 123\textsuperscript{T} =NBRC 100419\textsuperscript{T} is not the type strain.

- The genus *Rhodothalassium* is considered as a genus *incertae sedis*, in second edition of Bergey’s Manual of Systematic Bacteriology (2005). It is also given in the editorial note that, based on 16S rRNA gene sequence information, *Rhodothalassium salexigens* forms a distinct phylogenetic lineage, which is separate from other phototrophic members of the *Alphaproteobacteria*. Further, the separation is so significant that it does not fit into any of the families of the *Alphaproteobacteria* allowing this species to be considered as a separate family. However, as the 16S rRNA gene sequence evidence is only available from a single strain, further data should be obtained before assigning this genus to a family.

### 2.1.7 Polyphasic taxonomy

Bacterial taxonomy comprises the interrelated areas of classification, nomenclature, and identification and is supposed to reflect phylogeny and evolution. Characterization changed from simple procedures, in which a limited number of features of the bacterial cell (mainly morphological and physiological aspects) were studied, to a
multidisciplinary approach using phenotypic, genotypic, and chemotaxonomic techniques. Determination of phylogenetic relationships (which at this time is essentially synonymous with 16S and/or 23S rRNA gene sequence similarities) became a routine procedure in bacterial taxonomy.

While the rules of bacterial nomenclature remain largely unchanged (Lapage *et al.*, 1992; Stackebrandt and Goebel, 1994; Murray and Stackebrandt, 1995), the term “polyphasic taxonomy” was introduced 30 years ago by Colwell (1970) to refer to a taxonomy that assembles and assimilates many levels of information, from molecular to ecological, and incorporates several distinct, and separable, portions of information extractable from a nonhomogeneous system to yield a multidimensional taxonomy. Nowadays, polyphasic taxonomy refers to a consensus type of taxonomy and aims to utilize all the available data in delineating consensus groups, decisive for the final conclusions (Gillis *et al.*, 2005).

### 2.1.8 Description of novel purple non sulfur bacteria

For the detailed description of a new Purple non sulfur bacterium, careful physiological studies are required, including the utilization of substrates, relations to oxygen, the ability to grow in darkness, respiratory / fermentative growth, vitamin requirement, pH, temperature ranges and optima of salt concentration. In addition to the phenotypic characterization, information on the genetic relatedness of a new isolate has to be obtained. To achieve a phylogenetic classification, 16S rDNA sequences are used to distinguish closely related strains and species and DNA-DNA hybridization studies are required for the description of novel species of purple non sulfur bacteria (DeBont *et al.*, 1981; Imhoff and Caumette, 2004).
Recommended standards for the description of new species of the anoxygenic phototrophic bacteria are proposed in accordance with Recommendation 30b of the International Code of Nomenclature of Bacteria. These standards (Table 4) include information on the natural habitat, ecology and phenotypic properties including morphology, physiology and pigments and on genetic information and nucleic acid data. The recommended standards were supported by the Subcommittee on the taxonomy of phototrophic bacteria of the International Committee on Systematics of Prokaryotes. They are considered as guidelines for authors to prepare descriptions of new species (Imhoff and Caumette, 2004; Tindall, 2010).

Numerous studies have been done in recent years to isolate and characterize purple non sulfur bacterial members from various marine environments. Even though it is a well established fact that PNSB members are prevalent in marine shrimp ponds, a systematic survey of PNSB members prevalent in those artificial man made marine environments, remains uncharted and no work to the best of our knowledge, is available on PNSB population from shrimp ponds rearing Tiger prawn (*Penaeus monodon*), along the south east coast of Tamilnadu.

The present work aims in surveying the shrimp pond samples for PNSB, on course of cultivation of shrimps, starting from seed stocking till harvesting with the following objectives.

1. To isolate and characterize Purple non sulfur bacterial members from brackish and direct sea water shrimp ponds.
2. Description of new taxa, if any, based on polyphasic taxonomic approach.
2.2 MATERIALS AND METHODS

To accomplish the objectives of the present study as outlined in the introductory chapter the following experiments were carried out.

- Enumeration of Purple non sulfur bacteria (PNSB) from shrimp pond samples.
- Enrichment of PNSB from shrimp pond samples.
- Isolation and purification of PNSB strains.
- Characterization of PNSB Strains.
- Description of new taxa, if any, based on polyphasic taxonomic approach.

2.2.1 Area of study and sampling locations

The study area comprised of four shrimp ponds and their water inlets, located in Nagapattinam, Thanjavur, and Ramanathapuram districts. While the nature of water source was brackish in the shrimp ponds of Nagapattinam district, it was direct sea water in the shrimp ponds of Thanjavur and Ramanathapuram districts. The location of shrimp ponds/inlets, with their geographical positions are given below:

1) Vadakkupoygainallur shrimp pond (Nagapattinam Dist.)
   10°44'5.80"N, 79°50'12.96"E
2) Vedaranyam canal inlet (Vadakkupoygainallur)
   10°44'18.84"N, 79°50'0.68"E
3) Pappakovil shrimp pond (Nagapattinam Dist) 10°44'38.66"N, 79°49'39.25"E
4) Kaduvaiauru canal inlet (Pappakovil) 10°44'42.02"N, 79°49'37.81"E
5) Sethubavachatram shrimp pond (Thanjavur Dist.)
   10°15'24.50"N, 79°17'19.05"E
6) Sea water pump house (Sethubavachatram) 10°15'17.27"N, 79°17'26.24"E
7) Karankadu shrimp pond (Ramanathapuram Dist.) 9°38'49.88"N, 78°57'11.46"E
8) Sea water inlet canal (Karankadu) 9°38'43.28"N, 78°57'5.54"E

2.2.2 Collection of samples

The sub surface water samples from the shrimp farms were taken from locations like, water source /inlet (brackish canals/pump house), culturing ponds and draining channel in the study area using sterilized 1000ml plastic bottles and 300 ml glass stoppered bottles. Shrimp pond soil sediment samples were collected in fresh zip-lock polythene covers, from the above mentioned locations.

2.2.3 Enumeration of purple non sulfur bacteria (PNSB) from shrimp ponds

Water and soil sediment samples from locations of the shrimp pond like inlet, culture pond and draining channel were used for the enumeration of PNSB. The enumeration of PNSB was done by Paraffin wax overlay of pour plate method (Archana et al., 2004).

2.2.3.1 Paraffin wax overlay of pour plate (Archana et al., 2004)

The water and sediment samples were serially diluted by taking, one ml of the water sample mixed in 9 ml saline (0.7% NaCl w/v) and one gram of soil sample suspended in 9 ml saline (0.7% NaCl w/v) mixed by vortexing and tenfold dilutions in the saline were made to give dilutions upto 10^-6. 0.5 ml from each of the dilution were used as inoculum, and pour plated with 20 ml of modified Biebl and Pfennig’s (1981) agar medium (Appendix) at 40–45°C. The medium was allowed to solidify. The plates were then over layed with molten paraffin wax (55–60°C), which solidified upon layering. The plates were rotated gently in a circular motion while pouring the wax in order to spread it evenly over the agar surface. The plates were kept open for
a period of 10 min after pouring the paraffin wax in order to radiate the heat of the wax before closing the lid and incubated at a temperature of 30±2°C with the agar side of the plate exposed to a light intensity of 2400 lux, for 12-15 days. At the end of the incubation period the brownish red-pink coloured colonies were counted.

2.2.4 Enrichment of water and sediment soil samples

The soil and water samples were enriched separately in 100 ml screw capped bottles, using modified Biebl and Pfennig’s medium (1981) where 1 gm of soil sample and 1mL of water sample were introduced aseptically into 100 ml screw capped bottles and the medium was poured up to the brim, screw capped tightly and sealed with parafilm. Initially the tubes were incubated in dark for 24 hours and later the tubes were kept under constant illumination (2,400 lux) at 30 ± 2°C for 7 to 12 days and observed for brown/brownish-red/purple colour, which indicates the presence of PNSB. Enrichment of the water and soil samples from each station was done during different periods of the shrimp culture cycle (i.e. from seed stocking to harvest).

2.2.5 Purification of PNSB (Archana et al., 2003)

Enrichment cultures were purified by repeatedly streaking on Biebl and Pfennig’s agar slants (1981) (Appendix) prepared in 25 x 150 mm. rim-less test tubes and sealed with polybutyrate rubber stoppers (suba seals). The gas phase in the test tube was replaced by flushing with argon for 2 min by using a pair of hypodermic needles and incubated under constant illumination (2400 lux) at 32°C. The purification was performed till the colonies appearing on two successive slants were all identical. Purity of the culture was checked by streaking on Nutrient agar (Appendix) plates and incubated anaerobically under illumination at 2400 lux. Contamination from other phototrophic bacteria was checked by monitoring the cultural
characters like color of the culture, colony morphology and by microscopic observation.

2.2.6 Maintenance of PNSB stock cultures

Stock cultures of PNSB were maintained as agar stabs or as broth cultures. Stabs were prepared using modified Biebl and Pfennig’s (1981) medium with 2% (w/v) agar as solidifying agent, filled to ¾ volume of 5 ml capacity screw cap test tubes. The culture was stabbed into the agar deeps and incubated at 2,400 lux and 30 ± 2°C. After 4-6 days of growth, the stab cultures were preserved under refrigeration at 4°C until further use. The stabs were sub-cultured every 90 days and contamination from other bacteria was checked periodically by microscopic examination and by streaking on Nutrient agar plates.

2.2.7 Characterization and identification of Purple non sulfur bacterial strains

The purified PNSB strains were characterized and identified based on the Bergeys manual of systematic bacteriology (2005). The PNSB strains that could not be identified up to species level based on the phenotypic characters were subjected to electron microscopic studies, molecular characterization and 16s rRNA sequencing, for establishing their identity.

2.2.7.1 Morphological and cultural characterization of PNSB

2.2.7.1.1 Light microscopic observation

Morphological properties such as cell shape, size, cell division and motility were visualized through phase contrast microscopy (Olympus- B201).
2.2.7.1.1 Gram staining

A loopful of logarithmically growing culture was taken on a neat clean slide, smear was prepared, dried and was fixed by heat, and performed Grams staining and the slide was observed under light microscope (Olympus B201) at 100X magnification.

2.2.7.1.2 Flagella staining

The air dried smear of the culture was flooded with reagent A (Appendix) for 2-4 minutes and rinsed with distilled water. Reagent B (Appendix) was flooded and after 30 seconds immediately washed with distilled water. Air-dried slide was observed under light microscope in oil immersion.

2.2.7.1.2 Electron microscopic observation

2.2.7.1.2.1 TEM (negative staining for flagella)

Five ml of well grown culture was centrifuged at 4000 rpm for 5 min and the pellet was suspended in 0.1 M phosphate buffer, centrifuged at 4000 rpm for 5 min. The supernatant was replaced with 1 ml of fresh phosphate buffer. A small drop of sample was placed on a piece of Para-film and a carbon coated EM grid was placed on that drop. After 20 min the grid was removed and the excess sample was drained with filter paper. The grid was washed with distilled water and stained with 2% uranyl acetate and allowed for air drying. The grid with sample was observed under transmission electron microscope (Model: Hitachi, H-7500).

2.2.7.1.2.2 TEM (sectioning) for ICM structures

Ultrathin section preparation (of bacteria) and observation under TEM was done at RUSKA Lab, College of Veterinary Sciences, SVVU, Rajendranagar, Hyderabad. For microscopic studies samples were transferred to vials and fixed in 2.5% gluteraldehyde in 0.05 M
phosphate buffer (pH 7.2) for 24 hr at 4°C and post fixed with 2% aqueous osmium tetroxide in the same buffer for 2 hr. After the post fixation samples were dehydrated in a series of graded alcohol, infiltrated and embedded in Spurr’s resin. Both Semi thin and Ultra thin sections were cut with a glass knife on a Leica Ultra cut UCT-GA-D/E-1/100 ultra microtome, Semi thin sections of 200-300 nm were stained with toluidine blue and ultra thin sections (50-70 nm thickness) stained with saturated aqueous Uranyl acetate and counter stained with 4% lead citrate and mounted on grids. Now the sections were observed at various magnifications under transmission electron microscope.

2.2.7.2 Determination of chemical composition

2.2.7.2.1 Pigment analysis

2.2.7.2.1.1 Whole cell absorption spectrum

Absorption spectrum of whole cells was measured by the sucrose method of Pfennig and Trüper (1981). To 3.5 ml of the liquid culture, 5 g of sucrose was added and mixed thoroughly on a vortex spinner. The absorption spectrum from 300-1100 nm was measured using uv spectrophotometer using sucrose in the medium as blank.

2.2.7.2.1.2 Separation of pigments by column chromatography

Ten times concentrated cell pellet was eluted with acetone in 10 \( \times \) 200 mm column packed with aluminium oxide. Various eluted fractions were collected at regular intervals and the pigments were analyzed spectrophotometrically from 400-550 nm with acetone as blank. The spectral values obtained were compared with standard carotenoid analysis data from Britton et al., 2004.

2.2.7.2.1.3 Carotenoid composition

For determination of carotenoid composition, 1000 ml of the 48-72 h grown culture was harvested by centrifugation (16,000 rpm for
10 min). The supernatant was discarded and the pellet was washed twice with 0.8 % NaCl solution and lyophilized. Pigments were extracted with methanol: acetone (7:2, v/v) from the lyophilized cells. Carotenoid composition was analyzed by using HPLC [Solvent system used: 50:40:10 –acetonitrile: methanol: ethylacetate; Flow rate: 1ml min-1; column: luna 5μm, C18, 250 x 4.6 mm phenomenex; Photo Diode Array (PDA) detector (400-800nm); wavelengths monitored at 450, 500 and 550 nm].

2.2.7.2.2 Cellular fatty acid composition

Cellular fatty acid methyl esters were analyzed gas chromatographically according to the instructions of the Microbial Identification System (Microbial ID, MIDI; Sasser, 1990), which was outsourced at Royal Life Sciences Pvt. Ltd., Secunderabad. Photoheterotrophically grown culture on agar slants was scraped with a loop to harvest about 40 mg of bacterial cells. The cells were placed in a clean 13 x 100 mm culture tube. The fatty acids were extracted by a procedure which consists of four steps.

i. Saponification

One ml of 15 % NaOH in 1:1, methanol: distilled was added to the tube containing cells. The tubes were sealed with teflon lined caps, vortexed briefly and heated in a boiling water bath for 5 minutes, at which time the tubes were vigorously vortexed for 5-10 seconds and returned to the water bath to complete the 30 minute heating. (At this step, the cells were lysed to liberate fatty acids from the cellular lipids).

ii. Methylation

To the cooled tubes, 2 ml of 13:11, 6.0 N HCl:Methanol was added which drops the pH of the solution below 1.5 and causes methylation (for the increased volatility in a partially polar column) of
the fatty acid. The fatty acid methyl ester is poorly soluble in the aqueous phase at this point. The tubes were capped and briefly vortexed. After vortexing, the tubes were heated for 10 ± 1 minute at 80° ± 1°C.

iii. Extraction

1.25 ml of 1:1, hexane:methyl tertiary-butyl ether was added (this will extract the fatty acid methyl esters into the organic phase for use with the gas chromatograph) to the cooled tubes which was followed by recapping and gentle tumbling on a clinical rotator for about 10 minutes. The tubes were uncapped and the aqueous (lower) phase is pipetted out and discarded.

iv. Base Wash

About 3ml of dilute NaOH solution (1.08 g NaOH in 90 ml distilled water) was added to the organic phase remaining in the tubes, the tubes were recapped, and tumbled for 5 minutes (this procedure reduces contamination of the injection port liner, the column, and the detector). Following uncapping, about 2/3 of the organic phase is pipetted into a GC vial which is capped and ready for gas chromatographic analysis.

v. Chromatographic conditions

Agilent 6850 make gas chromatograph (GC) equipped with Flame Ionization Detector (FID) and N₂ gas (“makeup” gas) were used for Ultra 2 Column (a 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column). The operating parameters of GC were: carrier gas- Hydrogen; the temperature program ramps from 170°C to 270°C at 5°C per minute. With the help of external calibration standard (which consists of a mixture of the straight chained saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0) and five hydroxy
acids) developed and manufactured by Microbial ID, Inc., the Equivalent Chain Length (ECL) values of various fatty acids were identified. Retention time data obtained by injecting the calibration standard is converted to ECL data for bacterial fatty acid naming. The ECL value for each fatty acid can be derived as a function of its elution time in relation to the elution times of a known series of straight chain fatty acids.

2.2.7.3 Determination of Growth

Increase in optical density (OD) (turbidity) was used to monitor the growth of PNSB. Optical density of the bacterial suspension was directly measured in a Systronics make (model 112) colorimeter at 660 nm (filter 8) against un-inoculated medium blank.

2.2.7.4 Physiological and Biochemical characterization

2.2.7.4.1 Growth modes

2.2.7.4.1.1 Photolithoautotrophy

Photolithoautotrophy of the culture was tested by inoculating the culture in fully filled screw cap tubes (10 × 100 mm) with modified Biebl and Pfennig’s (1981) medium [anaerobically in the light (2400 lux) with Na₂S·9H₂O (0.5 mM)/Na₂S₂O₅·5H₂O (2 mM)/elemental sulfur (0.05% w/v)/sulfite (2 mM)/Ferrous iron (10 mM) as the electron donor and NaHCO₃ (0.1%, w/v) as sole/principal carbon source] and incubated at 30±2°C.

2.2.7.4.1.2 Photoorganoheterotrophy

Photoorganoheterotrophy of the culture was tested by inoculating the culture in screw cap tubes (10 × 100 mm) fully filled with modified Biebl and Pfennig’s (1981) medium [with pyruvate (0.3%, w/v) as carbon source] and incubated anaerobically in the light (2400 lx) at 30±2°C.
2.2.7.4.1.3 Chemolithoautotrophy

Chemolithoautotrophy of the culture was tested by inoculating the culture in to 100 ml of modified Biebl and Pfennig’s (1981) medium in the 250 ml conical flask plugged with cotton plug [aerobically in the dark with Na$_2$S$_2$O$_3$·5H$_2$O (2 mM) as the electron donor and NaHCO$_3$ (0.1 %, w/v) as carbon source] and incubated in both incubator (micro aerobic conditions) and orbital shaker (aerobic – 100 rpm) at 30±2°C.

2.2.7.4.1.4 Chemoorganoheterotrophy

Chemoorganoheterotrophy of the culture with pyruvate as carbon source was tested by inoculating the culture in to 100 ml of modified Biebl and Pfennig’s (1981) medium in a 250 ml conical flask plugged with cotton plug [aerobically, in the dark] and incubated in both incubator (micro aerobic conditions) and orbital shaker (aerobic – 100 rpm) at 30±2°C.

2.2.7.4.1.5 Fermentative mode

Fermentative growth mode of the culture was tested by inoculating the culture in the fully filled screw cap tubes (10 × 100 mm) with modified Biebl and Pfennig’s (1981) medium [with glucose/fructose/pyruvate (0.3%, w/v) as carbon source] and incubated anaerobically in the dark at 30± 2°C.

2.2.7.4.2 Utilization of organic/inorganic compounds as electron donor and/or carbon source

One percent inoculum of culture was inoculated into modified Biebl and Pfennig’s (1981) basal medium with NH$_4$Cl (0.12% w/v) and yeast extract as source of nitrogen and growth factors, respectively with the test organic or inorganic compound serving as the electron and/or carbon source. Growth was monitored turbidometrically
(OD_{660}) in fully filled 10 x 100 mm screw cap test tubes after phototrophic (2,400 lux) incubation at 30±2°C. Various organic carbon sources viz. sugars, sugar alcohols, fatty acids, alcohols, tri carboxylic acid cycle (TCA) intermediates, some amino acids, aromatic compounds and a few inorganic compounds such as sodium sulfide, sulfite, elemental sulfur, H₂ and thiosulfate were tested for their utilization as electron and/or carbon source. Sugars, sugar alcohols and TCA cycle intermediates were used at a concentration of 0.3% w/v. Fatty acids, alcohols (filter sterilized on 0.45 μm cellulose acetate membrane) and thiosulfate (filter sterilized on 0.45 μm cellulose acetate membrane) at 0.1% w/v, benzoate at 1 mM, sodium sulfide at 2 mM (unless and other wise mentioned), while H₂ was used at a concentration of 20% v/v of gas phase. In addition, 0.1% w/v bicarbonate was supplemented in the medium in case of fatty acids, alcohols, thiosulfate, sodium sulfide, benzoate, H₂, elemental sulfur and thioglycolate.

2.2.7.4.3 Utilization of sulfur sources

Modified Biebl and Pfennig’s (1981) medium with pyruvate (0.3% w/v each) was used (unless otherwise mentioned) as the electron donor and carbon source in the presence of various inorganic compounds (magnesium sulfate [0.2% w/v], sodium sulfite [0.2% w/v], elemental sulfur [0.05% w/v], sodium sulfide and sodium thiosulfate [1 mM]), and organic compounds (thioglycolate and cysteine [1 mM]) serving as sources of sulfur. Media without any sulfur source but with magnesium chloride [0.2% w/v] served as the control. Growth of the culture, inoculated in to media were monitored turbidometrically in fully filled 10 x 100 mm screw cap test tubes after phototrophic incubation at 2,400 lux and 30±2°C.
2.2.7.4.4 Utilization of nitrogen sources

Modified Biebl and Pfennig’s (1981) medium with pyruvate (0.3% w/v each) was used as the electron donor and carbon source in the presence of various inorganic (sodium nitrite, sodium nitrate, NH₄Cl and urea, 0.12% w/v), organic (glutamine, glutamate, 0.12% w/v) or N₂ (100% v/v of gas phase) serving as sources of nitrogen. Media without any nitrogen source served as the control. Growth of the culture, inoculated into media were monitored turbidometrically in fully filled 10 x 100 mm screw cap test tubes after phototrophic incubation at 2,400 lux and 30±2°C.

2.2.7.4.5 Vitamin requirement

One percent inoculum of the culture was inoculated in modified Biebl and Pfennig’s (1981) medium with pyruvate (0.3% w/v) and NH₄Cl (0.12% w/v) as carbon and nitrogen source, respectively, devoid of yeast extract and replaced with the test vitamin solutions (Biotin [15 µg/l], Thiamine [500 µg/l], Nicotinic acid [500 µg/l], para-Aminobenzoic acid [300 µg/l], Pyridoxal Phosphate [15 µg/l], Calcium pantothenate [10 µg/l], Riboflavin [5 µg/l], B₁₂(cyanocobalamin) [15 µg/l]) filter sterilized with 0.45µm cellulose acetate membrane. Growth was monitored in 10 x 100 mm fully filled screw cap test tubes under phototrophic conditions at 2,400 lux and 30±2°C. Repeated subculture without the vitamins was carried out to determine absolute requirement.

2.2.7.4.6 Saline requirement and tolerance

To the prepared modified Biebl and Pfennig’s (1981) medium with pyruvate (0.3% w/v) and NH₄Cl (0.068% w/v) as carbon and nitrogen source, respectively, different concentrations of sodium chloride (0.05, 0.1, 0.5, 1, 2, 3, 4, 5, 6, 8, 10 %, w/v) were added before autoclaving. Media without NaCl served as control. A one
percent of the culture was inoculated and incubated under phototrophic conditions at 2,400 lux and 30±2°C.

2.2.7.4.7 Growth at different temperatures

Growth of the strains at different temperatures (10, 20, 25, 28-30, 35, 40 and 45°C) was monitored by incubating the inoculated cultures in the growth media under phototrophic conditions at 2,400 lux.

2.2.7.4.8 Growth at different pH

Growth of the strains at different pH (4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10 and 11) was monitored by inoculating the cultures in the modified Biebl and Pfennig’s (1981) medium and by incubating under phototrophic conditions at 2,400 lux and 30±2°C.

2.2.7.4.9 Gelatin liquefaction

Gelatin liquefying ability of the strains is tested by inoculating 10% of culture into fully filled screw cap test tubes containing modified Biebl and Pfennig’s (1981) medium supplemented with pyruvate (0.22% w/v) as carbon source, ammonium chloride (0.06%, w/v) as nitrogen source and gelatin (12% w/v), uninoculated test tube served as control. The test tubes were incubated in light (2, 400 lux) at 30±2°C. After growth of the culture the test tubes along with control were refrigerated at 4°C for 10 min and the liquefaction of the gelatin is tested by comparing with the control.

2.2.7.4.10 Indole production from L-tryptophan

Indole formation from L-tryptophan was studied for the purple bacterial strains by inoculating logarithmically growing cultures, using Biebl and Pfennig’s (1981) basal salts with pyruvate (0.3%, w/v) as carbon source and ammonium chloride (0.06%, w/v) as nitrogen
source, along with L-tryptophan (3 mM) in fully filled screw cap test tubes (10 × 100 mm) incubated phototrophically (2,400 lux) at 30 ± 2°C. After 48 h of incubation, grown culture was directly tested for the indole formation by adding 5 ml of Kovac’s reagent. Appearance of cherry red colour indicates the presence of indole.

2.2.7.5 GENETIC CHARACTERIZATION

2.2.7.5.1 DNA mol % determination (Marmur, 1961 modified)

2.2.7.5.1.1 DNA extraction and purification

The purple bacteria were harvested by centrifugation (10,000 rpm for 15 minutes) and their genomic DNA was isolated by the method of Marmur (1961) modified as illustrated below. Except the solvents used, glassware, buffers, solutions were all sterilized by autoclaving.

Protocol for DNA extraction and purification

2 to 3 g wet packed cells

↓

Suspended in 50 ml Saline – EDTA (0.15M NaCl + 0.1M EDTA, pH8)

↓

Centrifuged (10,000 rpm, 10 min)

↓

Suspended the cells in a total volume of 25ml of Saline – EDTA

↓

*Lyzed the cells

[*2 ml of 25 % (w/v) Sodium Lauryl Sulfate (SLS) was added to the cell suspension and the mixture was placed at 60ºC in a water bath for 10 min and then cooled to room temperature.

↓

Sodium Perchlorate (5M) was added to a final concentration of 1 M to the viscous, lysed suspension

↓
Equal volume of chloroform – Isoamyl alcohol (24:1 v/v) mixture was added to the lysed cell suspension and shaken for 30 min.

Centrifuged (10,000 rpm for 15 minutes) formation of three layers.

Pipetted out upper aqueous layer which contained nucleic acids.

Added 95% (v/v) ethyl alcohol to precipitate nucleic acids.

Removed the precipitated nucleic acids by stirring with the help of a glass rod (removed excess alcohol by pressing the glass rod against the container).

Transferred and dissolved the precipitate into 10 – 15ml of dilute saline citrate (0.015M NaCl + 0.015M Tri sodium citrate; pH 7).

Adjusted the above solution approximately to standard saline citrate concentration by adding concentrated saline citrate solution (1.5M NaCl + 0.15 M Tri sodium citrate, pH 7).

Shaken well with an equal volume of chloroform – Isoamyl alcohol (24:1 v/v) for 15min.

Centrifuged and removed the supernatant (Repeated three times to remove all the proteins).

Added 95% (v/v) ethyl alcohol to the supernatant; dispersed the precipitate in ½ to ¾ of the supernatant volume.
Added ribonuclease [(50 μg. ml⁻¹) ribonuclease was dissolved in 0.15M sodium chloride pH 5 and heated at 80 °C for 10 min to inactivate any DNAase present]

↓

Incubated for 30min at 37°C

↓

Added chloroform – Isoamyl alcohol (24:1 v/v) and centrifuged at 10,000 rpm for 10 min (Repeated three times to remove all the proteins)

↓

To the supernatant 95 % (v/v) ethyl alcohol was added to precipitate the nucleic acids. Dissolved the precipitate in 9 ml dilute saline citrate

↓

Added 1.0 ml acetate – EDTA solution (3M sodium acetate + 0.001M EDTA, pH7)

↓

While stirring the solution rapidly with glass rod isopropyl alcohol was added dropwise into the vortex.

↓

DNA as fibrous material was collected on glass rod.

↓

Washed the DNA isolated with ethyl alcohol (70% and 80% v/v) for 5 min.

↓

DNA was dissolved in dilute saline citrate (0.015M NaCl + 0.0015M Tri Sodium citrate) and then stored in refrigerator.

Twenty five microlitres of the DNA stock in concentrated buffer solution was diluted to standard saline buffer concentration with diluted saline citrate buffer (0.015M NaCl + 0.0015M tri Sodium citrate) and the absorption at 25°C was noted.
2.2.7.5.1.2 HPLC

i. Lysis of the DNA

Five mg of purified DNA was lysed to bases with 0.1ml of perchloric acid in glass sealed bottles at 100°C in water bath for 1 hour. Standard DNA obtained from Himedia chemicals was also lysed by the same method. After the hydrolysis the black char was homogenized with sterile glass rod, diluted to 0.5 ml. The DNA lysate was centrifuged; supernatant was collected and filtered with 0.22 μm cellulose nitrate filters.

ii. Standard DNA bases preparation and chromatographic conditions

Standard bases A, T, G and C (obtained from HIMEDIA) were dissolved in 0.1 N HCl to a concentration of 1 mM. The flow-rate of the solvent is 1.0 ml/min at a temperature of 37°C. The solvent was prepared by combining 40 ml of 0.5 M triethylamine phosphate (TEAP), pH 5.1, with about 750 ml of Milli-Q water. HPLC-grade methanol (120 ml) was added, and the volume was adjusted to 1L (Mesbah et al. 1989). The solvent was then filtered through a 0.2 μm Nylon membrane filter. To prepare 0.5M TEAP solution, triethylamine was diluted with water, the pH was adjusted to 5.1 with 85% phosphoric acid, and the solution was brought to its final volume.

iii. HPLC Analysis

Detector was adjusted to 254 nm. 25 μl of the sample was injected with 25 μl HPLC Hamilton syringe. The peaks were compared with the standard bases, the peak heights were noted down. The % G+C was calculated using the formula given:

$$mol \% G+C = \frac{G+C}{A+G+C+T} \times 100.$$
2.2.7.5.2 16S rRNA gene sequence analysis

2.2.7.5.2.1 DNA extraction

i. DNA extraction by modified Marmur (1961) method

Cell material for 16S rDNA sequencing was taken from 5 ml of well grown liquid cultures. DNA was extracted and purified as described earlier (2.2.7.5.1.1).

ii. Agarose gel electrophoresis

10 μl of genomic DNA, 10 μl of standard genomic DNA (as marker) are electrophoresed (Bangalore GENEI) in 0.8% (w/v) horizontal agarose gel in TAE buffer at 15V/cm, stained in 0.5 μg/ml ethidium bromide and visualized on UV transilluminator (Bangalore GENEI).

2.2.7.5.2.2 Amplification of 16S rRNA gene

Amplification is routinely performed on 50 μl volumes in 0.2 ml microfuge tubes using a DNA thermal cycler (MJ Mini Personal Thermal Cycler – BIO-RAD). All plastic ware was autoclaved and ultraviolet irradiated. The primers used for the amplification of the 16S rRNA gene are Eub27F (5’-GAGTTTGATCCTGGCTCAG-3’) and Univ1492R (5’-GGTTACCTTGTTACGACT T-3’). (Or) F-27 (5’– GTTTGATCCTGGCTCAG-3’) and R-1489 (5’– TACCTTGTTACGACTTCA–3’).

The concentration and volume of the reaction mixture are as follows.
1. Primers: 2 μl of each primer (10 pmol),
2. Template: 2 μl of DNA template, (25 ng μl⁻¹),
3. DNase free Water: 19 μl and
4. Master mix: 25 μl (Obtained from Bangalore GENEI [Cat. No. 105908]).
The thermo cycling parameters

<table>
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<th>Denaturation</th>
<th>Annealing</th>
<th>Elongation</th>
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Agarose gel electrophoresis

Five µl of amplified DNA, 5 µl of 1Kb DNA marker are electrophoresed in 2% (w/v) horizontal agarose gel as described earlier in TAE buffer at 15V/cm, stained in 0.5 µg/ml ethidium bromide and visualized on UV transilluminator.

2.2.7.5.2.3 PCR amplicon purification

The amplified product was purified by using the QIAquick PCR Purification Kit (Cat. No.28104) and the quality and concentration of the purified product were checked by agarose gel electrophoresis as described above.

2.2.7.5.2.4 16S rRNA gene sequencing and assembling of the 4 partial sequences

The 16S rRNA gene sequence was obtained by sequencing with primers *viz.*, Eub27F (5’-GAGTTTGTACCTGGCTCAG-3’), Univ1492R (5’-GGTTACCTTGGTACGACTT-3’) (or) F-27 (5’-GTTTGATCCTGGCTCAG-3’) R-1489 (5’-TACCTTGTTACGACTTCA-3’). The 16S rRNA gene amplicon was outsourced for sequencing at MWG, Bangalore, India. The sequences
obtained as *.scf format were assembled using software Seq Man in the DNA STAR Lasergene 6 package.

2.2.7.5.2.5 BLAST search

The obtained sequences was submitted in the NCBI-BLAST search in order to know the nearest phylogenetic relative. EzTaxon server (web based database of 16S rRNA gene sequences) was more useful for comparison 16S rRNA gene sequences with type strain sequences.

2.2.7.5.2.6 Collection of 16S rRNA gene sequences of the type strains

Based on the blast search results, type strain sequences of the closely related members and an out group sequence were obtained in FASTA format from National Center for Biotechnology Information (NCBI) – Nucleotide search or from Ribosomal Database Project-II (RDP-II) Release 9.56. The type strain numbers were obtained from Bergey’s Manual of Systematic Bacteriology (2005) and from, List of prokaryotic names with standing in nomenclature (LPSN – http://www.bacterio.cict.fr/index.html).

2.2.7.5.2.7 16S rRNA gene Sequence Alignment

The identification of phylogenetic neighbors and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the NCBI-BLAST search (Altschul et al., 1990) and EzTaxon server (Chun et al., 2007). The CLUSTAL-W algorithm of MEGA 4 was used for sequence alignments and MEGA 4 (Tamura et al., 2007) software was used for phylogenetic analysis of the individual sequences. Distances were calculated by using the Jukes and Cantor correction in a pair-wise deletion manner (Tamura et al., 2007). Neighbour-joining (NJ), minimum evolution (ME) and maximum
parsimony (MP) methods in the MEGA4 software (http://www.megasoftware.net/mega4.pdf) were used to construct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure.

2.2.7.5.3 DNA-DNA Hybridization

The taxonomic relationship between strains were examined using DNA-DNA hybridization. DNA-DNA hybridization was outsourced to DSMZ, Germany. Genomic relatedness was determined using a membrane filter technique (Seldin and Dubnau, 1985). Using a DIG High Prime DNA labeling and Detection Starter Kit II (Roche). Hybridization was performed with three replications for each sample (control: reversal of strains was used for binding and labeling) and the mean values are quoted as DNA-DNA relatedness.
2.3 RESULTS

2.3.1 Enumeration of purple non sulfur bacteria (PNSB) from shrimp ponds

Appearance of brownish red-pink coloured colonies in the Paraffin wax overlay of pour plates containing water and sediment samples from various locations of shrimp ponds in the study area revealed the occurrence of PNSB (Fig.1). The details regarding the number of colonies are given in table 5 and 6.

2.3.2 Enrichment of water and sediment soil samples

Out of (84) water samples kept for enrichment, 71 samples showed positive enrichment. However some of the enrichments of water samples from inlet (8), culture ponds (3) and draining channel (2) were negative and all the soil sediment samples (84) showed positive enrichment, with the appearance of brown / brownish-red to purple colour (Table 7 – 7g; Fig.2a-3).

2.3.3 Purification of PNSB

All the (155) total positive enrichments obtained from water and soil sediment samples, were subjected to purification. After repeated streaking on agar slants, using modified Biebl and Pfennig’s (1981) medium, 210 pure cultures of purple nonsulfur bacteria were obtained (Table 8). Based on morphological and cultural characterization, the purified colonies were grouped under 12 PNSB strains (Fig. 4-5j) with alphanumeric strain code viz. BRP (Brown, Red, Purple) and numbers ranging from 1 to 12 viz. BRP1 to BRP12. The details regarding the number of purified colonies representing different strains of PNSB are given in table 8. Among the strains BRP5 alone was found in all the four stations, BRP4 and BRP8 were found in 3 stations excepting Sethubavachatram. While the shrimp ponds containing brackish water (Vadakkupoygainallur and Papakovil) registered more number of
colonies, they were comparatively lesser in ponds containing direct sea water (Sethubavachatram and Karankadu).

2.3.4 Characterization and identification of Purple non sulfur bacterial (PNSB) isolates

PNSB positive enrichments when purified revealed 12 different strains. The strains were characterized, morphologically, culturally, physiologically as well as biochemically based on the Bergey’s manual of systematic bacteriology (2005). On that basis attempts were made to identify the PNSB strains up to species level. However, all the strains could not be identified up to species level. In such cases molecular characterization was employed i.e. 16s rRNA gene sequences were analyzed using appropriate bioinformatics tools to arrive at conclusions regarding the identity of the strains. The characters and identity of the 12 PNSB strains are summarized in tables 9 to 15.

Among the 12 PNSB strains, the identity of BRP3, BRP4, BRP5, BRP7, BRP8, BRP9 and BRP11, could be established based on Bergey’s Manual of systematic bacteriology (2005). These 7 strains were identified as Rhodobacter sphaeroides (BRP3, BRP4 and BRP9), Rhodovulum sulphidophilum (BRP5), Rhodobium orienties (BRP7), Rhodobium marinum (BRP8), and Rhodovulum strictum (BRP11).

The PNSB strains that could not be identified up to species level based on phenotypic characters (BRP1, BRP2, BRP6, BRP10 and BRP12), were further subjected to 16s rRNA gene sequence analysis which helped in establishing the identity of BRP6 and BRP12 as belonging to Rhodobacter capsulatus, based on 100% 16s rRNA gene sequence similarity with the nearest type strain and BRP10 was identified as Rhodobacter maris., based on 100% 16s rRNA sequence similarity. However, there was ambiguousness with regard to BRP1 and
BRP2 strains even at the genera level, where their 16srRNA sequences, showed only 92.2 % sequence similarity with *Rhodospirillum sulfurexigens* and 91.5 % with *Rhodospirillum rubrum*. It is a well known fact that the members of *Rhodospirillum* spp., have a spiral shaped cell morphology, but the strains BRP1 and BRP2, exhibited “curved rod” shaped cell morphology. As the sequence comparison results obtained contradicted with the cellular morphology of the strains BRP1 and BRP2, this ambiguousness necessitated the isolates to be probed further. Hence these two strains were subjected to detailed characterization based on polyphasic taxonomic approach.

2.3.4.1 Polyphasic characterisation of BRP1 (JA480T) and BRP2 (JA481)

2.3.4.1.1 Habitat

The strain BRP1 (= JA480T) was isolated from a sediment sample collected from a brackish shrimp pond (water pH was 8.2, temperature 30°C.) at Vadakkupoygainallur, a village near Nagapattinam, Tamilnadu, India. (GPS positioning of the sample collection site; 10°44′06.09″N and 79°50′12.96″E) on 9th march 2003. Strain BRP2 (= JA481) was isolated from brackish water sample collected near another village Pappakovil, Nagapattinam (GPS positioning of the sample collection site; 10°44′39.75″N and 79°49′39.61″E) on 12th September 2006.

2.3.4.1.2 Colonial characteristics of the strain BRP1 and BRP2

Colonies of strain BRP1 were round, convex, large, sticky and reddish- brown pigmented (Fig.6a). The colonies of the strain BRP2 were round, convex, small, sticky, reddish- brown pigmented colonies (Fig.11a).
2.3.4.1.3 Morphology and fine structure

Cells of strain BRP1 were vibrioid shaped (Fig.6c), 1-2 μm long and 0.3-0.5μm wide. The cells of BRP2 were vibrioid, 2.1x0.7 μm wide. The cells of both the strains stained gram negative. The cells of BRP1 and BRP2 strains are non-flagellated and if present (1 in 100) are monopolar biflagellate. Flagellar motility could not be demonstrated at any stage of phototrophic growth under phase contrast microscope and the transmission electron microscopic analysis also showed cells without flagella. A more careful search confirmed the presence of monopolar biflagellate cells (Fig.6d) in these strains, however, their number was very low (1 out of 100). Soft agar (0.5% w/v) stabs confirmed the motility of the cells, where the strain diffused throughout the medium (Fig.8). Phototaxis was observed in BRP1 and BRP2 where the tubes incubated in dark (completely covered with an aluminum foil) had no growth, while the cells (after 5 days of incubation) moved in the direction of light (Fig.9) in the tube where illumination was provided only from the bottom. Transmission electron micrographs of ultrathin sections of both strains revealed chimeric type of intracellular cytoplasmic membrane (ICM) structures (Fig.7 and 12b), where both lamellar stacks and vesicles are present in a single cell.

2.3.4.1.4 Pigment composition

The colour of the photosynthetically (anaerobic under light 2,400 lux) grown cell suspension is light brown. Whole cell absorption spectrum of BRP1 = (BRP2) showed absorption maxima at 488 (491), 524 (527), 593, 794 and 863 nm (Fig.10a), indicating the presence of bacteriochlorophyll a. Acetone extracted spectrum of both the strains showed absorption maxima at 360, 474, 504, 579 nm (Fig.10b), indicating rhodopinal series of carotenoids. Carotenoid composition of both the strains as determined by C18-HPLC analysis, showed the
presence of rhodopin (80 mol%; t<sub>R</sub>=9.37 min; absorption maxima - 294, 443, 470, 501 nm), 4,4′-diapolyycopene (17 mol%; t<sub>R</sub>=5.41 min; absorption maxima - 225, 445, 470, 502 nm) and tetrahydrolycopin (3 mol%; t<sub>R</sub>=18.3 min; absorption maxima - 294, 361, 447, 471, 502 nm). Carotenoid glycosides could not be detected from the acetone extracts of both strains using HPLC.

2.3.4.1.5 Cellular fatty acid composition

The predominant fatty acids present in the whole cells of BRP1 were C<sub>18:1</sub> ω7c and C<sub>16:0</sub>, whereas minor quantities of C<sub>16:1</sub> ω7c/C<sub>16:1</sub> ω6c were observed and summarized in the table 16.

2.3.4.1.6 Physiological characteristics

The strains BRP1 and BRP2 were able to grow photoheterotrophically (2,400 lux) with pyruvate (0.03% w/v) as carbon sources/electron donor. Photolithoautotrophic growth was observed at [2400 lux], by the utilization of sodium sulfide, sodium thiosulfate [1mM] and sodium bicarbonate [0.1% w/v]), chemolithoautotrophy (aerobic, dark, sodium thiosulfate [1mM] and sodium bicarbonate[0.1% w/v]). Chemoheterotrophy and fermentative growth could not be demonstrated in these strains. Not many organic substrates supported growth of the strains (Tables 16-20). The substrates that were utilized as carbon/electron donors under photoheterotrophic conditions included acetate, pyruvate and succinate. Those which could not be utilized included glucose, fructose, glutamate, lactate, malate, methanol, ethanol, caproate, caprylate, glycerol, butyrate. BRP1 and BRP2 strains could be grown with ammonium chloride and glutamine as nitrogen sources while, molecular nitrogen, urea, glutamate, aspartate, nitrite and nitrate did not support phototrophic growth. Biotin and p-amino benzoic acid are required as growth factors. Sulfide is obligatory and used as sulfur
source, while thiosulfate and cysteine did not support phototrophic growth of BRP1. There was no requirement of NaCl for growth and this strain tolerated up to 0.5% NaCl. pH range is from 7.0-8.0 with an optimum at 6.8. The optimum growth temperature ranged from 25-30°C.

2.3.4.1.7 mol% G + C content of DNA

The G+C content of the genomic DNA of strains BRP1 and BRP2 was 67.8 and 68.8 mol% (by HPLC), respectively.

2.3.4.1.8 16S rRNA gene sequence similarity

16S rRNA gene sequences of BRP1 were obtained, and the identification of phylogenetic neighbors and calculation of 16S rRNA gene sequence, revealed that the isolates belonged to the family Rhodospirillaceae of the class Alphaproteobacteria. Highest sequence similarities were found between BRP1 strain, and the type strains of Rhodospirillum sulfurexigens (92.2%), Rhodospirillum rubrum (91.5%) and Rhodospirillum photometricum (91.4%). The similarity to other species of the genera Rhodocista, Phaeospirillum, Rhodovibrio, Rhodospira and Roseospira was 80-91%. Between strains BRP1 and BRP2, the 16S rRNA gene sequence similarity was 100%.

2.3.4.1.9 16S rRNA gene sequence deposition

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the strain BRP1 (= JA480T) is FN 391894.1 and BRP2 (= JA481): FN543109.1.

2.3.4.1.10 Culture deposition of Strain

BRP1 (= JA480T): KCTC 5825T = NBRC 106163T = DSM23193T.
2.4 DISCUSSION

PNSB are known to live in aquatic habitats with significant amounts of soluble organic matter and low oxygen tension, and also utilize various nitrogenous substances for their growth (Gobel, 1978; Imhoff, 2006). In the shrimp ponds there is excessive accumulation of organic matter, leading to intensive organic matter degradation at the pond bottom and high sediment oxygen demand exceeds the oxygen renewal rate. This leads to the development of anoxic conditions in the sediments and at the sediment water interfaces (Avnimelech and Ritvo, 2003). Besides the organic matter accumulation, the release of nitrogenous substances takes place on the course of cultivation of shrimps. The nitrogenous substances are released into the pond from the feed pellets which nearly contain, 80% nitrogenous matter (Briggs and Funge smith, 1994). The other sources of nitrogen in the pond are, the excretory products let out from the growing shrimps in the form of ammonia (Regnault, 1987; Hargreaves, 1998). Other than organic nitrogenous sources, nutritional elements from the soil are also released into the water column of the shrimp ponds (Sun and Zhaoyang, 1997). This makes the shrimp pond an ideal habitat for the PNSB members to thrive there.

In the present endeavor to screen the prevalence of PNSB in the brackish and direct sea water shrimp ponds, the water samples and soil sediments were collected from various locations (inlet, culture pond and draining channel) of each shrimp pond. The sampling was carried out at regular intervals during various cycles (days) of shrimp growth (Seed stocking till harvest). The collected samples (water and soil sediments) were subjected to enumeration and enrichment. The enumeration of PNSB from shrimp pond samples, yielded brown to red pigmented colonies. The PNSB cell numbers in the soil sediment as well as water samples ranged from $0.9 \times 10^2$ to $1.0 \times 10^4$ cells/gram and $1.2 \times 10^2$ to $1.88 \times 10^4$ cells/mL respectively.
During the enumeration of PNSB from various shrimp pond locations like inlet, culture pond, draining channel, all the soil sediment samples showed a sizeable population of PNSB members, but in water samples from the inlet of shrimp ponds showed negative presence of PNSB colonies in many instances (Table 5). In the inlet water samples collected from Karankadu, during the entire period of shrimp culture cycle the PNSB colonies were very few to non-existent. In the water samples collected from culture ponds from Vadakkupoygainallur, Pappakovil and Karankadu on certain days of culture cycle the PNSB colonies were absent, except Sethubavachatram (Table 5).

Among the stations, Vadakkupoygainallur, registered cent percent presence of PNSB colonies in the water samples collected from draining channel of the shrimps ponds followed by Sethubavachatram (85.7%), Pappakovil (71.42%) and Karankadu (28%). On the whole the inlet water samples collected from brackish ponds harbored more PNSB colonies than its direct sea water counterpart. All the soil sediment samples kept for enrichment gave 100% positive enrichment, but water samples gave only 58.2% positive enrichment. The percentage negative enrichments observed in the water samples were 28.57%, 10.71% and 7.14% respectively in various locations like inlet, culture pond and draining channel. Low prevalence of PNSB members and negative enrichments in the water samples may due to the absence of anoxic niches in the water inlet/ marine water sources, as phototrophic bacteria occur at all kinds of marine environment and they prefer anoxic zones, but some of this bacteria live in anoxic niches of the bulk oxic environment, and the tidal fluctuations in the marine environment also influence the presence of photosynthetic bacteria, where smaller the water exchange with the tides the better the
conditions for the establishment of an anoxic water body, as rightfully observed by Imhoff (2002).

During certain sampling cycles, the culture pond water samples (Vadakkupoygainallur, Pappakovil, and Karankadu) showed absence of PNSB colonies and negative enrichments were exhibited from the sample collected from two stations (Vadakkupoygainallur, Pappakovil). This may be attributed to the usage of aerators in the shrimp ponds, thereby disturbing their anoxic niche.

The draining channel samples (Pappakovil, Sethubavachatram, and Karankadu) showed absence of PNSB during enumeration, and negative enrichments were observed from stations like Karankadu and Sethubavachatram. This may be attributed to the nature of water samples collected from the draining channel i.e whether it is free flowing or stagnant. In the draining channel with stagnant pool of water positive enrichments could be observed because of its anoxic nature with sufficient quality of light. But in the case of water samples collected from the draining channels where the culture waste water discharge was free flowing the conditions will be oxic (rather than anoxic) which might not have favored PNSB. Such observations were also made by Imhoff (2002) in other types of marine environment.

2.4.1 Isolation and characterization of PNSB

The positive enrichments were Brown/red colour. The positive enrichments were subjected to purification based on the purification protocols of Archana et al. (2003), which helped in the successful isolation and purification of PNSB colonies. The resulting PNSB colonies were grouped into 12 Strains (BRP1 to BRP12), and identified up to genera level based on Bergey’s manual of systematic bacteriology (2005). Accordingly the strains BRP3, BRP4, BRP6,
BRP9, BRP10 and BRP12 were identified as belonging to the Genus, *Rhodobacter* spp., the strains BRP5 and BRP11 as *Rhodovulum* spp., and BRP7 and BRP8 as *Rhodobium* spp. The 10 strains that were identified up to genera level were subjected to characterization based on Bergey’s manual of systematic bacteriology (2005) for species identification.

The strains BRP3, BRP4 and BRP9 identified as belonging to the genera *Rhodobacter* spp., was further tentatively identified to their closest species as *Rhodobacter sphaeroides*, showing minor variations in cell size and carbon utilization. These strains (BRP3, BRP4, BRP9), lacked capsulation of cells, absence of “Zig-zag” cell arrangement and did not have obligate requirement for NaCl. However the strains BRP3, BRP4, BRP9 showed tolerance to NaCl at concentrations of 3%, 2% and 4% respectively. The characteristic feature of saline tolerance existing among the members of *Rhodobacter sphaeroides* has been reported by Imhoff (2005a). Variation was observed in the carbon utilization pattern also. While one strain of *Rhodobacter sphaeroides* (BRP4) tested negative for citrate utilization, the other two strains (BRP3 and BRP9) tested positive. This can be considered as minor strainal variation. Such type of variation in carbon utilization pattern has been reported by Sasikala (1995) from the fresh water strains of *Rhodobacter sphaeroides* too.

The species identity of the strains BRP6 and BRP12 under the Genus *Rhodobacter* spp., could not be established because they differed from the ideal biotypes of suspected species of *Rhodobacter* spp., (viz., *R.capsulatus*) with regard to carbon utilization. Hence they were subjected to 16s rRNA sequencing. The sequence results obtained for BRP6 and BRP12 showed sequence similarity of 100% with *Rhodobacter capsulatus* strains viz., ATCC11166 and *Rhodobacter*
capsulatus XJ-1. This kind of unusual pattern in carbon utilization has been observed in *Rhodobacter capsulatus* strains by Weaver et al., (1975), where two strains among all the other test strains of *Rhodobacter capsulatus* namely P12F1 and YW1 which were isolated from a lake and forest environment respectively, showed negative for the utilization of propionate and a single strain of *Rhodobacter capsulatus* isolate marked as (SCJ) strain, which was isolated from sugar cane field soil showed moderate growth in tartarate. Hence this unusual pattern of carbon utilization in BRP6 and BRP12 can be considered as a minor variation and their identity as *Rhodobacter capsulatus* can be confirmed based on 100% 16s rRNA sequence similarity. Strains BRP6 and BRP12 showed saline tolerance up to 4%. Though the members of *Rhodobacter* spp., are termed as fresh water species without the obligate requirement for Nacl, this character of saline tolerance makes them to be present in brackish environment also.

The strain BRP10 which was characterized as belonging to *Rhodobacter* spp., upto genera level, could not be characterized at species level based on Bergey’s manual of systematic bacteriology (2005), as this strain could not utilize majority of carbon sources as utilized by other members of *Rhodobacter* spp. Hence this strain (BRP10) was subjected to 16srRNA sequencing, and the sequence results, showed 100% sequence similarity with *Rhodobacter maris* JA276ᵀ. Based on the sequence data and physiological properties of this strain, BRP10 was characterized as *Rhodobacter maris*.

The strains BRP5 and BRP11 which showed phenotypic similarity with *Rhodovulum* spp., at the genera level, was tentatively identified up to their closest species as *Rhodovulum sulphidophilum* (BRP5) and *Rhodovulum strictum* (BRP11), based on Bergey’s manual
of systematic bacteriology (2005). All the strains of BRP5 and BRP11 required NaCl for growth. The strain BRP11 was slightly halophilic and slightly alkalophilic and showed a peculiar narrow optimal NaCl and pH growth range of 0.5-1% NaCl, and pH of 8.0-8.5. Thiosulphate and sulphide utilization and tolerance are exhibited by these strains. This specific characteristic feature among the members of *Rhodovulum* spp., was also observed by Hiraishi and Ueda (1995) where their strains of *Rhodovulum* spp., obtained from tidal sea water pools also showed a narrow NaCl conc. (0.8%) and pH (8.0-8.5) and with high sulfide tolerance.

The PNSB strains BRP7 and BRP8 which were identified as belonging to the members of the Genera of *Rhodobium* spp., showed rod shaped budding cells, actively motile and were halophilic requiring NaCl for their growth. In the strains of BRP7 thiosulfate served as an electron donor with obligatory requirement of NaCl and they tolerated NaCl conc. upto 8%, with an optimal growth pH of 7.5. In the strain BRP8, thiosulfate could not be utilized but utilized sulphide, and tolerated NaCl conc. up to 5%, the optimal growth pH was 7.0. Both these strains utilized a variety of carbon, nitrogen and vitamin sources for the growth. These strains were further characterized upto species level as *Rhodobium orientis* (BRP7) and *Rhodobium marinum* (BRP8) based on Bergey’s manual of systematic bacteriology (2005).

The strains namely BRP1 and BRP2, though showed a phenotypic character like sulphide utilization as in *Rhodopseudomonas* spp., and *Rhodobacter* spp., respectively, the definitive morphological characteristic feature viz., budding cells and rosette formation prevalent in *Rhodopseudomonas* spp., was absent in BRP1 and BRP2 and these strains were not actively motile. However in BRP2 most of the strains were non motile, and the cells were not ovoid or straight rods as
commonly observed in *Rhodobacter* spp. Instead they were curved rods/vibriod in nature and both these strains showed poor growth in many organic substrates. The strains BRP1 and BRP2 were not metabolically versatile and they were strictly anaerobic and obligately phototrophic. As these two strains (BRP1 and BRP2) showed ambiguity in identification at the genera level based on Bergey’s manual of systematic bacteriology (2005), they were subjected to 16s rRNA sequencing, and the sequence results showed (92.%) sequence similarity with *Rhodospirillum sulfurexigens*, and (91.5 %) with *Rhodospirillum rubrum*. It is a well known fact that the members of *Rhodospirillum* spp., have a spiral shaped cell morphology, whereas the strains BRP1 and BRP2, exhibited “curved rod” shaped cell morphology. This ambiguousness necessitated the strains (BRP1 and BRP2) to be probed further, where these two strains were subjected to detailed characterization based on polyphasic taxonomic approach.

### 2.4.2 Polyphasic characterization of the strains BRP1 = JA480<sup>T</sup> and BRP2 = JA481

The phylogenetic relationship of strains BRP1 and BRP2 with other purple non-sulfur bacteria, as examined by near complete (1450 bp) 16S rRNA gene sequence, revealed that the strains belong to the family *Rhodospirillaceae* of the class *Alphaproteobacteria* (NJ tree is shown in Fig.13a; while other [ME, MP] trees have similar tree topologies; Fig.13b,13c, respectively).

The highest sequence similarities were found between the strain BRP1 = JA480<sup>T</sup> with the type strains of *Rhodospirillum sulfurexigens* (92.2%), *Rhodospirillum rubrum* (91.5%) and *Rhodospirillum photometricum* (91.4%). The similarity to other species of the genera *Rhodocista, Phaeospirillum, Rhodovibrio, Rhodospira* and *Roseospira* was only 80-91%. Between strains BRP1 and BRP2 the 16S rRNA
gene sequence similarity was 100% with a DNA-DNA relatedness of 89±1% (triplicate analysis).

The strain BRP1= JA480ᵀ was distinct from its phylogenetically close relatives (Rhodospirillum/Phaeospirillum), due to presence of vibriod cell shape, chimeric ICM structures, presence of significant levels of C₁₆:₀ over C₁₆:₁ω₇c/C₁₆:₁ω₆c and having limited (pyruvate, succinate, acetate) utilization of carbon/donor for growth. The morphological, physiological and molecular traits of strain BRP1=JA480ᵀ are sufficient to place it in the new genus Phaeovibrio. Phylogenetically the genus Phaeovibrio (Lakshmi et al., 2011) belongs to the family Rhodospirillaceae (Imhoff, 2005b) of the order Rhodospirillales belonged to the class alphaproteobacteria of the phylum Proteobacteria (Garrity et al., 2005) of the domain Bacteria.

2.4.3 Description of Phaeovibrio gen. nov.

Phaeovibrio (Pha.e.o.vi'br.i.o. Gr. adj. phaeos, brown; L. v. vibro, to set in tremulous motion, move to and fro, vibrate; N.L. masc. n. vibrio, that which vibrates, and also a bacterial genus name of bacteria possessing a curved rod shape (Vibrio) N.L. masc. n. Phaeovibrio, brown vibrio), encompasses gram-negative, vibrioid, mesophilic and freshwater phototrophic bacteria. Cells are motile by polar flagella and multiply by binary fission. Chimeric internal membranes of lamellar stacks and vesicles present in a single cell. This is the first report of chimeric ICM structures among the members of phototrophic bacteria. Bacteriochlorophyll a and carotenoids of rhodopinal series are the major photosynthetic pigments. Metabolism is strictly anaerobic and obligately phototrophic. Limited number of organic substrates can be photoassimilated. Growth factors are required. C₁₈:₁ω₇c and C₁₆:₀ are the major fatty acids. The G+C content of genomic DNA is 67.8-68.8 mol% (HPLC). Delineation of the genus is determined primarily by the
phylogenetic information from 16S rRNA gene sequences. The type species is *Phaeovibrio sulfidiphilus*.

### 2.4.3.1 Description of *Phaeovibrio sulfidiphilus* sp. nov.

*Phaeovibrio sulfidiphilus* (sul.fi.di'phi'lus. N.L. n. sulfidum, sulfide; N.L. masc. adj. philus (from Gr. masc. adj. philos), friend, loving; N.L. masc. adj. sulfidiphilus, sulfide loving).

Cells are 0.3-0.5 μm wide and 1.2-2.5 μm long. Phototrophically grown cultures are light brown. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 377-380, 488-491, 524-527, 593, 794 and 863 nm. The type strain is mesophilic, NaCl is not required for growth but up to 0.5 %NaCl is tolerated, pH optima at 7 (range: 7-8), obligate photoheterotroph. Growth occurs with acetate, pyruvate and succinate. Ammonium salts and glutamine are used as good nitrogen sources. Sulfide is required as sulfur source. Biotin and *p*-amino benzoic acid are required as growth factors. The predominant components of cellular fatty acids are C\textsubscript{18:1} ω7c and C\textsubscript{16:0}. The G+C of genomic DNA is 67.8-68.8 % (by HPLC). The type strain JA480\textsuperscript{T} = BRP1 (=KCTC 5825\textsuperscript{T} = NBRC 106163\textsuperscript{T} = DSM23193\textsuperscript{T} ) and its additional strain BRP2= JA481 were isolated from the brackish waters of Nagapattinam district, Tamilnadu, India.

Thus characterization based on polyphasic approach of both BRP1 and BRP2 yielded novel genera belonging to the family Rhodospirillaceae, proposed as *Phaeovibrio* spp., gen. nov., and a novel type species of this genera, *Phaeovibrio sulphidiphilus* gen nov. sp. nov. Among the 210 strains of purple non sulfur bacteria isolated from brackish and direct sea water shrimp ponds, the majority of the strains belonged to the genera *Rhodobacter* spp., (34.28%), *Phaeovibrio* spp., nov. (30%), *Rhodovulum* spp., (26.19%) and *Rhodobium* spp., (9.52%). Among this 4 genera isolated from shrimp
ponds, the species that were prevalent in each type of shrimp pond are as follows. *Phaeovibrio sulphidiphilus* gen. nov., sp. nov., was prevalent only in brackish shrimp ponds of Vadakkupoygainallur and Pappakovil. The presence of *Rhodobacter sphaeroides* was observed in both brackish and direct sea water shrimp ponds excepting the station Sethubavachatram. *Rhodobacter capsulatus* was prevalent in both brackish and direct sea water shrimp ponds of Pappakovil and Sethubavachatram only but not in Vadakkupoygainallur and Karankadu. *Rhodobacter maris* was present only in direct sea water shrimp ponds of Sethubavachatram and karankadu. *Rhodovulum sulphidophilum* was present in all the shrimp ponds both brackish and direct sea water. *Rhodovulum strictum* was present in one brackish shrimp pond of Vadakkupoygainallur alone. *Rhodobium orienties* was present in the direct sea water shrimp pond of Sethubavachatram only. *Rhodobium marinum* was present in both brackish and direct sea water shrimp ponds excepting Sethubavachatram.