PHOTOSYNTHETIC BACTERIA FOR BIOREMEDIATION OF HYDROGEN SULPHIDE IN AQUATIC SYSTEMS

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Photosynthetic Bacteria for Bioremediation of Hydrogen Sulphide in Aquatic Systems

Ph.D. Thesis under the Faculty of Environmental Studies

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Certificate

This is to certify that research work presented in the thesis entitled is "PHOTOSYNTHETIC BACTERIA FOR BIOREMEDIATION OF HYDROGEN SULPHIDE IN AQUATIC SYSTEMS" based on the original work done by Mrs. Ammu Thomas under my guidance at National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition. All the relevant corrections and modifications suggested by the audience and recommended by the doctoral committee of the candidate during the pre-synopsis seminar have been incorporated in the thesis.

Cochin-682016
August 2017

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Declaration

I hereby do declare that the work presented in this thesis entitled "PHOTOSYNTHETIC BACTERIA FOR BIOREMEDIATION OF HYDROGEN SULPHIDE IN AQUATIC SYSTEMS" is based on the original work done by me under the guidance of Prof. I. S. Bright Singh, UGC BSR Faculty, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin- 682016, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Chapter 1

GENERAL INTRODUCTION

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1.1 Hydrogen sulphide in aquatic systems

Hydrogen sulphide is an important substance in the aquatic environment. Its presence and reactions affect photosynthesis, algal respiration, and the iron cycle in the establishment of the electron (E*h*) and proton (pH) activity of the aquatic environment (Bass Becking *et al.*, 1960). Hydrogen sulphide is an extremely active chemical and biochemical participant and it can easily reduce biogeochemical pathways.

Hydrogen sulphide constantly evolved from organic sludge in streams and to be a normal product found in hypo limnetic situations during various seasons of the year. A variety of industrial effluents contain hydrogen sulphide or their break-down products resulting hydrogen sulphide generation in aquatic systems. Although, hydrogen sulphide

oxidizes rapidly, its continued evolution from bottom muds and formation from break-down products may result substantial concentration occurring at all times in some fish habitats. Hydrogen sulphide affects oxygen relationships in the organism and also may affect mitochondrial systems. Besides, in aqueous solutions, total sulphide quantity is found as dissolved hydrogen sulphide (H_2S) and as HS^- and S^{2-} ions, depending on proteolytic equilibrium and its increased toxicity for higher order organisms. Similarly hydrogen sulphide plays an important role in biogeochemical processes at oxic-anoxic interfaces, such as in the formation of heavy metal precipitates and oxidation by phototrophic or chemolithotrophic bacteria, respectively. (Jeroschewski, *et al*., 1996).

Basic sulphide chemistry must be explained at the outset of this chapter. Hydrogen sulphide (H_2S) in aqueous solution dissociates into the hydrosulphide anion (HS) and the bisulphide anion (HS^-) and their proportions of the three species vary with pH. At physiological and environmental pH around 7, gaseous H_2S and HS anion are the important forms. H_2S is the form that freely diffuses across membranes.

The possible products of the oxidation of sulphide (oxidation state 2-) include: polysulphide (oxidation state 2^-), elemental sulphur S (0), thiosulphate, sulfone and inner sulfone sulphur atom, sulphite SO^{4-} (4+) and sulphate SO^{2-} (6+). Production of sulphate leads to highly acidic conditions, whereas thiosulphate is non-acidic, non-toxic, soluble, and still contains a large amount of energy. 'Thiols' is a collective name for reduced sulphur compounds, including sulphide, cysteine, methionine, glutathione, and disulphides. Sulphide chemistry illustrates the formation of hydrogen

sulphide, dissociation of hydrogen sulphide and toxic non toxic element of hydrogen sulphide in the aquatic system.

1.2 Formation of hydrogen sulphide in aquatic systems

It has generally been accepted that the only significant route of hydrogen sulphide production in the aquatic environment is sulphate reduction, the microbial reduction of higher oxidation state inorganic sulphur, primarily sulphate, which serves as the terminal electron acceptor from the anaerobic oxidation of organic matter in the respiratory process. The principal sulphate reducing bacterium is *Desulfovibrio desulphuricans.* However, it is common knowledge that hydrogen sulphide is a product of the microbial-mediated anaerobic decomposition of the sulphur fraction of proteinaceous matter (putrefaction) and there is evidence that putrefaction can play a very significant role in hydrogen sulphide production in anoxic sediment under some conditions (Nriagu, 1968; Gunkel and Oppenheimer, 1963; Koyama and Sugawara, 1953; Ivanov and Terebkova, 1959). In sediments, sulphate and H_2S are constantly recycled between oxidation and reduction step, due to microbial action.

While the seafloor hydrothermal vents are certainly the most spectacular sulfidic habitats (Corliss *et al*., 1979; Spiess *et al*., 1980; Desbruyeres *et al.,* 1982; Chase *et al*.. 1985; Tunnicliffe *et al*., 1986; Rona *et al*., 1986) and have propelled the current interest in sulphide-related metabolism and physiology, it is important to realize that sulphide occurs in many other habitats (Brooks *et al*., 1979; Paul1 *et al*., 1984;Kennicutt *et al*., 1985) and is ubiquitous in marine sediments worldwide (Fenchel and Riedl, 1970; Goldhaber and Kaplan, 1974). High-temperature interactions between seawater and rock produce sulphide at the seafloor vents (Edmond *et*

*al.,*1982; Johnson *et al.,* 1988), whereas the activity of sulphate-reducing bacteria generates the sulphide at the brine seeps and in marine sediments in general (Brooks *et al.,* 1979; Jorgensen, 1982, 1984). Once oxygen is depleted by aerobic heterotrophic bacteria in sediments, alternative electron acceptors such as nitrite, nitrate, metal oxides, carbon dioxide and sulphate are used by anaerobic bacteria in decomposing the remaining organic matter. The presence of high concentrations of sulphate (20-30 mM) indicates that sulphate reduction is the dominant process in marine sediments.

An estimated 30 million tons of hydrogen sulphide is produced annually from aquatic areas, 60-80 million tons from natural land sources (e.g., volcanoes, sulphur springs) and 3 million tons from industries and pollution sources (Beauchamp *et al*., 1984). Salt marshes, coastal lagoons, mangrove swamps, stagnant basins and anoxic sediments are some of the habitats where sulphide is produced in large amounts. The 2000m deep black sea has a more or less permanent sulfidic layer below 150-200 m (Sorokin, 1972). Other stratified water bodies with a sulfidic lower layer include the Cariaco Trench off Venezuela (Baird *et al*., 1973) and Solar Lake in the Sinai (Jorgensen, 1982). Occurrences of sulphide in the open sea beneath areas with intense productivity have also been reported (Dugdale *et al*., 1977). Anthropogenic inputs of organic carbon from sewage plants, paper mills, other factories, and aquaculture farms have led to increase in hydrogen sulphide, heavy metals and other xenobiotics in stream and coastal habitats (Ellis, 1937; Southern California Coastal Water Research Project, 1989; Thompson *et al*., 1989). Sediments under Atlantic salmon cages and intensive mussel farms can have sulphide levels at an order of magnitude greater than background (Gunnarson *& Ronnow*, 1982; Lieffrig, 1985).

Sulphide concentrations show gradients with depth into the sediment, or in the water column (Fenchel, 1969; Jorgensen, 1982, 1984). Sulphide concentrations in salt marshes show die1 fluctuations (Ingvorsen and Jorgensen, 1979), and vary with season, being typically low in winter and high in summer and early fall, in consonance with the seasonal fluctuations in rates of organic matter production and sulphate reduction (Groenendaal, 1979; Senior *et al*., 1982; Jorgensen, 1984; Luther *et al.*, 1986; Hines *et al.*, 1989).

It is important to realize that oxygen is also present in sulphide-rich habitats, and the interface between the two can vary in scale from meters to millimeters (Sorokin, 1972; Jorgensen, 1982). In the shallow waters of tidal flats and lagoons under certain conditions, 30-50 µM sulphide has been measured in the presence of 125 µM oxygen (Bella *et al.*, 1972; lngvorsen and Jorgensen, 1979). At the hydrothermal vents with 0.6-330, µM sulphide, l0-125, µM oxygen has been measured (Johnson *et al.*, 1988). In the Santa Barbara basin where pockets of black mud contain about 22 µM sulphide, 18-20, µM oxygen occurs 1 cm above the sediment-water boundary (Cary *et al.*, 1989). Oxygen is crucial to the survival of sulphide-tolerant organisms (Jorgensen, 1982, 1984; Nelson and Jannasch. 1983; Fox and Powell. 1986, 1987; Julian *et al.*, 1990) that they shift to anaerobic metabolism and enzymatic oxidation by mitochondria.

1.3 Toxicity of hydrogen sulphide –general view

Sedimentary production of hydrogen sulphide can increase the oxygen demand rate of sediment leading to a reduction in dissolved oxygen in overlying waters. Hydrogen sulphide is toxic to fish and macro invertebrates particularly at pH < 7 when 50-100ppm of the hydrogen

sulphide present is in the undissociated form, H_2S. Acute toxicity to rainbow trout and sensitive macro invertebrates occurs at 0.0087 and 0.20mg hydrogen sulphide respectively (Smith *etal.,* 1976). Hydrogen sulphide has been associated with corrosion of concrete sewers (Parker, 1945), cooling water towers stone and metal pipe (Postgate, 1959). There has been considerable recent environmental health interest in the role of natural sources of hydrogen sulphide in the formation of sulphur oxides and sulphuric acid aerosol near coastal environments.

Oxidation of H_2S is very important in ponds, which otherwise will lead to blackening of sediment and from sediments it will diffuse into overlying water column, triggering deleterious reactions. Recommended safe hydrogen sulphide concentration in shrimp culture is less than 0.02mg/L, preferably not detectable (Whetstone *et.al.,*2002). Exposure to H_2S is extremely toxic to aquatic fauna, it may affect health, survival and productivity of aquatic organisms. H_2S readily diffuses across membranes and inhibit respiratory enzymes and blood pigments (Phillips *et al*. 1997). H_2S is recognized as a potent inhibitor of cytochrome "c" oxidase, the terminal enzyme of oxidative phosphorylation. This involves the binding of the heme of the enzyme, with the greatest affinity being for the oxidized ($Fe3^+$) state. This process completely inhibits the aerobic metabolism, followed by ATP depletion and finally hypoxia condition is developed in the aquatic system (Eghbal *et al.* 2004). Hydrogen sulphide toxicity, its consequences and how much it would effect in aquatic systems are described below.

1.3.1 Fishes

Acuteexposure of freshwater and marine fishes to sulphide also results in an initial stimulation of both ventilation and circulation, followed

by a repression of metabolic pathways especially respiration as mentioned earlier (Torrans and Clemens. 1982; Bagarinao and Vetter, 1989). In the channel catfish *Ictalurus punctatus,* the heart rate increases from 88 to 128 beats.min^{-1}within 1 min of exposure to 16 µM H_2S at 20 °C, while the ventilation rate slows down from 140 to 128 cycles min^{-1} but becomes deeper (Torrans and Clemens, 1982).

Documented effects of sulphide on freshwater fishes include: (1) enhanced survival and growth at low sulphide concentrations between 0.02 and 0.4ppm H_2S (attributed to the antibiotic effect of sulphide); (2) reduced survival and growth at sulphide concentrations greater than 0.45 µM H_2S; (3) lower swimming endurance; (4) tissue irritation and necrosis; (5) lower food consumption and conversion: (6) inhibited spawning behaviour and reduced egg production; and (7) lower survival of eggs, and smaller size and higher incidence of deformities in newly-hatched larvae (Adelman and Smith, 1970; Oseid and Smith, 1972; Smith and Oseid, 1976; Smith *et al.*, 1976; Reynolds and Haines, 1980). A study in the Ganges River (India) shows that 30-day exposure of sexually maturing common carp *Cyprinus carpio* to a sub lethal concentration of 280 µM total sulphide (about 28 µM H_2S) causes a gradual decrease in the gonadosomatic index (% body weight as gonads) due to hepatic malfunction (Kumar and Mukherjee, 1988).

1.3.2 Plants & Algae

Sulphide has been reported as a toxic component and it may affect growth and survival of plants including algal growth *eg.,* physiological disorders of rice, and identified as the primary cause of straight head disease and mild sulphide disease (Allam, 1972; Joshi *et al.*, 1975; Joshi and Hollis, 1977). Sulphide inhibits respiration, oxygen release, and nutrient uptake by

rice roots. Disease-resistant cultivars show higher tolerance to sulphide. Likewise, sulphide inhibits the growth of the salt marsh plants, *Puccinellu martima, Atriplex patula* and *Festuca rubra* (Ingold and Havill, 1984). *Spartina alterniflora* the cord grass that dominates the salt marshes of the US east coast, takes up sulphide without acute toxicity effects over a long growing season (Carlson and Forrest, 1982). Sulphide has complex effects on multiple metabolic parameters eg. Photosynthesis, cell division, respiration, assimilation and fermentative ability in cyanobacteria and unicellular algae. Some species and strains of algae could multiply in the presence of 250-500 µM sulphide (Cyanobacteria.spp), whereas others are inhibited in 30-60 µM sulphide.

1.3.3 Invertebrates

The toxic effects of sulphide on aquatic invertebrates have not been much studied, but metabolic pathways may affect with the presence of sulphide concentration beyond the limit as discussed earlier as above, and at the biochemical level (see below).

1.3.3.1 Toxic effects at the biochemical level

The effects of sulphide at the whole-organisms, level have their basis at the cellular and molecular level. Khan (1990) categorizes the in vivo biochemical effects of sulphide in mammals to be of the primary type: inhibition of cytochrome "C" oxidase and oxidative phosphorylation, resulting in histo-toxic hypoxia and loss of energy; and of the secondary type: metabolic impairment due to changes in enzyme activities, metabolites and cofactors; and generation of reactive radicals and alteration of membrane permeability, causing edema and organ-specific functional disorders. Enzyme inhibition by sulphide may be due to direct interference

with essential metal cofactors or the obligatory metal cofactor, modification of disulphide groups, or other interactions with cellular components.

1.3.3.2 Inhibition of Cytochrome "C" oxidase and mitochondria

Sulphide like cyanide is a slow-binding high-affinity inhibitor of cytochrome "C" oxidase, reversible and non-competitive with respect to both ferrocytochrome "C" and oxygen (Nicholls, 1975; Petersen, 1977; Degn and Kristensen, 198 I); the mechanism of inhibition of this enzyme by sulphide has been studied over several years and the current model is the following (Wever *et al.,* 1975; Nicholls, 1975; Nicholls and Kim, 1981, 1982; Hill *et al.,* 1984). Sulphide, cyanide and azide all compete for an oxidized binding site on the enzyme, apparently the ferricytochrome center. Addition of excess (mM) sulphide causes reduction of cytochrome "a" and ferricytochrome subsequent ligation to both the cytochrome "a", "c" and ferricytochrome, forming a partially reduced enzyme-sulphide complex and inhibit the enzyme action (Nicholls and Kim, 1982) Repercussions arising from sulphide inhibition of cytochrome "c" oxidase maybe great enough to account for the lethality of sulphide (Khan 1990). Likewise, freshwater and marine fishes killed by sulphide exposure show cytochrome oxidase activities lower than control values in various tissues (Torrans and Clemens. 1982: Bagarinao and Vetter, 1989; Bagarinao. 1991). During sulphide exposure, ATP concentrations decrease and affect the metabolic pathways. Since the enzyme is located in the mitochondria. Mitochondrial respiration is also gets inhibited by H_2S in different organisms. This is because lower sulphide concentrations are effectively oxidized by mitochondria and the enzyme is inhibited only when it is overloaded with sulphide.

1.3.3.3 Modification of Oxygen transport proteins

Sulphide at high concentrations in vitro causes the formation of sulfhemoglobin and sulfmyoglobin, derivatives in which sulphide is covalently bound in to the pyrrole group of the porphyrin ring, in mammals and marine fishes (Hoppe 1863; Morel1 *et al.,* 1967; Nichol *et al.,* 1968; Berzofsky *et al.,* 1972; Carrico *et al.,* 1978; Park *et al.,* 1986; Bagarinao, 1991). Sulfhemoglobin is non-functional in oxygen transport (Carrice *et al.,* 1978). However, mammals and fishes poisoned by sulphide rarely contain sulfhemoglobin in the bloodstream, and impediment of oxygen transport does not seem to play a role in acute sulphide poisoning (Park and Nagel, 1986; Curry and Gerkin, 1987; Bagarinao, 1991). Bagarinao (1991) found that fish hemoglobin does not convert to sulfhemoglobin, and thus remains fully functional in oxygen transport, unless sulphide concentrations reach l-5 mM, some time has been allowed, and pH is on the acid side. The dependence of sulfhemoglobin formation on sulphide concentration, time and pH may explain the observations that rat and fish blood do not form sulfhemoglobin in vivo (Sorbo, 1958; Bagarinao, 1991), and that the extracellular hemoglobins of the marine polychaetes *Arenicola marina* and *Abarenicola afjnis* and the vent tubeworm *R. pachyptila* do not convert into sulfhemoglobin (Patel and Spencer, 1963; Wells and Pankhurst, 1980; Arp *et al.,* 1985, 1987). On the other hand, hemoglobin in turn affects sulphide in terms of immobilization and oxidation. Less is known of the effects of sulphide on other oxygen transport proteins.

Sulphide toxicity is dose-dependent for any one species, and differs somewhat according to the particular biological system (whole organism, isolated mitochondria or enzyme preparation) under study. Toxic level of

sulphide in vertebrates and invertebrates indirectly depend on the mechanism of diffusion, the rate of penetration of sulphide into cells is directly proportional to the concentration of hydrogen sulphide in the external solution, and is best explained by diffusion of across H_2S the non-aqueous cell membrane (Jacques, 1936).

1.4 Hydrogen sulphide removal in aquatic systems

Based on the observation of above data, in aquatic systems remediation of hydrogen sulphide play an important role in the health and natural condition of living flora. Remediation continues to improve, with use of microorganisms (both genetically modified organisms (GMOs) and naturally occurring ones, including extremophiles) to clean contaminated areas (David *et a1*., 1995; Tebo, 1995) and conventional methods commonly adopted for the removal of sulphide are based on physio-chemical processes like oxidation and chemical precipitation are commonly used strategies but are expensive in terms of energy, chemicals and disposal of spent chemicals (Buisman *et al.,* 1990). As the aquatic system develops, efficient, cost-effective and environment friendly preventive and bioremediation methods of improving effluent water quality prior to discharge into receiving waters of sensitive areas will be necessary (Jones *et al.,* 2001). Two types of remediation of hydrogen sulphide are described below.

1.4.1 Physiochemical methods

Once formed, sulphide may be removed from or bound in the system by volatilization, chemical oxidation, metal sulphide precipitation, and biological oxidation. Oxidation of sulphide in stratified water bodies such as the Black Sea and Solar Lake appears to be largely chemically mediated

(Sorokin, 1972; Jorgensen, 1982, 1984). In sediments, about 90% of the sulphide generated from sulphate reduction is oxidized, mostly through microbial activity (Hansen *et al.,* 1978; Jorgensen, 1982, 1984).

1.4.2 Chemical oxidation

The products of the chemical oxidation of sulphide in natural waters include polysulphides, elemental sulphur, thiosulphate, and sulphite and sulphate as discussed earlier (Cline and Richards, 1969; Chen and Morris, 1972). Chemical oxidation of sulphide in seawater obeys pseudo-first order kinetics, with reaction rates that decrease exponentially with increasing initial sulphide concentration, and half-times that decrease exponentially with increasing oxygen: sulphide ratios (Almgren and Hagstrom, 1974). Spontaneous oxidation of sulphide is often considered rapid, but in fact the reported half-times of 0.4 to 65 h in air saturated seawater (Almgren and Hagstrom, 1974; Millero, 1986; Millero and Hershey, 1989) are slow relative to biological rates of catalysis. Oxidation of hydrogen sulphide require use of oxidizing agents like air, oxygen, chloride, potassium ferricyanide, quinine, manganese sulphide, manganese dioxide, hypochloride, chlorine dioxide and nitric oxide (Kobayashi *et al.,* 1983). Natural conditions were essential for the successful chemical oxidation that are described below.

Millero's (1986) review of the thermodynamics and kinetics of the H_2S system in natural waters indicates that: (1) the solubility of H_2S decreases with increasing temperature and is lower in seawater than in freshwater; (2) the ionization constants vary with temperature and salinity, the best estimates being pK = 6.6 and pK =13.8 at 25^0C in seawater; and (3) the kinetics of oxidation is affected by pH, temperature and the presence of metal and organic catalysts. Overall conclusion is that chemical oxidation of

hydrogen sulphide is directly depends on temperature, salinity, and pH. Any changes in these three parameters will leads to the decrease of the oxidation rate of H_2S in aquatic systems.

1.4.2.1Air stripping

There are three types of aeration used to remove H_2S from water: (a) spray aeration where pressurized water is forced into the air through spray nozzles, (b) forced draft aeration where sheets of water fall over a series of wood baffles from a predetermined height and (c) air diffusion aeration where air bubbles are forced through a body of water. However, removal of H_2S by aeration is not necessarily ideal for all situations. It requires an acidic pH, long contact times and high energy input (Lemley *et al.*, 1999). Furthermore, H_2S is difficult to remove in alkaline water because most of H_2S is present in the form of HS^- and H^+ ions. The removal of hydrogen sulphide by aeration is not an efficient method because the presence of sulphur in the form of hydrogen sulphide depends on the pH and chemical oxidation of bisulphide with chlorine is significantly slower than the oxidation of hydrogen sulphide and results in high turbidity.

The other method for the removal of H_2S is ion exchange, reverse osmosis and ozonation. Both ion exchange and reverse osmosis are complicated and do not produce the desired results (Brimblecombe and Lein, 1989). Ozone oxidation of H_2S is very effective treatment but it is very costly to set up a treatment facility (Kato *et al.*, 2005).

1.4.2.2Precipitation

Insalt marsh sediments, 594% of the sulphide formed is precipitated as pyrite, depending on the sediment type (Howarth. 1984; Jorgensen,

1984). Rather than being simply accumulated, pyrite is a dynamic constituent, present mostly as microcrystals with a high surface-to-volume ratio, and thus more reactive and easily oxidized. Pyrite forms throughout the year, but there is a net loss during the summer when the marsh grasses are most able to oxidize the sediments, and a net accumulation during fall, winter and spring when the grasses are less active (Howarth, 1984). Other metal sulphides (Mn, Hg, Pb, Cd, Zn. Cu) which can be precipitate the hydrohen sulphide in the marine sediments as discussed earlier but are generally of little significance (Howarth, 1984; Jorgensen, 1984).

Use of precipitants like Mn, Cd, Zn, Cu and Fe leads to the formation of insoluble metal sulphide it is less toxic than sulphide. Interestingly, it has been proposed that the oxidative formation of pyrite from ferrous sulphide and insoluble metal sulphide may have provided the energy source for chemoautotrophic bacteria. (Wachterhauser, 1990a).

1.4.2.3 Disadvantages of chemical treatments

Various methods have been reported for the removal of sulphide as described earlier. The relatively high energy requirements or the high chemical and disposal costs besides environmental problems constitute important drawbacks of these methods. Therefore, alternative techniques for the hydrogen sulphide removal are required. Partial biological oxidation of sulphide to sulphur is a cheap alternative, which also allows sulphur reclamation, since sulphur is non-soluble and thus, it can be removed from the wastewaters well as various aquatic systems. The partial oxidation of H_2S to elemental sulphurinstead of sulphate has several advantages. Elemental sulphuris non-toxic, non-corrosive solid containing more sulphur per unit mass. Moreover, the elemental sulphur generated during the process can be

used as a feedstock for the chemical, fertilizer and materials manufacturing industries.

1.4.3 Biological oxidation

Sulphide oxidation in sediments is for the most part done by microbial mediated. Two physiologically different groups of bacteria have specialized in sulphide oxidation: the phototrophic green and purple sulphur bacteria and the chemotrophic colourless sulphur bacteria (Jorgensen, 1982, 1984). Pathways of oxidation of sulphur compounds by bacteria are varied and complex, and are elucidated by Kelly (1988). Radiotracer studies in marine sediments show that thiosulphate constitutes 68 to78% of the immediate sulphide oxidation products, and is concurrently reduced back to sulphide, oxidized to sulphate, and fermented or disproportionate to sulphide and sulphate by different groups of bacteria (Bak and Cypionka, 1987; Jorgensen, 1990). Sulphide may be oxidized by eukaryotes as well as by bacteria. Meiofaunal invertebrates oxidize sulphide to various products depending on oxygen availability in the sediment layer; Turbellarians and gastrotrichs produce thiosulphate (Powell *et al.,* 1980). Several species of macro-invertebrates and fishes produce thiosulphate during sulphide exposure of whole animals and of mitochondria (Vetter *et al.,* 1987; Bagarinao and Vetter, 1989, 1990; Cary *et al.,* 1989; Vismann, 1991). (Microbial mediated sulphide oxidation is discussed in following sessions)

Biological oxidation of sulphide and thio sulphate takes plays in different ways in different organisms. These reactions take place in anaerobic conditions.

$CO_2+2H_2S \xrightarrow{light} (CH_2O) + H_2O + 2S$

$S+ CO_2+3H_2S \xrightarrow{light} (CH_2O) + H_2SO_4$

$CO_2+Na_2S_2O_3 + 3H_2O \xrightarrow{light} 2(CH_2O) + NaS_2O_4 + H_2SO_4$

1.4.3.1 Advantages of biological oxidation

As a component of anthropogenic waste water, hydrogen sulphide often causes problems because of its high toxicity even at low concentrations and its unpleasant odors. In biological sewage plants sulphide concentrations can be found which are toxic not only to man, but also to the microorganisms clarifying the water. Therefore, operation of sewage plants can be inhibited by sulphide. In addition, sulphide can be oxidized to sulphuric acid by microorganisms which inhabit biofilms inside waste water tubes. The resulting sulphuric acid leads to corrosion of metallic or concrete material. In order to remove sulphide from liquid and gaseous effluents, biotechnological applications have been developed using photochemotrophic bacteria (Midha *et al.*, 2008). It can also be removed by chemical methods, such as chemical oxidation or precipitation as discussed earlier but these methods are expensive and may cause new problems because of the chemicals used. Several microorganisms have been studied for application in biotechnological hydrogen sulphide removal processes. In this point of view applied, sulphide utilizing bacteria for addressing the bioremediation of hydrogen sulphide in various aquatic systems.

H_2S removal by microbial methods (photosynthetic as well as non photosynthetic microorganisms) is a good alternative to physico-chemical treatment because of its economic feasibility. The biotechnological sulphide removal system is based on the oxidation of sulphide to sulphur.

Photosynthetic purple and green sulphur bacteria utilize reduced sulphur compounds, such as sulphide, as electron donors (Dahl *et al.,* 1999).

As per Buisman *et al.,* (1990) biotechnological Sulphide removal has several merits:

1) Catalyst and oxidants are not used, making the process economical.
2) No hassles of chemical sludge disposal.
3) Biological sludge production is at bare minimal level.
4) Low energy consumption.
5) Possible reuse of sulphate, which is a byproduct of the process.
6) Low sulphate and thiosulphate discharge.
7) Fairly fast and high removal efficiency.

1.5 Sulphide utilizing phototrophs- General view

Biological oxidation of hydrogen sulphide to sulphate is one of the major reactions of the global sulphur cycle. Reduced in organic sulphur compounds are exclusively oxidized by prokaryotes, and sulphate is the major oxidation product. (Friedrich *et al.,* 2001) Sulphate generated during the process also gets converted into sulphide during anaerobic treatment. In the absence of dissolved oxygen and nitrate, sulphate reducing bacteria converts sulphate into sulphide. In order to remove sulphide from wastewater stream by using several aerobic and anaerobic photosynthetic bacteria.

Aerobic microorganisms or chemotrophs used for the oxidation of hydrogen sulphide are the species of *Thiobacillus, Pseudomonas, Beggiatoa and Thiothrix* which have been thoroughly studied by various researchers

(Midha *et al.,* 2008). These microorganisms use inorganic carbon as a carbon source and chemical energy from the oxidation of reduced inorganic compounds. The simpler nutritional requirements and higher sulphide tolerance of chemotrophic organisms favoured their application in biological sulphide oxidation. Various *Thiobacillus* species are widely used in conversion of hydrogen sulphide to elementalsulphur on the laboratory scale.

Bioreactors with anoxygenic phototrophic bacteria have been successfully tested for sulphide removal. Lee & Kim (1998) states that's *Chlorobium thiosulphatophilum* consume hydrogen sulphide at 0.87µ mols/min.mg protein in a photo bioreactors. The advantages of such systems are the almost complete removal of sulphide from the effluents and no addition of chemical oxidants. The phototrophic bacteria acting in these systems are species of the genera *Chlorobium* and *Rhodobacter*. The above mentioned species convert sulphide mainly to sulphate, therefore the sulphur is not removed completely from the system and can be reduced to sulphide by sulphate-oxidizing bacteria again. In order to circumvent this problem and to obtain elemental sulphur as the main product, it could be useful to replace these organisms by other phototrophic ones which only can oxidize sulphide to elemental sulphur, e.g., *R. capsulatus* non sulphur bacteria. Overall these data demonstrates that most of the phototrophs and some chemotrophs can utilize hydrogen sulphide as an electron donor for photosynthesis. This study mainly focused on phototrophic bacteria with H_2S mediated photosynthesis and itsused as a bio augmenters for hydrogen sulphide in various aquatic systems.

1.6 Occurrence and distribution of phototrophs based on limiting factors

Phototrophic bacteria use light as the energy source to produce phosphate bond energy (ATP) and reductants [e.g., NAD(P)H and reduced ferredoxin] through photosynthetic electron transport (Tang *et al.*, 2011). Purple sulphur bacteria and non sulphur bacteria are anoxygenic phototrophs belonging to the phylum *Proteobacteria*, which occur primarily in stratified environments, where sufficient light reaches sulphide-containing water or sediment layers. In lakes harbouring these bacteria, an average of 28.7% of primary production is anoxygenic (Overmann, 1997).

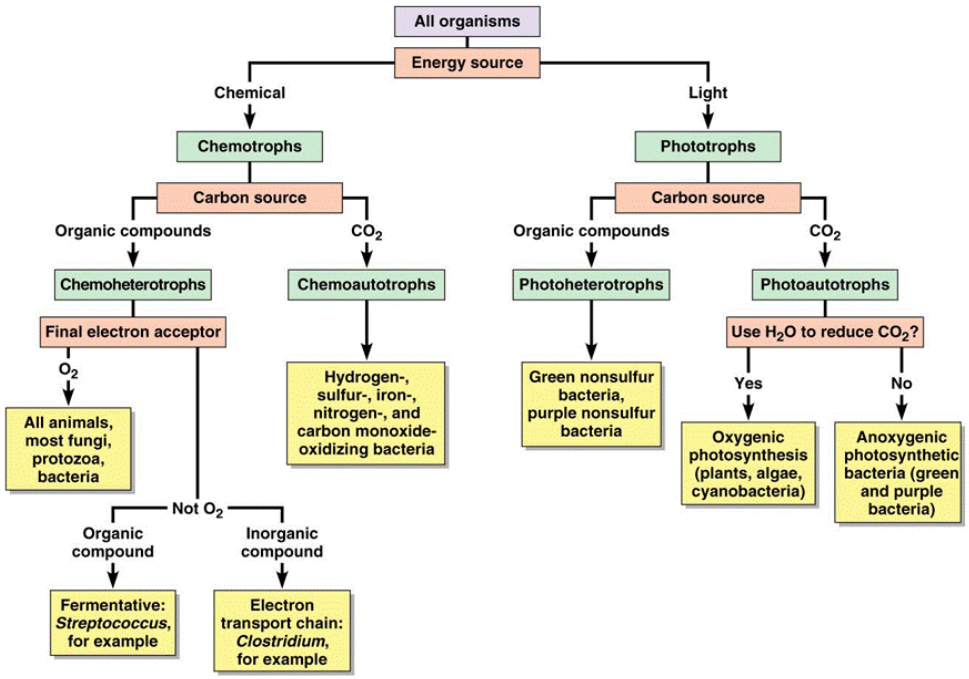


Fig 1.1 Classification of bacteria based on the nutritional requirements. Hoa *et al.*, 2008*)*

The dependence of this pathway on reduced inorganic sulphurcompounds which originate from the anaerobic degradation of organic carbon, previously fixed by oxygenic photosynthesis, has led to the term secondary primary production (Pfennig, 1978). Besides the limiting factors such as the availability of reduced sulphur compounds, organic carbon substrates, absence of oxygen, temperature and availability of nutrients, the availability and quality of light reaching these niches is another major determinant of the presence and composition of anoxygenic phototrophic communities. Depending on the position of these specific habitats, penetrating light lacks certain regions of its spectrum, due to scattering or absorption in the layers above. Being constrained to these anoxic environments, anoxygenic phototrophs has to adapt to performing photosynthesis with light that reaches their habitats. At sea level, light of the wavelength between 400 to 700 nm represents 50% of this irradiation mainly due to water vapour absorbing infrared light. In aquatic habitats, water is the major light-absorbing component only in very clear Open Ocean and inland lakes. Blue light is predominant, but between the wavelengths where water molecules absorb light (for example between 750 and 900nm) there are still niches available (Stomp *et al.,* 2007).

This limited wavelength range available at great depth selects for species of anoxygenic phototrophic bacteria with complementary absorption spectra. The competition for light lead to the prevalence of species using different pigments and light harvesting antenna for the absorption of available light spectra. Another habitat for anoxygenic phototrophs are intertidal marine sandy sediments, which harbour complex, fine-layered microbial mats that predominantly consist of a vertical sequence of different types of phototrophic microorganisms (Nicholson *et al.,* 1987). The top layer is formed by a layer of diatoms, followed by a layer of cyanobacteria.

Within the anoxic region below, purple sulphur bacteria and purple non sulphur bacteria (*Chromatiaceae*) containing bacteriochlorophyll (BChl) "*a"* with a typical long-wavelength Qy absorbance peak at 870-890 nm overlie *Chromatiaceae* containing BChl "*b"* (Qy at 1020 nm). The lowermost phototrophic layer contains green sulphur bacteria (*Chlorobiaceae*) that typically contain BChl "*c"* (Qy at 735-775 nm). In contrast to pelagic systems, absorption of infrared radiation by the embedding matrix is very low within the euphotic zone of sandy sediments. As a consequence, mainly far-red and near-infrared light reaches the anoxygenic phototrophic bacteria in the anoxic layers of multilayered microbial mats. The vertical distribution of the different bacteria can therefore be related to their differential absorption of far-red and near-infrared radiation as well as their affinity for sulphide (Pierson *et al.*, 1990).

While most of the BChl "*a*" containing anoxygenic phototrophs described so far exhibit long-wavelength absorption maxima between 800 and 900 nm, three proteobacteria possess Qy absorption bands that are red-shifted to wavelengths above 900 nm. The alphaproteobacterium *Roseospirillum* was isolated from a microbial mat (Glaeser and Overmann, 1999) and has a Qy at 909 nm. The thermophilic *T. tepidum*is a member of the *Chromatiaceae* that was isolated from a hot spring (Madigan, 1984; Garcia *et al*., 1999) and has an absorption maximum at 915 nm. To date, the phototropic bacterium exhibiting the most extreme red shift for a BChl "*a"* containing LHC1 complex is strain 970. This photolithoautotrophic purple sulphur bacterium belongs to the *Gammaproteobacteria*, also originates from a marine microbial mat and absorbs light at a wavelength of 963 nm (Permentier 2001). All mentioned limiting factors were deeply influencing the geographical distribution of phototrophs in natural environment.

Hydrogen sulphide utilizing phototrophic bacteria, its classification and habitats has been described in the following sections.

1.6.1Anoxygenic phototrophic bacteria (APB)

Anoxygenic phototrophic bacteria (APB) are physiologically and phylogenetically diverse group of photosynthetic bacteria which can perform photosynthesis without producing oxygen and in the absence of air with an exception of aerobic APB which require oxygen. As discussed earlier, most phototrophic bacteria uses reduced inorganic sulphur compounds as electron donors during anoxygenic photosynthesis. Principally, sulphide is oxidized via sulphite to sulphate. Elemental sulphur may appear as intermediary storage product (inside: *Chromatium, Thiocapsa;* outside: *Chlorobium ,Ectothiorhodospira;* not in: *Rhodopseudomonas sulfidophila*).

Acoording to Truper (1981) the utilization of sulphur compounds (at oxidation levels below that of sulphate) as electron donors in anoxygenic photosynthesis is thought to a different extent common to most groups of phototrophic prokaryotes, in this respect are the purple and green sulphur bacteria (*Chromatiaceae* and *Chlorobiaceae)* all of which utilize reduced sulphur compounds as electron donors. But also a number of classical "purple-nonsulphur" bacteria *(Rhodospirillaceae*) and the filamentous flexible green bacteria (*Chloroflexaceae)* are able to oxidize reduced sulphur compounds during photosynthesis. Even certain species of the cyanobacteria can perform anoxygenic photosynthesis at the expense of sulphide as electron donor.

1.6.1.1Classification of APB

APB are comprised of the members of the groups of purple sulphur bacteria (PSB), purple nonsulphur bacteria (PNSB), and green sulphurbacteria (GSB) (Overmann, 2001). Based on 16S rRNA gene sequence analysis, they were distributed in five distantly related phyla; *Proteobacteria* (which includes the purple bacteria**),** *Chloroflexi* (which includes the filamentous green nonsulphur bacteria), *Chlorobium* (which includes the green sulphur bacteria), and *Firmicutes* (which includes the Gram-positive *Heliobacteria*).The phototrophic members were also discovered in the phylum *Acidobacteria* which was represented by a single species –*Candidatus, Chloracidobacterium thermophilum* (Bryant *et al.,* 2007). *Proteobacteria*is again classified in to 3 classes; *Alphaproteobacteria, Betaproteobacteria* and *Gammaproteobacteria.* Phylogenetic analyses of purple bacteria based on comparative 16S rRNA sequencing have shown that purple sulphur bacteria are species of *Gammaproteobacteria* while purple nonsulphur bacteria are of either *Alpha*proteobacteria or *Betaproteobacteria* (Imhoff *et al*., 2005).

1.6.1.1.1 Purple non-sulphur Bacteria (PNSB).

Purple non-sulphur (PNS) photosynthetic bacteria constitute a non-taxonomic group of versatile organisms which can grow as photoheterotrophs, photoautotrophsor chemoheterotrophs—switching from one mode to another depending on available conditions such as: degree of anaerobiosis, availability of carbon source (CO_2 for autotrophic growth, organic compounds for heterotrophic growth), and availability of a light source (needed for phototrophic growth).The term ‘‘non-sulphur’’ arose because PNS bacteria were thought not to use hydrogen sulphide as an electron donor while growing photoautotrophically. However, PNS bacteria can use sulphide as an electron

donor but not at high concentrations like sulphur bacteria (Brock *et al.,* 2003). They give purple to deep red pigments in facultative anaerobic conditions, whereas in aerobic conditions no pigments can be observed. Compared to water splitting by algae, PNS bacteria require much less free energy to produce hydrogen by completely decomposing organic substances.

The purple nonsulphur bacteria *Rhodospirillum rubrum*, *Rhodopseudomonas capsulata* R. *sphaeroides* and R. *palustris* were tested for a possible utilization of sulphide. The first three strains were found to oxidize sulphide to extracellular elemental sulphur only, whereas R. *palustris* converted sulphide into sulphate without intermediate accumulation of elemental sulphur. Growth ceased at lower sulphide concentrations than usually found with purple sulphur bacteria. In consequence of the low sulphide tolerance information on the specific growth rates obtained with sulphide as photosynthetic electron donor could not be provided by cultivation in batch cultures. Sulphate was the only product of the conversion of sulphide. Some purple nonsulphur bacteria may play a role in the dissimilatory sulphur cycle in nature.

1.6.1.1.2 Purple sulphur bacteria (PSB)

Purple phototrophic members of Anoxygenic phototrophic bacteria were classified under the phylum *Proteobacteria,* which is in fact, a phylum proposed for all phototrophic purple bacteria and their purely chemotrophic relatives (Stackebrandt *et al.,* 1988). Among all the five phyla of photosynthetic prokaryotes (i.e, *Proteobacteria*, *Acidobacteria*, *Chlorobi*, *Chloroflexi*and *Firmicutes*), the purple *Proteobacteria* are the most metabolically diverse. Photosynthesis in these purple bacteria depends on anoxic or oxygen deficient conditions, because the synthesis of the photosynthetic pigments is repressed by

oxygen. These bacteria are unable to use water as an electron donor and instead need more reduced sulphur compounds such as sulphide. These bacteria can also utilize hydrogen and a number of small organic molecules as photosynthetic electron donors and use a diverse number of metabolic pathways for energy generation, carbon assimilation, as well as nitrogen, sulphur and phosphorous metabolism (Imhoff *et al*., 2005; Madigan and Jung, 2005). Growth with reduced iron as electron donor has been demonstrated in some phototrophic purple bacteria (Widdel *et al*., 1993; Ehrenreich and Widdel 1994). Purple sulphur bacteria are strong photoautotrophs and capable of limited photoheterotrophy, but they are poorly equipped for metabolism and growth in the dark. Several purple bacteria inhabit extreme environments of temperature, pH, and salinity. Collectively, purple bacteria are important phototrophs because they (1) consume a toxic substance H_2S and contribute organic matter to anoxic environments by their autotrophic capacities; (2) consume organic compounds, primarily non fermentable organic compounds in their roles as photo heterotrophs; and (3) offer scientists for the study of diverse photosynthetic metabolisms. Purple sulphur bacteria differ from purple non-sulphur bacteria on both metabolic and phylogenetic grounds. In certain habitats, particularly, favourable for their development, purple bacteria have also been shown to be significant primary producers. The phototrophic sulphur bacteria classified in to three they are ***Chromatiaceae***, ***Ectothirhodospiraceae*** and ***Rhodospirillaceae.*** *Chromatiaceae* can grow with sulphide and some representatives also use in elemental sulphur, sulphite or thiosulphate as electron donors during oxidation of sulphide to sulphate, elemental sulphur is deposited inside the cells. Photoheterotrophic, chemoautotrophic and/or chemoheterotrophic growth has been demonstrated for several species. Some species require vitamin B12 as the only growth factor. Various type of

carotenoids and in most species, bacteriochlorophyll 'a' serve as photosynthetic pigments. These are located in cytoplasmic membrane that are formed as vesicle.

***Ectothiorhodospiraceae* spp** have been isolated from marine sources and alkaline condition are more preferable than other natural sources. *Ectothiorhodospiraceae*are distinguished from the *Chromatiaceae* by the intermediate deposition of the elemental sulphur outside the cells. Its Growth and survive depends on saline and alkaline condition. Other important feature is that intracytoplasmic membranes are present as lamellar stacks.

***Rhodospirillaceae* -** include purple non sulphur bacteria. Which are the most diverse and best studied group of phototrophic bacteria. Most species are motile, require growth factors and have various type of carotenoids and bacteriochlorophyll an as photosynthetic pigment. These bacteria grow preferably under photoheterotrophic growth conditions and many representatives are aerotolerent and can grow as chemohetrotrophs in the dark. Some species are also able to use sulphide and elemental sulphur to sulphate. Globules of elemental sulphur never appear inside the cells.

1.6.1.1.3 Green sulphur bacteria (GSB).

The green sulphur bacteria are *Proteobacteria* which can be grow in sulphide rich conditions e.g. *Chlorobium limicola* but higher concentration of hydrogen sulphide is extremely toxic to green sulphur bacteria.

The phototrophic green sulphur bacteria require strictly anaerobic condition for growth, and can assimilate only a limited number of organic compound sulphide, CO_2, and light dependent metabolism, elemental sulphur globules are formed outside the cells, in the medium. The multicellular

filamentous green bacteria (*Chloroflexaceae*) can also utilize hydrogen sulphide during photosynthesis it can preferably grow in alkaline photoheterotrophic condition, and are tolerant towards oxygen. Hydrogen sulphide mediated photosynthesis of green sulphur bacteria no oxygen is formed. During photosynthesis of GSB the light is absorbed by photochemical reaction centres (PRC) and carried out non cyclic phosphorylation at that time the electron is moved from the excited PRC are replaced by those from an electron donor like sulphide (Stainer *et al.,* 1986). In these ways green sulphur bacteria play an important role in biogeosulphur cycle

1.6.2 Cyanobacteria

Cyanobacteria grow in a wide range of habitats from lakes, streams, oceans, soil, glaciers, and deserts, in endolithic communities, and in hot springs below 72 ^{0}C. In the microbial world, they play a major role as primary producers (Waterbury *et al*., 1979; Chisholm *et al*., 1988). The oxygen they produce as a result of oxygenic photosynthesis plays fundamental roles in the global biogeochemical cycling of nitrogen, sulphur, carbon, and redox sensitive metals. Thus, the Cyanobacteria have had a profound impact on microbial communities and biogeochemical cycles past and present. Cyanobacteria considered as an oldest known autotrophic organisms inhabiting the Earth. Some cyanobacteria can perform anoxygenic photosynthesis during the presence of sulphide. Myers *et al.,* (2007) found different species of *Geitlerinema* and *Leptolyngbya* strains to prefer low light conditions and to carry out a sulphide-resistant oxygenic photosynthesis, which is relatively uncommon among cyanobacteria, thus most of them follow a strategy of sulphide-sensitive oxygenic photosynthesis, which involves an inhibition of photosynthesis even when low amounts of sulphide are present and, thereby,

are considered to be obligate oxygenic phototrophs (Cohen *et al.,* 1986). All the above mentioned organisms play an important role in sulphur cycle in the aquatic systems.

1.7 Sulphur cycle in biogeochemical level

Sulphur cycling in aquatic sediments involves both reductive and oxidative processes (Jorgensen, 1990) (Fig.1.2). Several bacteria have been participating the sulphur cycle. The major events in the sulphur cycle are described below.

Major steps in the sulphur cycle include:

1. **Assimilative reduction** of sulphate (SO_4^{2-}) into -SH groups in proteins.
2. **Release of -SH** to form H_2S during excretion, decomposition, and desulphurylation.
3. **Oxidation of H_2S** by **chemolithotrophs** to form sulphur (S^o) and sulphate (SO_4^{2-})
4. **Dissimilative reduction** of sulphate (SO_4^{2-}) by anaerobic respiration of sulphate-reducing bacteria.
5. **Anaerobic oxidation** of H_2S and S by **anoxygenic phototrophicbacteria**(purple and green)

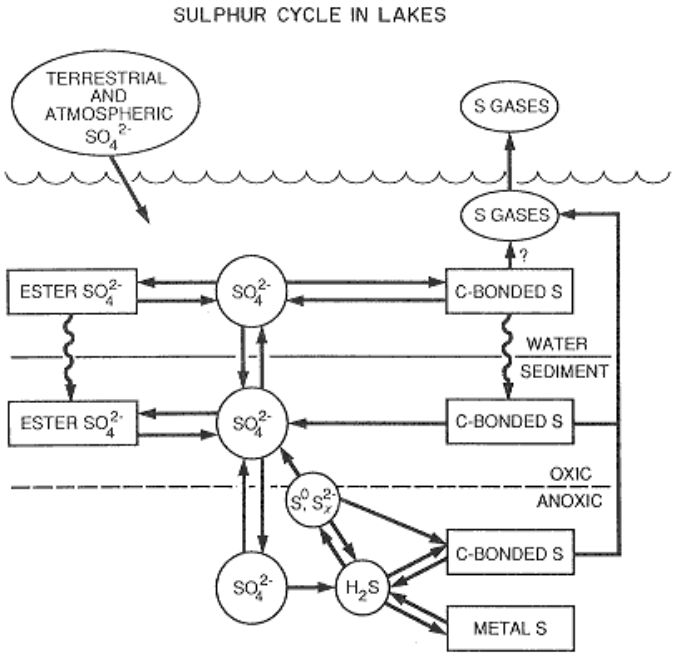


Fig.1.2 Demonstrate the normal sulphur cycle and its pathways in and out of the water sediments.

Microbial sulphur oxidation is a key process for the biogeochemical sulphur cycle in marine sediments and closely linked to the cycling of other elements like oxygen, nitrogen, and carbon. The process is one of the oldest types of biological energy conservation (Wachtershauser, 1990b). Sulphur compound oxidizing prokaryotes are phylogenetically and metabolically highly diverse (Lane *et al.,* 1992; Brock *et al.,* 2006). Many bacteria and some archaea can carry out dissimilatory sulphur oxidation of reduced inorganic sulphur compounds, e.g. sulphide, thiosulphate, polythionates, elemental sulphur and sulphite (Brüser *et al.,* 2000) .

Sulphur chemolithotrophs grow primarily aerobic, i.e. using molecular oxygen as terminal electron acceptor. However, some species (*Beggiatoa sp.,*

Thioploca sp.,Thiobacillus denitrificans, Thiomicrospira denitrificans) oxidize H_2S anaerobically coupling it to nitrate reduction (Brock *et al.,* 2006).

The oxidation of sulphide, sulphur and polythionates is linked to thiosulphate oxidation and can therefore easily be included into the existing thiosulphate oxidation pathways (Brüser *et al.,* 2000). So far several biochemical models for thiosulphate and sulphide oxidation have been proposed: (1) Thetetrathionate pathway (e.g. *Thiobacillus thioparus*, *Acidithiobacillus* sp. and *Acidiphilum* sp.) (2) the multi-enzyme-complex or SOX pathway (e.g. *Paracoccus denitrifcans*, *Starkeya novella*) and (3) the branched thiosulphate oxidation pathway (e.g.*Allochromatium vinosum*, *Thiobacillus denitrificans*, *Thiocapsa roseopercicina*) (Brüser *et al.,* 2000).

The best studied archaeal sulphur oxidizer is the aerobic *Acidianu sambivalens*, the oxidation of sulphide, sulphur and polythionates is linked to thiosulphate oxidation and can therefore easily be included into the existing thiosulphate oxidation pathways (Brüser *et al.,* 2000). The multi-enzyme-complex or SOX pathway (e.g. *Paracoccus denitrifcans*, *Starkeya novella*) and the branched thiosulphate oxidation pathway (e.g.*Allochromatium vinosum*, *Thiobacillus denitrificans*, *Thiocapsa roseopercicina*) (Brüser *et al.,* 2000).

In the tetrathionate pathway of *Acidithiobacillus* and *Acidiphilum* spp. thiosulphate oxidizes to the stable intermediate tetrathionate prior to cleavage by a hydrolase (Brüser *et al.,* 2000). For this pathway extra cellular elemental sulphur or persulphide in the outer membrane oxidized and form sulphite which is further oxidized to sulphate. In this pathway free hydrogen sulphide in the periplasam get reduced by SQR enzyme which are present in the cytoplasmic membrane of *Acidithiobacillus* .and *Acidiphilum spp.* (Fig.1.3) (Rohwerder and Sand, 2003).

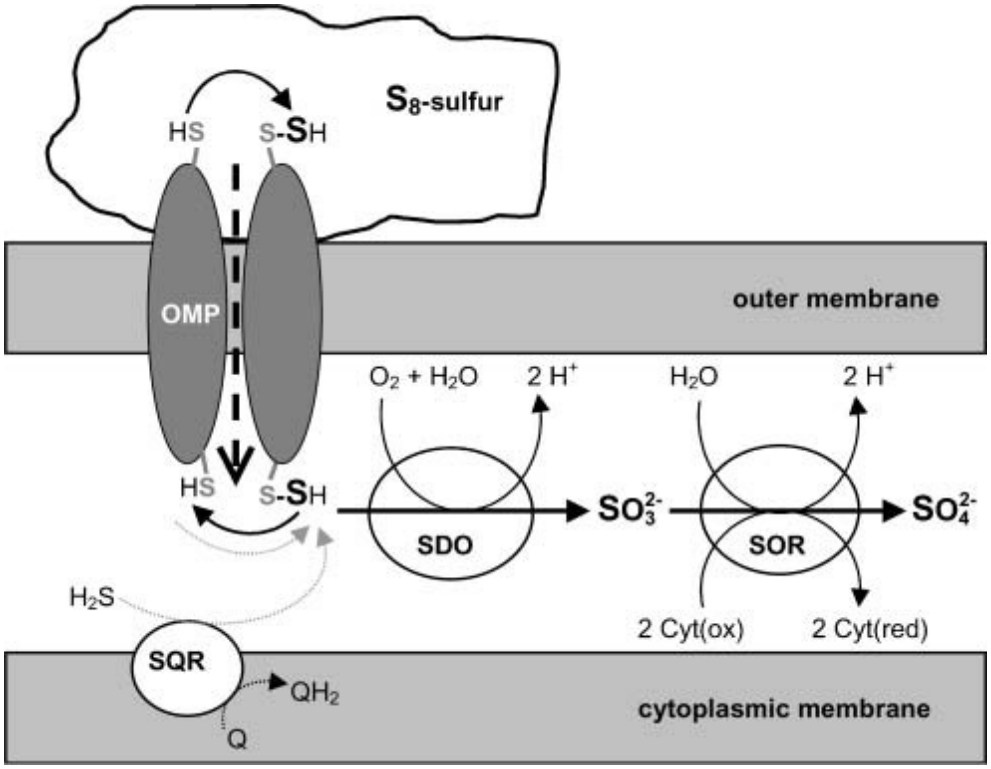


Figure 1.3: Model for sulphur and sulphide oxidation in *Acidithiobacillus*and *Acidiphilum*spp. Extracellular elemental sulphur (S8) is mobilized as persulphidesulfan sulphur by outer membrane proteins (OMP) and oxidized by a periplasmatic sulphur dioxygenase (SDO). The resulting sulphite is further oxidized to sulphate by sulphite:acceptor oxidoreductase (SOR). Free sulphide is oxidized to elemental sulphur by a separate sulphide:quinon oxidoreductase. Cyt Cytochromes, Q Quinonens (Rohwerder 2003).

Among the sulphur chemotrophs members of the genera, *Thiobacillus* and *Paracoccus*are the best studied, whereas *Allochromatium vinosum*is the best studied phototrophic sulphur oxidizer. The "**s**ulphur oxidation (SOX) pathway" or "*Paracoccus* sulphur oxidation (PSO) pathway**"** (Kelly *et al.,* 1997) is widespread among Alphaproteobacteria. The latter harbors a complete thiosulphate-oxidizing multi-enzyme system (Kelly *et al.,* 1997). The encoding SOXgene cluster (Friedrich et *al.,* 2001) comprises 15 genes, seven of which (*soxXYZABCD*) encoding four essential proteins, and was first described for *Paracoccus panthotropus* (Friedrich *et al.,* 2005). Catalytic activities of the SOX proteins involve the oxidation of sulphite, thiosulphate, sulphur and hydrogen sulphide coupled to cytochrome "C" reduction without the formation of any free intermediate (Fig.1.3).

Sulphur-oxidizing members of the *Beta-* and *Gammaproteobacteria* as well as the Chlorobiaceae (e.g *Thiobacillus denitrificans, Allochromatium vinosum* and *Chlorobium tepidum*) possess an incomplete SOXgene cluster and lack the sulphur dehydrogenase (Friedrich *et al.,* 2005). This points at a different pathway for oxidation of sulphur to sulphate. In this pathway several genes were actively participating the oxidation of sulphide and thisulphate to elemental Sulphur .e.g. DSR, SQR, SOX, Rhodanase, APS reductase, ATP Sulphurylase and Sulphite dehydrogenase. All these genes playes very important roles in Sulphur cycle. (Fig1.4)

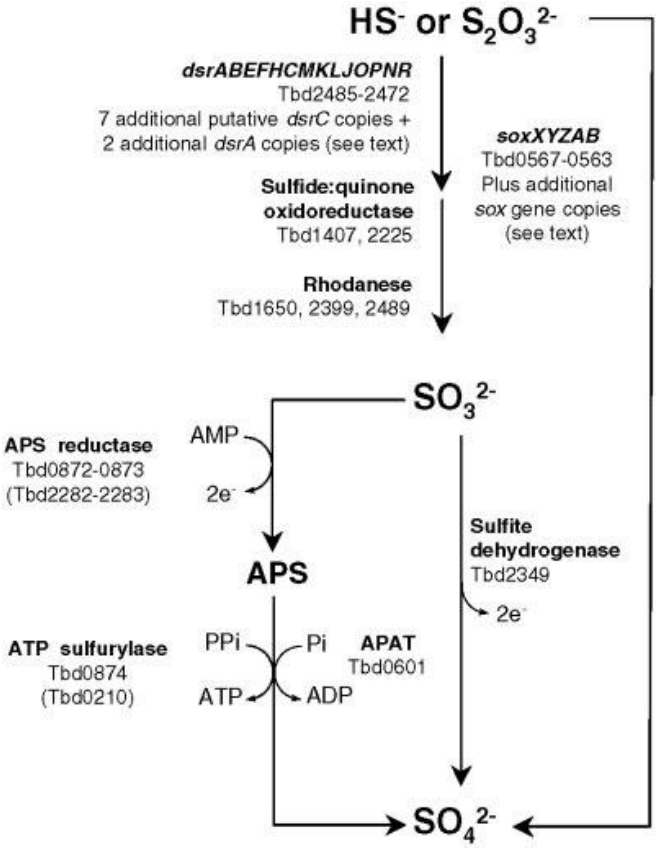


Figure 1.4: Schematic overview of key genes/enzymes putatively associated with sulphur compound oxidation in *Thiobacillus denitrificans*. The presence of SOXand DSR genes suggests that different pathways enable the oxidation of thiosulphate and sulphide to sulphate. Moreover different enzymes may be involved in sulphur oxidation under aerobic versus anaerobic conditions (Beller *et al.,* 2006).

For phototrophs of the gamma-subclass and obligate chemotrophs of the beta subclass of *Proteobacteria*the branched thiosulphate oxidation pathway is established. Dahl and Trüper (1994) proposed a general scheme for dissimilatory sulphur metabolism in anaerobic phototrophic bacteria, which includes three essential steps: (1) oxidation of sulphide or thiosulphate

resulting in the formation of elemental sulphur, (2) oxidation of sulphide or elemental sulphur to sulphite and (3) formation of sulphate as the final product. Moreover, pioneering work on *Allochromatium vinosum* identified dissimilatory sulphite reductase (DSR A, B) to be involved in the anaerobic oxidation of sulphide and sulphur (Dahl and Trüper, 1994; Pott and Dahl, 1998; Dahl *et al.*, 2005). The respective enzyme is well-known from sulphate-reducing prokaryotes (SRP), mediating the reduction of sulphite to sulphide (Hatchikian and Zeikus, 1983; Wolfe *et al.*, 1994; Molitor *et al.*, 1998). In the entire SRP, DSRoperon contains 15 genes, with DSR A, B encoding proteins that catalyzes the oxidation of sulphide to sulphite. Importance of DSR genes and its expression in the presence of sulphide is well described in *Allocromatium vinosum* (Dhal *et al.*, 2005).

All the models of sulphide and thiosulphate pathways and genes with respective organisms mentioned above play a vital part in the sulphide or thiosulphate mediated photosynthesis. The genes are functional as an operon concept and the main inducer of the operon is sulphide and thiosulphate.

1.8 Genes responsible for sulphide mediated photosynthesis-general view

Green sulphur bacteria, purple non sulphur bacteria, purple sulphur bacteria, cyanobacteria, colourless sulphur bacteria (aerobic photosynthesis) use various reduced sulphur compounds such as sulphide, elemental sulphur, and thiosulphate as electron donors for photoautotrophic growth mentioned earlier. This section briefly summarizes what is known about the inorganic sulphur oxidizing systems of bacteria with emphasis on the biochemical aspects. Enzymes that oxidize sulphide in green sulphur bacteria, purple sulphur bacteria and some purplenon sulphur bacteria eg., *Rhodospeudomona s*are membrane-

bound sulphide-quinone oxidoreductase (SQR) periplasmic (sometimes membrane-bound) flavocytochrome "C" sulphide dehydrogenase (FCSD) monomeric flavocytochrome c (SOX, Sulphur oxidation multi enzyme). A fewgreen sulphur bacteria oxidize thiosulphate by the multienzyme system called either the TOMES (thiosulphate oxidizing multi-enzyme system) or SOX (sulphur oxidizing system) composed of the three periplasmic proteins: SOX B,SOX Y and Z, and SOX A,X and K with a soluble small molecule cytochrome "C" as the electron acceptor. Another enzyme which including in sulphur oxidation path way is Dissimilatory sulphite reductase (DSR). The oxidation of sulphide and thiosulphate by these enzymes in vitro is assumed to yield two electrons and result in the transfer of a sulphur atom to persulphide, which are subsequently transformed to elemental sulphur. The elemental sulphur is temporarily stored in the form of globules attached to the extracellular surface of the outer membranes. The oxidation pathway of elemental sulphur to sulphate is currently unclear, although the participation of several proteins including those of the dissimilatory sulphite reductase system are known.

Flavocytochrome *c*-sulphide dehydrogenase (FCSD), an enzyme that catalyzes the reversible conversion of sulphide to elemental sulphur in vitro, is common to bacteria that utilize reduced sulphur compounds as electron donors in the process of carbon dioxide fixation. FCSD is a heterodimer containing two different cofactors, a flavin (FAD) and one or two heme *c* groups, located on the separate protein subunits. Finally FCSD can play an important role in Sulphur cycle.

Guanylate cyclase (c-di-GMP) is considered to be a regulatory protein that play an important role in the regulation of SOX and DSR of *Allochromatuim vinosum* (Grimm *et al.,* 2011). However the gene act as a

marker gene for sulphide oxidizer. Sulphide mediated photosynthesis mentioned above has been hypothesized that with respect of the study of Grimm *et al* (2011) state that in the purple sulphur bacterium *Allochromatium vinosum*, regulation of SOX X, and Kis probably performed by di-guanylate cyclase activity and di-guanylate gene is essential for sulphide /sulphite assimilation in respective organisms.

1.9 Photosynthesis in presence or absence of hydrogen sulphide

Sulphide is an inhibitor of oxygenic photosynthesis, while it is absent from stably oxygenated environments, sulphide is either permanently or periodically present in many ecosystems as a result of its presence in source water and/or biological sulphate reduction. Cyanobacteria and other sulphur and non sulphur bacteria likewise vary in sulphide tolerance, and strains that typically are not exposed to sulphide in nature. And these organisms are extremely sensitive to sulphide. In contrast, those from sulfidic habitats exhibit one or more adaptations for maintaining their photoautotrophic metabolism under such conditions. There are three strategies to explain the sulphide mediated photosynthesis. The first strategies include the maintenance of oxygenic photosynthesis by the resistance of photosystem (PS) II to sulphide and the ability to perform PS II-independent, anoxygenic photosynthesis with sulphide as an electron donor to PS I sulphide-tolerant organisms may represent a unique radiation within an otherwise sensitive group. Sulphide is known to bind metalloproteins and could conceivably interfere with one or more components involved in photosynthetic electron transport. These include the manganese-containing, water-splitting complex on the donor side of PS II as well as cytochromes on the acceptor side. The second strategies that some organism like cyanobacteria *Oscillatoria amphigranulata* (Castenholz 1984) conduct sulphide-resistant oxygenic

photosynthesis, which allows the continuation of oxygenic photosynthesis during exposure to sulphide. In this case, partial inhibition of oxygenic photosynthesis combined with stimulation of anoxygenic photosynthesis, in which sulphide is an electron donor to photosystem I (PSI). The ability to conduct simultaneous oxygenic and anoxygenic photosynthesis allows for efficientCO_2 assimilation by cyanobacteria in environments that exhibit diel transitions between oxic and anoxic conditions.

The third strategy is the total inhibition of PSII (oxygenic photosynthesis) and stimulation of PSI to allow anoxygenic photosynthesis using sulphide as an electron donor (Cohen, 1984; Miller & Bebout, 2004). An example of a cyanobacterium that exhibits this strategy is *Oscillatoria limnetica* (Cohen, 1984), which has been reclassified as*Geitlerinema* PCC9228 (Castenholz *et al.*, 2001).

1.10 Molecular approach for the identification of phototrophs.

Analysis of DNA (protein) sequences and other molecular markers have become key methods for understanding the evolution of organisms. A similar trend evolved in the molecular systematics and population genetics of cyanobacteria and other phototrophs (e.g. Giovanonni *et al.* 1988, Boyer *et al.* 2001, Castenholz *et al.,* 2001, Komárek 2010). The most widely used gene is 16S rRNA, Inter Transcribed Spacer, RBC-L, Cpc-BA-IGS, PUFM, etc. All these region contain highly conserved region for identification purpose. Other technique rather than gene level identification is DNA-DNA hybridization, and MLST (Multilocus Sequence Typing) was developed for typing pathogenic bacterial strains (Maeda *et al.,* 1998). Generally, MLST requires use of several housekeeping genes (ribosomal operon, circadian genes, and cytochrome b6) together. This approach was also found suitable for the identification of cyanobacteria.

Another one easy and accurate method for the identification of phototrophs is identification of PUFM gene in natural isolates. According to Achenbach, *et al.* (2001) application of PCR primer sets PUFM phylogenetic gene targeted for the detection of green sulphur bacteria, green non sulphur bacteria, and heliobacteria and primers specific for a conserved photosynthesis gene for the detection of purple phototrophic bacteria.

1.11 Significanceof sulphur oxidizing bacteria and H_2S removal in various aquatic systems.

H_2S in aquatic system is one of the real issues emerging in now a days and it will affect the health and sustainability of a system as discussed earlier. Several microorganisms have been studied for application in biotechnological hydrogen sulphide removal processes. Based on the above understanding on bioremediation of hydrogen sulphide and its importance, significance in various aquatic systems the following objectives have been identified for the present work.

1.12 Objectives

- Isolation, purification and characterization of photosynthetic bacterium, MCCB147 as bioaugmentor for bioremediating hydrogen sulphide in aquatic systems.
- Isolation, purification and characterization of photosynthetic bacterium MCCB146 as bioaugmentor for removal of hydrogen sulphide in aquatic system
- Isolation, purification and characterization of photosynthetic Bacterium, MCCB234, as bioaugmentor for bioremediating hydrogen sulphide in Aquatic Systems.

Chapter **2**

ISOLATION, PURIFICATION AND CHARACTERIZATION OF PHOTOSYNTHETIC BACTERIUM, MCCB147 AS BIOAUGMENTOR FOR BIOREMEDIATING HYDROGEN SULPHIDE IN AQUATIC SYSTEMS

Contents

2.1 General introduction

Bioremediation, an integral part of Environmental Biotechnology Programmer, explores the use of biological mechanisms to protect the environment. Use of living organisms, primarily microorganisms, is emerging as one of the most powerful alternative technologies for remediation of contaminants, restoration of contaminated sites and prevention of spread of pollution. The natural biological processes can be explored to remediate nutrient-rich water by converting nutrients in to forms that can be more easily removed (Panigrahi *et al*., 2004).

With regard to sulphide oxidation, oxic–anoxic transition zone (OATZ) of freshwater as well as marine sediments, where opposing gradients exist of reduced iron and sulfide with oxygen, creates a suitable

environment for microorganisms that derive energy from the oxidation of iron or sulfide. In these environmental conditions phototrophic organisms are dominated and use reduced compound as electron donors during photosynthesis.

Photosynthetic bacteria are isolated from various environments such as aquatic, marine, and terrestrial habitats. Identification of microorganism is fundamental of biological life science, classification systems of organisms are historically based on observable characteristics. The distinguishing morphological characteristics of photosynthetic bacteria are frequently too limited to allow its identification. The increased application of electron microscopy resulted in a number of significant findings such as the internal photosynthetic membranes of a photosynthetic bacterium and its lamellar structure, numerous gas vacuoles, vesicles and sulphur granules are the characteristic feature of photosynthetic bacteria which aid in the systematic identification. Simplified biochemical methods have also been developed based on assimilation characteristics. These techniques have contributed significantly to the advancement of taxonomy of photosynthetic bacteria, but on occasions, fluctuated with changes in environment have provided uncertain results. The phenotypic approach has been largely criticized for its lack of standardized and stable terminology and for its high subjectivity. In this approach, molecular phylogenetic analysis could be the basic criteria for classification of genera and species, but the cytological and morphological traits and their ecology are an integral part of taxonomic identification.

Biochemical characterization is considered as the primary data of organism. For detailed study of an organism, the biochemical identification of microorganisms gives us an idea of what these microorganisms are able

to do, being possible the discrimination of different strains of the same species by specific biochemical profiles. In the case of photosynthetic bacteria biochemical characterization is in terms of growth of the organisms in the presence or absence of different carbon sources, light, and their pigment compositions, and at different culture conditions.

Scope of light microscopic observation is too limited for identification of a species owing to the limited differences in morphology among photosynthetic bacteria. However, TEM can make clear identification of the internal features like lamellar intra cytoplasmic membrane, sulphur granules, vesicles and gas vacuoles as the characteristic features of photosynthetic bacteria not common in other bacterial species. In this point of view, transmission electron microscopy aid in the characterization and identification at primary level.

This chapter deals with enrichment, purification, identification and characterization and development of a bioaugmentor MCCB147 (*Rhodospeudomonas julia*) for the removal of hydrogen sulphide in aquatic system.

2.2 Materials and methods

2.2.1 Sampling

Water samples were collected from different localities: Samples included shrimp pond water and sediments collected from Ponneri, Tamil Nadu, Kodungallore and Maraud in Kochi. Salinity in the sampling stations varied from 10-40. All the samples were enriched in Pfennig's medium 1 (Pfennig *et al.*, 1981) described in a previous study by Manju (2007). Specifically the photosynthetic bacteria used in this work came from a water

sample collected from Maradu, Kochi and enriched (Consortium) named as PF2. This medium was used subsequently for all studies.

2.2.2 Purification of photosynthetic sulphur bacteria by deep agar dilution method (Pfennig *et al.*, 1981)

Preparation of Pfennig's Medium

Solution 1-Mineral solution (Composition/1000ml)

Table.2.1

$CaCl_2 .2H_2O$	0.25g
KH_2PO_4	0.34g
NH_4Cl	0.34g
KCl	0.34g
$MgCl_2$	3.00g
$MgSO_4$	0.50g
NaCl	22.0g
Distilled water	964 ml

The solution was autoclaved for 15 min at 121°C together with a teflon coated magnetic bar and cooled to room temperature under nitrogen atmosphere with a positive pressure of 0.05-0.1atm. The cold medium was then saturated with magnetic stirring for 30 min under CO_2 atmosphere of 0.05-0.1atm. The following sterile solution was then added through the screw cap opened against stream of N_2 gas.

Solution 2-Vitamin solution

0.002% Vitamin B_{12} solution

Filter sterilized the solution and stored at 4º C.

Solution 3- Trace element solution

Table.2.2

Na_2-EDTA	3.00g
$FeSO_4$. $6H_2O$	1.10g
$COCl_2$. $6H_2O$	0.19g
$MnCl_2$. $2H_2O$	0.05g
$ZnCl_2$	0.042g
$NiCl_2$. $6H_2O$	0.024g
Na_2MoO_4. $2H_2O$	18.00 mg
H_3BO_3	300.00 mg
$CuCl_2$. $2H_2O$	2.00 mg
Distilled water	1000.00 ml

Adjusted pH to 6.0 and autoclaved at 121 ºC for 15min, stored at 4 ºC.

Solution 4-Sodium bicarbonate solution

Table.2.3

$NaHCO_3$	7.5g
H_2O	100 ml

The solution was flushed with CO_2 on a magnetic stirrer platform and after saturation, filter-sterilized and stored at 4 ºC.

Solution 5- Sodium Sulphide solution

Table.2.4

Na_2S	35g
Distilled water	1000.00 ml

After preparation, the solution was flushed withN_2 gas, autoclaved and stored at 4 ºC.

The Pfennig's 1 medium was constituted by mixing, after autoclaving 964 ml solution 1 with 1ml solution 2, 1ml solution 2, 1ml solution 3, 20ml solution 4 and 4ml solution 5. Final pH of the solution was adjusted to 7.3 by using sterile 1N HCl and NaOH.

Growth yield of photosynthetic bacteria can be increased by the addition of acetate as a readily assimilated carbon source. 0.05% acetate was regularly added to agar shake dilution culture. The ammonium and magnesium salts of acetate were used to avoid strong pH changes during growth.

Acetate solution was prepared by dissolving 2.5g each of Ammonium acetate and Magnesium acetate in 100ml distilled water. The solution was autoclaved and stored at 4 ºC. An aliquot of 1ml of the solution was added to 100ml culture medium.

Procedure

Granular agar was thoroughly washed several times with distilled water and then prepared at a concentration of 0.3% agar was prepared in distilled water. Depending on the salinity, 22g Sodium chloride and 3g Magnesium chloride were added per 1000 ml of agar preparation in distilled water, liquefied by autoclaving. After autoclaving, the agar containing flasks were kept molten in a water bath at 55 °C. At the same time, prepared Pfennig's medium was pre warmed to 40 °C. An aliquot of 10ml pre-warmed agar solution was added into 30ml screw capped tube, and at the same time added 20 ml pre-warmed Pfennig's medium with agitation to effect mixing.

Similarly, 5 screw capped bottles were prepared in the above manner and maintained at 40 ^{0}C in water bath. An aliquot of 1ml sample of enrichment (PF2) culture was inoculated into the 1st tube, the contents immediately mixed by turning the tube once upside down and back. From this tube, serial dilution was performed up to 10^{-5}. After inoculation, each tube was turned upside down to effect mixing and kept in cold water to harden the agar. After hardening, the tubes were immediately sealed with sterile, liquefied paraffin wax.

The agar shake dilution culture tubes thus prepared were incubated at 20 to 28 °C and 800-1000 Lux in 12 hour dark/light photoperiod. When colonies developed, the individual pigmented ones were isolated from the tubes of higher dilution. Well separated pinkish purple colonies were removed with sterile Pasteur pipettes. The cells were then suspended in 3 ml of anoxic medium and were used as the inoculum for subsequent agar shake culture. The process was repeated until pure culture was obtained.

2.2.3 Modification of Pfennig's medium for better growth of photosynthetic sulphur bacteria MCCB 147.

Habitually, phototrophic autotrophs, especially phototrophic purple sulphur bacteria, are slow growers (Pfennig *et al.,* 1981). For attainting better cell biomass production of the isolate MCCB147, modification of Pfennig's mineral medium (Pfennig *et al.,* 1981) was accomplished by incorporating 0.12% yeast extract as an organic component (Rees *et al.,* 2002), and pH adjusted to 7.5 and incubated under light/dark condition for 15 days. The growth was visually observed and recorded as photographic images.

2.2.4 Absorption spectra measurement (Sucrose method)

In the present study, 5g sucrose was mixed with 3.5 ml cell suspension until the dissolution of sucrose was complete, and the spectrum recorded against blank of 5 g sucrose in 3.5 ml distilled water in a UV- Visible spectrophotometer (Shimadzu UV-1601, Shimadzu Corporation,Tokyo Japan) (Truper and Pfennig, 1981).The characteristic spectrum was analyzed for specific absorption peaks with the documented ones.

2.2.5 Disaggregation of the cellular aggregate by chemical method

For enumeration and biochemical characterization, disaggregation of the cellular aggregate was required. As per the literature, aggregation is the characteristic nature of MCCB147 (Seitz *et al.*, 2006). Because of cellular aggregation, it was not possible to get a uniform distribution of cells, and to accomplish the same, following protocols were attempted.

An aliquot of 1 ml culture was mixed with aliquots of 100,200,300,400, and 500µl 1M and 2M urea, 100Mm EDTA, 1%Tween 80, 1%SDS and 1%TritonX respectively. These chemicals acted as detergents for disrupting the cellular hydrophobicity between cells to cell. (Seitz *et al.*, 1993). After overnight incubation, the cells were enumerated using Haemocytometer.

2.2.6 Cell hydrophobicity assay for calculating the percentage of cellular aggregation of MCCB147

For determining the extent of aggregation of the isolate MCCB147, percentage hydrophobicity was determined (Seitz *et al.*, 1993). An aliquot of 3 ml cell suspension was pipetted into 100ml glass test tubes containing 0.5 ml of hexadecane and the gas phase of each tube was flushed with nitrogen for 1 min and the tubes sealed with butyl-rubber stopper. After 10

min of pre incubation, and the tubes were vortexed vigorously for 2 min, the tubes were incubated for 15 minutes at room temperature to permit phase separation. Absorbance (lower phase) at 650nm was measured in a spectrophotometer (Shimadzu UV-1601, Shimadzu Corporation, Tokyo, Japan). Absorbance of untreated culture was maintained as control.

Hydrophobicity index (H)

$$H= 100(OD_{650} – OD'_{650}) \div (OD_{650})$$

where,

OD_{650} is the Absorbance of untreated cells

OD'_{650} is the Absorbance of treated cells

2.2.7 Absorbance Vs Biomass by determining packed cell volume

The culture of MCCB147 was MCCB147 was vigorously mixed and aliquoted 15 ml culture having 0.1, 0.2, 0.3, 0.4, and 0.5 absorbance, (at 620nm). 2ml aliquots of the culture was centrifuged at 12000*g* for 10 min in Microfuge (Eppendorf, India, PVT Limited), discarded the supernatant and repeated the process until the 15 ml culture was consumed and packed cell was obtained. Calculated wet and dry weight of cell biomass derived from 15 ml cell suspension having 0.1, 0.2, 0.3, 0.4, and 0.5 absorbance and determined dry weight of packed cells with respective optical density and calculated the factor value of absorbance vs biomass.

2.2.8 Biochemical characterization-Substrate Utilization

Biochemical characterization of the isolate MCCB147 was accomplished by incorporating different substrates following Shabeb *et al.*,

(2008) into modified Pfennig's medium and examining the ability to utilize them. The substrates and concentrations used were as follows:

Succinate (5mM), Tartrate (5mM), Citrate (5mM), Acetate (5mM), Glucose (2mM), Fructose (5mM), Glycerol (5mM), Valerate (5mM), Sucrose (5mM), Methanol (5mM), Propane (5mM), Butyrate (5mM), Sodium lactate (5mM), Ethanol (5mM), Benzoate (0.5mM), Thioacetamide (2mM), Pyruvate (5mM), Trehalose (5mM), Methionine (5mM), Butanol (5mM), Nicotinamide (2mM), Mannose (5mM), Glutamic acid (5mM), Formate (5mM).

Cell suspension of the isolate MCCB147 having absorbance 0.1 was inoculated into autoclaved 30 ml screw caped tubes containing modified Pfennig's medium with the respective carbon sources as mentioned above. Tubes without the substrates were used as control and all the experiments were conducted in triplicates. All the tubes were incubated at light and dark regime in a photochamber with 900 lux light intensity. After 15 days of incubation, the growth was measured as absorbance at 620 nm

2.2.9 Utilization of Ammonium chloride and Urea

Nitrogen Source

The cell suspension of MCCB147 having absorbance 0.1 was inoculated into 30ml screw capped tube containing modified Pfennig's medium (0.12% yeast extract) with 5 mM ammonium chloride and urea added separately. The same preparation of Pfennig's medium without the nitrogen source was used as the control 1 and Pfennig's medium was used as control 2. All the tubes were incubated in light and dark regime as mentioned above. After 15 days of incubation growth were measured in spectrophotometer at 620nm.

2.2.10 Utilization of other reduced sulphur compounds.

The selected reduced sulphur compounds were Cysteine (5 mM), Thiosulphate (5 mM) and Thioglycolates (5mM).

The isolate MCCB147 having absorbance 0.1was inoculated into 30ml screw capped tube containing modified Pfennig's medium with 5 mM Cysteine, Thiosulphate and Thioglycolate independently. The medium without substrate (reduced sulphur compound) served as control. All tubes were incubated in light and dark regime as mentioned above. After 15 days of incubation the growth was measured in spectrophotometer at 620 nm.

2.2.11 Growth of photosynthetic purple sulphur bacteria in the presence of nitrate, nitrite and glutamate

The isolate MCCB 147 having absorbance 0.1was inoculated into 30ml screw capped tubes containing modified Pfennig's medium with 5mM nitrate, nitrite and glutamate independently. Modified Pfennig's medium without substrate served as control1 and the one without ammonium chloride as control2. All tubes were incubated at light and dark regime as previously mentioned. After 15 days of incubation growth was measured as described above.

2.2.12 Growth of photosynthetic purple sulphur bacteria in the presence or absence of hydrogen sulphide.

The isolate MCCB147 having absorbance 0.1 was inoculated into 30 ml screw capped tube containing the basal medium with and without Sodium sulphide and incubated at light and dark regime as previously described. After 15 days of incubation, growth was measured as optical density in spectrophotometer at 620 nm.

2.2.13 Growth of MCCB147 under different modes of nutrition

This experiment was undertaken to evaluate growth and preliminary metabolic status of the isolate MCCB147 at different nutritional conditions (Pfennig's medium) as described in Table 2.5.

Table.2.5 Different nutritional and culture conditions employed

Mode of nutrition	Conditions
Photolithoautotrophy	Anaerobic, Light/dark, (5mM)$Na_2S.9H_2O$, $NaHCO_3$(0.1%w/v)
Photolithohetrotrophy	Anaerobic, Light/dark, Pyruvate (0.3%,w/v), (5mM)$Na_2S.9H_2O$,
Chemolithohetrotrophy	Microaerobic, Dark, Thiosulphate (1mM), Pyruvate(0.3% w/v)
Photoorganohetrotrophy	Anaerobic, Light/dark, Pyruvate (0.3%,w/v),Cysteine(1mM)

The isolate having 0.1 absorbance was inoculated into respective culture conditions as mentioned above. For creating anaerobic conditions respective tubes were tightly sealed by using parafilmfor attaining anaerobic conditions. To establish Chemolithohetrotrophic and Microareophilic conditions, the tubes were not sealed instead closed with screw caps. All the experiments were done in triplicates. The tubes were incubated under light and dark regime as previously mentioned, except for generating Chemolithohetrotrophic conditions were the tubes were incubated completely in dark, and after 15 days of incubation growth was visually examined.

2.2.14 Biomass production of the isolate MCCB147 at different physical conditions such as temperature, pH and salinity

Optimization of growth condition of the isolate MCCB147 was carried out in 30 ml screw capped tubes using modified Pfennig's medium.

2.2.14.1 Optimization of Sodium Chloride

Modified Pfennig's medium was prepared at 30 ml aliquots at different salinities such as 0, 5, 10, 15, 20, 25, 30, 35 and 40 ppt by addition of NaCl. The pH was adjusted to 7.2. The tubes were inoculated with 0.1ml cell suspension of the isolate MCCB147having absorbance of 0.1 at 620nm and were incubated at room temperature for 15 days in a photochamber having 1000lux unit. Growth was measured as absorbance at 620nm.

2.2.14.2 Optimization of pH

Modified Pfennig's medium was prepared in 30ml aliquots in saline water (25ppt). pH was adjusted to 3 ,4, 5, 6, 7, 8, 9, 10, 11,12 and 13 using 1N HC land 1N NaOH, inoculated with the culture having 0.1 absorbance. The tubes were incubated at room temperature for 15 days under light/dark regime and growth was measured.

2.2.14.3 Optimization of temperature

Pfennig's medium was prepared in 30ml aliquots in saline water (25ppt) having pH adjusted to 7.2 and inoculated with the culture having 0.1 absorbance. The tubes were incubated at temperature controlled illuminated shaker incubator (Scigenics Biotech orbital shaker, LETTD-1L) for 15 days. The tubes were incubated at 5, 15, 20, 25, 30, 35, 40 and 45^0C. Growth was measured using spectrophotometer at 620nm.

2.2.15 Determination of Hydrogen Sulphide utilization of MCCB147 following Methylene blue estimation.

Sodium sulphide was used as the substrate for the following analysis (Fonselius *et al.,* 1983).

When MCCB147 attained growth for 15 days of incubation in a photobioreactor under light/dark regime in the absence of sodium sulphide in the modified Pfennig's medium, added 100 µl sodium sulphide (3.25g/100ml) to 30ml culture. The 30 ml Pfennig's medium tubes without inoculum was maintained as control. Mixed well and added 1 ml medium (from both test and control) to 50 µl N-Dimethyl-P-Phenylene Diamine Dihydrochloride (4g/L) and 50 µl of the Ferric Chloride solution (16g/L). After the addition of reagents, blue color developed within a minute was read at 670nm in spectrophotometer (Shimadzu UV-1601, Shimadzu Corporation, and Tokyo Japan). After 24 hr incubation, the final reading was taken and calculated the percentage removal rate of the dye.

2.2.16 Transmission Electron Microscopy of the Photosynthetic Bacterium MCCB 147

The ultrastructure of MCCB147 was investigated through transmission electron microscopy. The culture MCCB147 was centrifuged at 8000*g* for 15 min, resuspended in PBS and centrifuged again at 8000*g* for 5 min, the process repeated three times. After centrifugation, supernatant was discarded and 1 ml Karnoskey's fixative was added to the pellet and kept for incubation overnight. The cell suspension was washed with sodium cacodylate buffer, centrifuged at 8000*g* for 15 min.The supernatant was discarded and the pellet was post fixed by addition of1ml of 1% Osmium

tetroxide and 3 ml sodium cacodylate buffer, and incubation at 4ºC for 3hrs. The cell suspension was embedded in epoxy resin, ultrathin sectioned, and positively stained with 2% uranyl acetate and viewed under TEM (Philip CM100) at the Electron Microscope Facility of All India Institute of Medical Science, New Delhi.

2.2.17 Molecular characterization of MCCB147

2.2.17.1 DNA based methods

To extract the DNA, a sample of 2 ml of bacterial cell suspension (20days old bacterial cell suspension grown in Pfennig's modified medium) was centrifuged at 15,000*g* for 10 min at 4^0C. The pellet was collected and re-suspended in 500µl of TNE buffer (10mM Tris-Cl, pH 8.0, 1 mM EDTA, and 0.15 mM NaCl) and centrifuged again at 15,000*g* for 10 min at 4^0C. Subsequently, the pellets were re-suspended in 500 µl lysis buffer (0.05 mM Tris-HCl, pH 8.0, 0.05 mM EDTA, 0.1 mM NaCl, 2%, SDS, 0.2 % PVP and 0.1% mercaptoethanol) (Lee *et al*., 2003) and 10µl of proteinase K (20mg/mL) were added and incubated for 1 hrs at 37^0C, followed by 2 hrs at 55^0C. Further extraction was carried out by phenol-chloroform method. The sample was deproteinated by adding equal volume of phenol (Tris- equilibrated, pH 8.0), chloroform and isoamyl alcohol mixture (25:24:1). The phenol and the aqueous layers were separated by centrifugation at 15,000*g* for 15 min at 4^0C. The aqueous phase was carefully pipetted out into a fresh tube and the process was repeated once. Following this, an equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, mixed by gentle inversion and centrifuged at 15,000*g* for 15 min at 4^0C to separate the aqueous phase, which was then transferred to a fresh tube. Then the DNA was precipitated

by adding equal volume of chilled absolute ethanol and incubating at -20 ^{0}Covernight. The precipitated DNA was collected by centrifugation at 15,000*g* for 15 min at 4^{0}C and the pellet washed twice with 70% ice cold ethanol. The supernatant was decanted and the tubes left open until the pellet got dried. The DNA pellet was dissolved in 100µl MilliQ (Millipore) grade water. The isolated DNA was quantified spectrophotometrically (Abs_{260}) and the purity of DNA assessed by calculating the ratio of absorbance at 260 nm and 280 nm (Abs_{260}/Abs_{280}). Electrophoresis was done using 1% agarose gel.

Concentration of DNA (µg/µl) = Abs_{260}×50×dilution factor.

Amplification of 1500bp bacterial rRNA gene fragment was performed using 16S rRNA gene, 16S -1F (5' GAG TTT GAT CCT GGC TCA 3') and 16S-1R (5' ACG GCT ACC TTG TTA CGA CTT 3') primer systems (Reddy *et al*., 2000). PCR amplifications from pure cultures were performed in a total volume of 25µl containing 1X PCR buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 20mM TrisHCl, pH 8.8), 500µM each deoxyribonucleoside triphosphate, 1 µl of both forward and reverse primer (7.5 pmol/µl), 1µl of template DNA (100ng/µl) and 1U Taq polymerase (New England Biolabs) was added. PCR was performed (Thermal cycler: Eppendorf Mastercycler Personal) with the following thermal profile: initial denaturation at 95^{0}C for 5 min followed by 34 cycles of denaturation at 94 ^{0}Cfor 20sec, annealing at 58^{0}C for 30sec and extension at 68 ^{0}Cfor 2 min followed by final extension at ^{0}C68 for 10 min.

Cloning to pGEMT easy vector

Fresh PCR product of 16S rRNA gene was cloned in to pGEM-T easy vector (Promega,USA). The ligation mix consisted 2X rapid ligation buffer - 5µl, pGEM-T easy vector (50ng/µl) -0.5 µl, PCR product -3.5 µl, T4 DNA ligase (3U/µl) -1 µl. It was incubated at 4 ^{0}Covernight.

The entire ligation mix was used for transformation into JM 109 high efficiency competent cells of *Escherichia coli* (transformation efficiency f≥ x10^{8} cells/ µg DNA). The ligation mix was added to 10 ml glass tube previously placed in ice to which 50 µl of competent cells were added and incubated on ice for 20 min, a heat shock at 42 ^{0}Cwas given for 90 seconds to facilitate the entry of recombinant DNA to the host cells and the tubes were immediately placed on ice for 2 min. An aliquot of 600 µl of SOC medium (2% tryptone, 0.5% yeast extract,10 mM sodium chloride, 2.5mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM glucose per liter) was added and incubated for 2 hrs at 37 ^{o}C in an incubator shaker at 250 rpm. The transformation mixture (200 µl) was spread on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml), IPTG (100 mM), and X-gal (80 µg/ml). The plates were incubated at 37^{0}C overnight. The clones/transformants were selected using blue/white screening. The white colonies were selected and streaked on LB-Amp + X-gal + IPTG plates and incubated overnight at 37^{0}C. Colony PCR was done to confirm the presence of the insert DNA (DNA fragment to be cloned). All individually streaked colonies were tested with colony PCR using universal vector primers T7 (5'-TAATACGACTCACTATAGGG- 3') and SP6 (5'GATTTAGGT GA CACTATAG-3') to confirm the presence of the gene of interest and electrophoresis was done on 1 % agarose gel

prepared in 1X TBE buffer and stained with ethidium bromide. White colonies (template) picked from the transformed plate were dispensed into the PCR mix (25 µl) containing 2.5 µl 10X PCR buffer, 2.0 µl of 2.5 mM dNTPs, 1 µl of 10 pmol/ µl of T7 and SP6 primers. The thermal cycling conditions were as follows: After an initial denaturation at 95^0C for 5 min, amplification was made through 35 cycles, each consisting of a denaturation at 94^0C for 15 sec, annealing at 57^0C for 20 sec, extension step at 72^0C for 1 min and a final extension at 72^0C for 10 min.

Plasmid extraction and purification

Plasmid extraction and purification was done using 'GenElute HP' plasmid miniprep kit (Sigma) Cells were harvested by centrifuging 2 ml overnight grown recombinant *E.coli* at 16000 x *g* for 20 min. The pellet was re-suspended in 200µl resuspension solution containing RNase followed by the addition of 200µl lysis buffer to permit cell lysis. This suspension was immediately mixed by gentle inversion until the mixture became clear and viscous. The cell debris was precipitated by adding 350µl the neutralization buffer. The tube was gently inverted and the cell debris pelleted by centrifuging at 16000 x *g* for 10 min. Column was prepared by inserting a Gen Elute HP Miniprep Binding column into a micro centrifuge tube. Contaminants were removed by spin wash step. Transferred the column to a fresh collection tube and added 100 µl elution buffer (10 mM Tris-HCl) to the column and centrifuged at 16000 x *g* for 1 min. The DNA present in the eluate (plasmid DNA) was stored at -20^0C Nucleotide sequencing was performed using SciGenom Labs Pvt.Ltd., Kochi, Kerala, India. The primers used were T7 and SP6. Sequenced DNA data was compiled and analyzed. The sequence obtained was first screened for vector regions using

'VecScreen' system accessible from the National Centre for Biotechnology Information (NCBI). The Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.,* 1990) was used to search the GenBank database for homologous sequences (http:// www.ncbi.nlm.nih.gov/). The sequences were multiple aligned using the programme Clustal W (Thompson *et al.,* 1994). The aligned 16Sr RNA gene sequences were used to construct a phylogenetic tree (Saitou and Nei, 1987) using the MEGA 6 package (Tamura *et al.,* 2007).

2.2.17.2 Specific primers of photosynthetic sulphur bacterial PUFM (Photosynthetic Unit Forming gene) gene encoding the M subunit of the photosynthetic reaction centersof MCCB147

Oligonucleotide primers for PUFM genes were synthesized by Sigma Aldrich, USA. Primer sets were selected to target specific Photosynthetic unit forming gene (PUFM gene encoding the M subunit of the photosynthetic reaction centres, which is universally distributed among purple sulphur bacteria (Achenbach *et al.,* 2001). PUFM gene was used for the identification of isolate in the species level.

Genomic DNA was amplified with the primer PUFM.55F –CGCACCTGGACTGGAC, PUFM750R –CCCATGGTCCAGCGCCAGAA (Achenbach *et al.,* 2001). The product size was expected at 229bp and PCR mix included 15 µl deionized water, 2.5 µl 10X Thermopol buffer (New England Biolabs, USA), 1 µl 10 mM dNTPs, 2 µl Taq polymerase (New England Biolabs, USA), 1 µl 10 pmol primers and 120 ng DNA template in a final volume of 25 µl. The amplification of PUFM gene was carried out in a thermal cycler (Eppendorf, Germany). The PCR condition was in the following manner, 94 ^{0}C3min followed by 30 cycles of 94^{0}C for 1 min and

annealing temperature was 55^{0}C for 1 min then 72 ^{0}Cfor 1 minutes and final extension was 72 °C for 10min. Amplified product was obtained at 229bp and was separated by cutting the gel and purifying using Sigma gel extraction kit (GenElute^{TM} Sigma Aldrich) and was documented (BIO-RAD-molecular imager Gel DOC^{TM}U.S.A) and sequenced at Scigenom Lab Pvt., Kochi, Kerala, India.

Gel purification of PCR product

Gel purification was carried out using (GenElute^{TM} Gel Extraction kit U.S.A Sigma Aldrich). For purifying the gene product, agarose gel that contained the DNA bands of appropriate size was excised and taken in a 1.5mL tube, weighed and added 3 gel volume (approximately 450 µl) of gel solubilization solution and incubated at 60^{0}C for 10 minutes with repeated vortexing in every 2 min. After incubation, 1 gel volume (approximately 150 µl) of 100% isopropanol was added and mixed gently until it became homogenous. This solubilizing gel solution was loaded in the binding column that was pretreated with column preparation solution, centrifuged at 12,000x *g* for 1 min. 700 µl wash solution was added and centrifuged for 1 min at 12000 x *g*, repeated the centrifugation, and residual wash solution was removed. The binding column was transferred in to a fresh collection tube (2 ml MCT) and added 50 µl of preheated (at 65 ^{0}C) 10 mM Tris-HCl (pH 9.0), centrifuged at 12000 x *g* for 1 min, stored at -20 ^{0}C. The concentration of DNA was measured spectrophotometrically at 260/280nm in a UV-VIS spectrometer (U2800, Hitachi, Japan). The purified PCR product of 229bpPUFM gene was sequenced at Scigenom Labs Pvt., Kochi, Kerala, India.

2.2.17.3 Screening of sulphide oxidizing genes in MCCB 147

Table.2.6. Primers used in the present study

Genes	Primer Sequence and reference	Annealing Temp	Product Size
"FCSD(designed primer)"	Forward- 5'GAGGAGCAACACCATGACCCAAAG 3' Reverse- 5'GGACAAGAAGCTGGTCAAGACTGC3'. (Versa Kotanjeveeki *et.al.,* 2010)	60+/-6^{0}C for 30sec.	1022bp
"SOX"	Forward 5'AAAATCTAGACCAATACCGTGAAAGTCAC CATCGGCGGCT-3' Reverse5'AAAAGGATCCAGATCTCGCGGCCC TTCTCCCAGG TCGACT-3' (Ayme *etal.,*2001)	60+/-6^{0}Cfor 30sec.	300bp
"SQR"	SQR-G3-199F 5'TBT AYS AGC CGG GWC TKC TBT3' SQR-G3-566R 5' GGY GCM ACS GGG CAT TTG3' (Pham *et al.,* 2008)	60+/-6^{0}Cfor 30sec.	322bp
Diguanulate cyclase.(designed primer)	F – TCAGTCCATGGCGGAAAT R - TCAGAAGCGGATATAGGACGA	57^{0}C for 20sec.	1000bp
DsrAgene (designed primer)	dsrA F - CGCAATGGCTATCGACAA dsrA R - CGATTCTTGGGGAGTACGAT	57^{0}C for 20sec.	1000bp

Oligonucleotide primers for sulphide oxidizing genes were synthesized by Sigma Aldrich, USA. The primers selected for the detection of genes included, FCSD **(**Flavocytochrom C-Sulphide Dehydrogenase), SQR (Sulphide Quinone Reductase), SOX (Sulphide Oxidizing Multi-enzyme System). DSR (Dissimilatroy Sulfite Reductase) and Diguanulate cyclase.

Primers for DSR A, Diguanulate cyclase and FCSD genes were newly designed using the primer 3 software, Gene tool from the genome

sequence of *Allocromatium vinosum* and *Rhodovulum sulfidophilum* respectively (Dahl *et al.*, 2005 & Kotanjeveeki *et al.*, 2008).

For amplification of all the genes from the culture MCCB147, PCR was performed in thermal cycler and total volume of 25µl containing 1X PCR buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 20 mM TrisHCl, pH 8.8), 500µM each deoxyribonucleoside triphosphate, 1µl of both forward and reverse primer (7.5 pmol/µL), 1µL of template DNA (100ng/µl) was used. Gradient PCR was performed (Thermal cycler: Eppendorf Mastercycler Gradient) with the following thermal profile: initial denaturation at 98 °C for 30 sec, 98 °C for 10 sec 60 +/- 6^0C for 30sec 68 ^{0}C for 45 sec, repeat 34 cycles and 68 ^{0}C for 5 min. Annealing temperatures as tabulated Table 2.6.

2.2.18 Evaluation of MCCB 147 as a bioremediator in the removal of sulphide in aquatic system (Simulated Microcosm Study)

Preparation of MCCB147 biomass

Biomass MCCB147 was generated in laboratory condition using modified Pfennig's medium and the culture conditions were as discussed earlier. pH was adjusted to 7.3 and all the tubes for biomass production were incubated at room temperature for 15 days under light/dark condition. After the incubation period cell biomass was divided in to three portions on the basis of absorbance 0.1, 0.3 and 0.5 OD. The experiment consisted three phases as described below.

First phase of experiment

Black sediment (Fig.No.2.37) was brought from a culture pond at Cherthala, Kerala and allowed to settle in a FRP tank for a couple of weeks. Subsequently, analyzed hydrogen sulphide in the sediment following Methylene blue spectrophotometry (Fonsalius *et al.,* 1983).Weighed 150g black sediment into 3.5L capacity plastic bottles with 1.5L sea water having 25 ppt and tightly closed with using polythene transparent cover which could allow light to pass through providing anaerobic conditions. The water quality parameters such as ammonia, E*h*, pH, nitrate and nitrite (Grasshoff *et al*., 1999) of the samples were analyzed before inoculation.10ml of the cell suspension of MCCB147(25ppt) at three optical densities (0.1, 0.3, 0.5) were applied into the bottles provided with 1200 lux natural dim sunlight light as measured by lux meter (PCE-EM886). The bottles without inoculum were taken as control and triplicates were kept for all the bottles. All the bottles were incubated for 4 days for the acclimatization of the culture with the environment. After incubation, 5ml of 3.25g/100 ml concentration of sodium sulphide was added to all the tubes and calculated the initial concentration (0 hrs) of H_2S by Methylene Blue Spectrophotometric method. Further, the removal rate of H_2S of test samples was checked from $1^{st}$to 8^{th} day of the experiment by comparing with the control.

2^{nd} phase of experiment

The water quality parameters were again checked (7^{th} day) before starting the second phase. Adequate volume of hydrogen sulphide and cell suspension of MCCB 147 could be added to the test bottles depending on the sulfide removal efficiency. Experiment could be continued same as first phase for a week for checking theH_2S removal potency.

3rd phase of experiments

Before starting the third phase, water quality parameters were again measured (Final day). Depending on the removal efficiency, hydrogen sulphide was added accordingly. Then the experiment proceeded for one more week as the other two phases.

2.2.19 Water quality – Physico -chemical analysis

2.2.19.1 pH and E*h*

pH was measured using digital pH meter (Scientific tech, India) similarly E*h* was measured by E*h* meter (Eutech instrument, Japan)

2.2.19.2 Estimation of Ammonia (Solorzano, 1969)

Ammonia reacts in moderately alkaline solution with hypochlorite to monochloramine, which, in the presence of phenol, catalytic amounts of nitroprusside ions and excess hypochlorite, gives indophenol blue. The formation of monochloramine requires a pH between 8 and 11.5. At a pH higher than 9.6, precipitation of Mg and Ca ions as hydroxides and carbonates occurs in seawater. However, these ions can be held in solution by complexing them with citrate. An aliquot of 10 ml sample was taken in a test tube, added 0.4 ml phenol solution (20g of crystalline phenol dissolved in 95% V/V ethyl alcohol), 0.4 ml sodium nitroprusside (1g dissolved in 200 ml distilled water) and 1.0 ml oxidizing solution [alkaline reagent (100g sodium citrate and 5g sodium hydroxide dissolved in 500 ml distilled water) and sodium hypochlorite 4: 1 ratio]. Absorbance was taken at 640 nm after 1 hr incubation at room temperature. A series of standards (4.714 mg ammonium chloride dissolved in 100 ml double distilled water gave 10µg/ml ammonia - nitrogen) were prepared and the factor value was calculated.

Ammonia nitrogen in mg/L = Factor Value x Absorbance of the sample

Factor Value = Concentration of standards/ absorbance

2.2.19.3 Estimation of Nitrite and Nitrate (Bendschneider and Robinson, 1952)

Under acidic condition (pH 2.0 to 2.5) nitrite ion (NO_2^-) as nitrous acid(HNO_3) reacts with sulphanilamide to form a diazonium salt, which combine with N- (1-naphthyl)-ethylene diamine dihydrochloride (NED dihydrochloride) to form a bright coloured pinkish red azo dye. The colour produced is directly proportional to the amount of nitrite present in the sample. Nitrate is determined by converting nitrate to nitrite using a mixture of phenol – sodium hydroxide and copper sulphate - hydrazine sulphate. Reagents were added and incubated at dark for 18 hours, added acetone and complexed with sulphanilamide and NED.

Nitrite analysis

An aliquot of 10 ml sample was taken in a test tube, added 0.2 ml sulphanilamide (5g dissolved in a mixture of 50 mL concentrated hydrochloric acid and 450 ml distilled water) and 0.2 ml of NED (0.5g dissolved in 500 ml distilled water). The absorbance was taken after 8 minutes at 543 nm. Series of standards (4.925 mg sodium nitrite dissolved in 100 ml gave 10µg/ml) were prepared and calculated the factor value.

Nitrate analysis

To the 10 ml sample added 0.4 ml phenol – sodium hydroxide solution [This solution was prepared by the mixing of Phenol solution (46 gm dissolved in 1 L distilled water) and sodium hydroxide (30g dissolved in 2 L distilled water) at 1:1 ratio] and 0.2 ml hydrazine sulfate – copper

sulphate solution [this solution was prepared by the mixing of hydrazine sulphate (14.5g hydrazine sulphate dissolved in 1 L distilled water) and copper sulphate (0.1g copper sulphate dissolved in 1 L distilled water) at 1:1 ratio], incubated in dark for 18 - 24 hrs.

After incubation 0.4 ml acetone, 0.2 ml sulphanilamide and 0.2 ml NED were added. Absorbance was measured after 8 minutes at 543 nm. A series of standards (6.0707 mg sodium nitrate dissolved in 100 ml gave 10 µg/ml Nitrate - nitrogen) were prepared and calculated the factor value.

Calculation

Concentration of nitrate in sample in mg/L = [(x-y) x 100/ efficiency]

Where x = Absorbance of nitrate x Factor value of nitrate

y = corresponding concentration of nitrite

Efficiency = (A/B) x 100

Where A = Observed concentration of standard (absorbance x factor value of nitrite)

B = Original concentration of standard prepared.

Efficiency measures the percentage of nitrate converted into nitrite.

2.3 Results

2.3.1 Enrichment and isolation

A consortium of purple sulphur bacteria was successfully developed and named as PF-2. This was subjected for further purification (Fig.2.1 & Fig.2.2).

2.3.2 Purification of photosynthetic sulphur bacteria MCCB147 by deep agar dilution method (Pfennig *et al.*, 1981)

Among different colonies developed in each tube, from the highest dilution one of the colony was picked and purified by deep agar dilution method. Purity was confirmed through microscopy. Purified culture was coded MCCB147 (Fig.2.2).

2.3.3 Modification of Pfennig's medium for better growth of MCCB147

Modified Pfennig's medium could support better growth compared to the original medium, as growth was visible within a week. This was based on visual observation of growth. This prompted to utilize modified Pfennig's medium for further studies (Fig.2.3 & Fig.2.4). Results of the study helped to conclude that MCCB147 exhibited photolithohetrotrophic as well as photolithoautotrophic mode of nutrition having light as the common requirement.

2.3.4 Absorption spectra measurement

Characteristics absorption maxima of bacteriochlorophyll of MCCB 147 is given in Table.2.7 (Prokayotes, 1991).

Table.2.7

Bacteriochlorophyll type	Absorption maxima
bcl "a"	280-375,590,805,830-890
bcl "b"	400- 490, 605,840,1020-1040
bcl "c"	745-755
bcl "d"	725-745
bcl "e"	710-725

The peak obtained in the absorption spectra of MCCB147 at 888, 217, 553, and 486 are presented in Fig.2.5 & Fig.2.5.1. According to prokaryote, 1991 the peaks represent those of bcl "a" and bcl "b"

2.3.5 Disaggregation of the cells by chemical method

The steps taken for disaggregation of the cellular biomass were not successful suggesting that cellular aggregation was one of the important features of MCCB147.

2.3.6 Cell Hydrophobicity assay for calculating the percentage of cellular aggregation

According to the formula applied, calculated hydrophobicity index of MCCB147 was 55%.

2.3.7 Optical Density Vs Biomass (packed cell volume)

Determination of biomass as absorbance with respective wave length was essential for calculating the initial cell count during an experiment. The culture having increased cell hydrophobicity index was very difficult to be dealt with to find out the initial cell count. Therefore, enumeration of MCCB147 was carried out by determining the packed cell volume. When the culture was at exponential phase, its respective absorbance was used for the present study, and its Biomass was calculated gravimetrically and standard graph was plotted and found 5.2817 as the factor value. (Fig.2.6)

2.3.8 Biochemical characterization-Substrate Utilization by MCCB147

Among the substrates tested sodium lactate supported the highest biomass output (9 µg/ml). In the presence of methionine and nicotinamide

the biomass output was less than that of the control, and in all other cases growth was comparatively better than that in the control (Fig.2.7) (P<0.05).

2.3.9 Utilization of Nitrogen Source

Utilization of nitrogen sources such as ammonium chloride and urea by MCCB147 indicated that higher biomass production could be obtained in the presence of urea than that with ammonium chloride on maintaining two control sets (Fig.2.8).(The same preparation without the nitrogen source was used as control 1 and Pfennig's medium as such was used as control 2) (P<0.05)

2.3.10 Utilization of other reduced Sulphur compounds.

On incorporation of reduced sulphur compounds such as cysteine, thiosulphate and thioglycolate in growth medium, MCCB147 exhibited negative growth in terms of biomass production (Fig.2.9) (P<0.05).

2.3.11 Growth of MCCB147 in the presence of nitrate, nitrite and glutamate.

On incorporation of nitrate and nitrite in to the medium, MCCB147 exhibited reduced growth; however, when glutamate was incorporated enhanced biomass production was noticed (Fig.2.10) (P<0.05).

2.3.12 Growth of MCCB 147 in the presence of hydrogen sulphide.

Biomass production in the presence of sodium sulphide was only marginally higher than that in its absence (Fig.2.11) (P<0.05).

2.3.13 Growth of MCCB147 under different mode of nutrition

On growing the organisms under different conditions, light was found essential concluding it to be obligatorily photosynthetic (Table.2.5).

2.3.14 Optimum temperature, pH and salinity

2.3.14.1 Optimum Sodium Chloride

MCCB 147 exhibited wide range of tolerance to sodium chloride and having the optimum ranging from 20 to 35ppt in terms of biomass production(P<0.05)(Fig.2.12).

2.3.14.2 Optimum pH

Biomass production of MCCB147 was found highest at pH 8, and a range from 7 to 8 could be ideal for practical purposes (P<0.05) (Fig.2.13).

2.3.14.3 Optimum temperature.

Highest biomass production of MCCB 147 was at 30 ^{0}C slightly higher than that at 25 ^{0}C (Fig.2.14). For all practical purposes 25 – 30 ^{0} C could be considered (P<0.05).

2.3.15 Determination of hydrogen sulphide removal ability of MCCB147 through methylene blue spectrophotometry

The utilization/removal efficiency of MCCB 147 was calculated in laboratory condition by methylene blue spectrophotometry from the difference between initial and final readings. The data presented (Fig.2.15) clearly demonstrated the significant removal (62%) of hydrogen sulphide over a period of 24 hrs compared with that of the control. This experiment was considered as a primary level assessment of the efficacy of the organism.

2.3.16 Transmission electron microscopy of MCCB147

One of the important characteristics of phototrophic bacteria of Alpha-2 *(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 *Rhodopseudomonas* species.Transmission electron micrograph of MCCB147 demonstrated the cells with stack of intracytoplasmic membranes, cytomembranes, lamellar and tubular cytoplasmic membranes with inclusion bodies, numerous vesicles, Polyhydroxy butyrate granules (Fig.2.17) and reproduction by way of budding (Fig.2.16).

2.3.17 Molecular characterization

2.3.17.1 DNA based identification

Genomic DNA was successfully extracted by phenol chloroform isoamyl alcohol method (Fig.2.18) and the quantity of purified DNA was determined spectrophotometrically.

2.3.17.2 16S rRNA amplification of photosynthetic MCCB147

DNA was isolated (Fig.2.18) and16S rRNA gene was amplified using universal primers and the PCR product of 1500bp (Fig.2.19) was cloned in to pGEMT easy vector and transformed in to *Escherichia coli* (JM109) and confirmed of the insert by colony PCR using T7 and SP6 vector primers which produced a product of 1500bp. Plasmid from the transformed organism was extracted and partially sequenced using T7 and SP6 primers, and sequence of the clones containing 16S rRNA region was matched with the GenBank database using the BLAST search algorithm

(Altschul *et al.,* 1990). The 16S rRNA gene sequences of all isolates available with the GenBbank were multiple aligned using ClustalW algorithm (Thompson *et al.,* 1994). To classify the isolate, 16S rRNA gene sequence obtained from the isolate with several non-sulfur alpha-2 *Proteobacteria* groups A, phylogenetic tree was constructed (Saitou and Nei., 1987) using MEGA 6 software (Tamura *et al.,* 2007). The sequence was submitted in the GenBank with the Accession number KU761996. Phylogenetic analysis of the isolate using the 16S rDNA sequence data suggested that the isolate was closely related to *Afifella marina* strain and *Rhodospedomonas julia* (Fig.2.20). Based on the morphological feature obtained in the TEM analysis, MCCB147 was closer to *R. julia* than to *Afifella* strain. Based on the present data the organism MCCB147 could be nomenclatured as *Rhodospedomona*s *julia.*

2.3.17.3 Specific primers of photosynthetic sulphur bacterial PUFM gene encoding the M subunit of the photosynthetic reaction centers.

DNA was isolated and amplified using PUFM primers and PCR product of 229bp (Fig.2.21) were excised from the gel as described earlier. Gel purification was carried out using Sigma extraction kit and the product has been sequenced and deposited in GenBank with Accession number KX-784507. The sequences were multiple aligned using ClustalW algorithm (Thompson *et al.,* 1994). The phylogeny reconstruction of the PUF M gene performed using Mega 6.0 software in which bootstrap phylogeny test of 1000 replication has been opted. The statistical method called maximum likelihood using Tamura-Nei model was selected in turn to explain the closeness/relatedness of the gene against the sequence from the NCBI database. Tree was constructed with 2 major clusters and sub clusters of

similar groups, of which >60% similarity the PUFM gene of *R. julia* branched as an unique representative. The maximum likelihood tree (Fig 2.22) indicated the closest match/similarity of the PUF M gene towards the *Marichromatium* photosynthetic PUF M gene with scale of 0.02.

2.3.17.4 Amplification of sulphide oxidizing genes from MCCB 147

Genomic DNA of MCCB147 was subjected to PCR amplification with 5 different primers of sulphide oxidizing genes namely FCSD, SQR, SOX, DSR A and Diguanylate cyclase genes. Among these genes only Diguanylate cyclase genes got amplified at 1000bp (Fig.2.23), and the sequence was converted into protein by using Expasy software. The sequences were multiple aligned using the ClustalW algorithm (Thompson *et al.*, 1994). The phylogeny reconstruction of the Diguanylate gene performed using Mega 6.0 software. It was found to be 100% similar with Diguanylate cyclase genes of *Caulobacter* sp AP07 (Fig.2.24). The sequence has been deposited in Genbank with Accession number KX-784509.

2.3.18 Evaluation of MCCB 147 as a bio-remediator in the removal of sulphide in aquatic system. (Simulated Microcosm Study)

1st phase of experiment.

Significant removal of H_2S was observed with all counts of the bacterial isolate MCCB 147 from the 2nd day onwards during first 8 days (P<0.05, Fig.2.25) and removal rate could be maintained till 12th day of phase one experiment (Fig. 2.26, P<0.05). Besides, 100% removal was obtained during the first phase of the experiment (Fig.2.27).

2nd Phase of experiment

On supplementing Na_2S on the 13th day of the experiment the same trend of significant sulphide removal was observed at all concentrations proving the efficacy of the culture in vitro in bioremediation of hydrogen sulphide (Fig. 2.28 & 2.29)(P<0.05).

3rdphase of experiment.

As presented in Fig.2.30 & Fig.2.31 sulphide removal by MCCB147 was significant during the 3rdphase of the experiment also (P<0.05, Fig.2.29). All cell counts showed the removal efficiency in all the three phases.

2.3.19 Major water quality parameters measured in the experimental system during the experimental period

Nitrification potential was seen in experimental and control systems and were statistically significant (P<0.05). The removal of total ammonia nitrogen noted in both the systems were alike (Fig.2.32). There was efficient removal of nitrate (Fig.2.33) and nitrite from control and tests. (Fig.2.34).

pH variations have been marginal but statistically significant (P<0.05) (Fig.2.35). Significant improvement (increase) (P <0.05, Fig.2.36) of E*h* was noticed in the experimental system.

2.4 Discussion

This chapter deals with the isolation, purification, biochemical and molecular characterization besides sulfide utilization ability of *Rhodospeudomonas julia* (MCCB147).*Rhodopseudomonas* species is short-rods to ovoid with vesicular type of intracytoplasmic membranes having

carotenoids as pigments. The genus *Rhodopseudomonas* consists of photosynthetic *Alphaproteobacteria* of extreme metabolic versatility reported by Oda *et al.* (2008). Couple of studies had addressed the use of *Rhodospeudomonas* as bioaugmentor for the removal of hydrogen sulphide in various aquatic systems. Nagadomi *et al.* (2000) postulated that simultaneous aerobic treatment of COD, phosphate, nitrate and H_2S could take place in synthetic sewage by using porous ceramic immobilized photosynthetic bacteria, *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris*. In a batch treatment, effective simultaneous removal of COD (89%), phosphate (77%), nitrate (99%) and H_2S (99.8%) could be observed after 48 h.

Robertson & Kuenen (1983) stated "The population in a sulphide-oxidizing effluent treatment system is likely to be complex, it is therefore necessary to start with pure culture studies of suitable model organisms". Hence, the approach to remediate sulphide by using pure culture MCCB147, *R. julia,* adopted in the present study has significance.

Rhodopseudomonas capsulata, *Rhodopseudomonas sphaeroides*, and *Rhodopseudomonas sulfidophil*a are able to use sulfide as electron donor as reported by Hansen and Gemerden (1972) and Hansen and Veldkamp (1973). Meanwhile *R. sphaeroides, R. capsulata*, *R. suldophila*, *Rhodopseudomonas sulfoviridis* (Keppen *et al* 1976), and *Rhodopseudomonas* strain 51 (Hansen *et al,* 1975), oxidized sulfide and thiosulfate to sulphate during photolithotrophic growth. *Rhodopseudomonas palustris*, which is capable of oxidizing thiosulfate or sulphide to sulphate, but nevertheless can reduce sulphate to acid-volatile sulphur compounds (Peck *et al*1974).

Several data are available with regard to sulphide utilization ability of *Rhodopseudomonas* during photosynthesis. Based on the previous

studies, a process has been developed for bioremediation of sulphide from an aquatic system by using *R. julia* (MCCB147).

Cellular aggregation is considered as the most important feature of sulphur and non sulphur bacteria and it has been considered the defensive mechanism of bacteria from extreme conditions such as oxygen toxicity, light with high intensity (Shabed *et al.,* 2008). According to Cary *et al.* (1998), the purple non-sulfur bacteria are the best studied and most diverse group of the phototrophic bacteria. All species grow best as anaerobic photoorganotrophs and have the capacity to grow also as facultative microaerophilic. According to recent taxonomic studies, the anoxygenic phototrophic bacteria have been presented in seven subgroups that can be differentiated by the characteristics. The genera *Rhodopseudomonas* has been presented in subgroup 3 (Purple non-sulfur bacteria), and eight species (*R. palustris, R. viridis, R. sulfoviridis, R. acidophila, R. blastica,**R. julia,**R. marina and R. rutila*) have been presented. In addition, a new species, *R. rosea*, has been isolated by Jansen and Harfoot, (1991).

In this chapter introducing first time, a guanylate cyclase (c-di-GMP) gene of *R. julia* could be amplified with newly synthesised primer which were obtained from *Allochromatium vinosum* complete genome (Dahl *et al.,* 2005). It was a secondary messenger protein having different functions intimately associated with biofilm formation such as polysaccharide biosynthesis, production and transport of biofilm matrix components, oxygen-dependent regulation, and motility. In addition, c-di-GMP can also control virulence and morphological development. Apart from this it can regulate the pathways of sulphide oxidation genes like DSR and SOX in *Allocromatium vinosum* in such a way that c-di-GMP can positively regulate

uptake and assimilation of sulphide in sulphide mediated photosynthetic organism (Grimm *et al.,* 2011).

In any process development for bioremediation, the organisms to be used as bioaugmentor has to be isolated, purified, and characterized and the bioaugmenting potency has to be confirmed. Having that as the objective, water samples were collected from a shrimp farm in Cochin at Maradu and enriched for photosynthetic sulphur bacteria, and the enrichment culture was named PF -2. Purification of the enriched organisms was done by deep-agar dilution method and brownish purple colonies were picked up which were found most dominant. According to Imhoff *et al.* (2001) and Mehrabi *et al.* (2001) purification of sulphur bacteria could be successfully carried out by deep agar dilution method employing Pfennig's medium. Meanwhile, Pfennig (1969), successfully isolated budding purple non sulphur bacteria *Rhodospeudomonas acidophilus* from Lake Urbana by deep agar dilution method. Photosynthetic sulphur bacteria in the mineral based medium took around one month for its growth. According to several published articles regarding purification of sulphur bacteria it was clearly postulated that it might be slow growers in mineral based media, perhaps by incorporating yeast extract and other organic constituents growth might be enhanced. Madigan *et al.* (1989) stated that illuminated anaerobic enrichments employing mineral salts media supplemented with fermentable organic compound as carbon source and N_2 as sole nitrogen source have been found to be highly selective for purple non-sulphur photosynthetic bacteria. In that point of view, 0.13% yeast extract could be incorporated as part of the medium. This type of modified media for the purification of sulphur bacteria have been reported by Bryantseva *et al.* (1999) and Rees *et al.* (2002).Yeast extract incorporated modified medium significantly affected

the growth of *R. julia* positively, the growth was visible within a week compared to the same in the original Pfennig's medium.

R. julia MCCB 147 was demonstrated to have photoheterotrophic and photolithoautotrophic mode of nutrition having light as the common requirement. Bacteriochlorophyll of *R. julia* MCCB 147 was measured based on absorbance and exhibited resemblance to bchl "a" and "b". The similar observation was reported by Rees *et al.* (2002) and this has been already established as characteristics of anoxygenic phototrophicbacteria by Truper and Pfennig (1981). According to Caumette *et al.* (2007), phototrophic bacteria containing BChl "a" have three major absorption bands in the near infrared region, at 800, 850–860 and 880–890 nm; the two first bands (800 and 850–860 nm) corresponds to light harvesting complex (LHC) II and the third (880 nm) to the low-energy LHC I. Based on the above statement it has been ascertained that *R. julia* (MCCB147) has (LHC -I) light harvesting system because of the presence of peak at 888 nm, as was observed in the absorption spectra of present study. The major carotenoid pigment present in *R. julia was* of spirilloxanthin series ascertained due to the presence of the peak obtained at 553 nm (Mehrabi *et al.,* 2001). According to Caumette *et al.* (2007) the peaks of carotenoids at 483, 512 and 548-557 nm, with the highest peak at 512 nm are typical of carotenoids of the normal spirilloxanthin series. Meanwhile, The absorption ratio at 800nm (originating from accessory BChl-a) and 280 nm (from aromatic amino acids in peptides), A280/A800 (in the present study it was 217 and 888 nm) is one of the reliable criteria by which the presence of bacteriochlorophyll "a" can be confirmed as done in the present isolate (Mizoguchi *et al.,* 2012).

Bacteria are ubiquitously found as surface-attached communities and cellular aggregates in nature (Fritts *et al.,* 2017). According to them, bacterial adhesion was coordinated in response to diverse environments and it could be found in *Rhodopseudomonas palustris*, one of the most metabolically versatile organisms ever described. Similarly, in the present study, we could find cellular aggregation in *R. julia* MCCB147. The basic reason for aggregation in *R. julia* could be its preference to live under anaerobic condition. However, when the oxygen concentration could be high it could take a defensive mechanism against oxygen intolerance, and it aggregate to each other and make a protective shield around them reducing intercellular space against oxygen toxicity. This phenomenon is well described in the study of Zeng *et al.* (2016), according to whom microbial extracellular polymeric substances (EPS) exhibit crucial effect on microbial adhesion and aggregation process in *Rhodospeudomonas acidophila* and promote the formation and stability of microbial community structure. Similarly, aggregate formation in *Amoebobacter purpureus* was attributed to the hydrophobic effect involving surface proteins, and it was considered as the defensive mechanism other than oxygen, for example virus invasion, pH change and light intensity (Seitz *et al.,* 1993). The aggregative nature was one of the characteristics of *Rhodospeudomonas* sp. (Xie *et al.,* 2013). Due to the aggregative nature, it was difficult to enumerate and quantify cells. For cell disaggregation to facilitate enumeration, several detergents, chemicals and physical methods such as sonication were tried, and none of the methods could provide satisfactory results. According to Seitz *et al.,* 1993, aggregate formation is an important feature of natural assemblages of bacteria. The aggregate formation of *Amoebobacter purpureus* in a meromictic salt lake was attributed to the hydrophobic effect involving

surface proteins. In this study the disruption of hydrogen bonding by urea disintegrated the aggregates but detergents, proteases and chaotropic agents had no effect. However, urea was not acting as chaotropic agent for disrupting cellular aggregates. Dense cell aggregates were formed after depletion of sulphide. Formation of aggregates was correlated with an increase in cell surface hydrophobicity (Overmann *et al.,* 2006). Thus the results indicated that cell-to-cell adhesion in *R. julia* was mainly caused by a hydrophobic effect and included a specific mechanism possibly mediated by a surface protein. Accordingly, calculated the percentage of hydrophobicity of MCCB147 and in the present study around 55% hydrophobicity index was expressed by *R. julia* (Seitz *et al* 1993). However, for enumeration of MCCB147, packed cell volume method was adopted and calculated the dry weight, and found out the factor value and growth was expressed in biomass µg/ml.

Generally photosynthetic sulphur bacteria are able to use a variety of organic acids, alcohols, sugars and aromatic compounds as carbon sources and/or electron donors during photoheterotrophic growth. Their metabolic and physiological diversity makes them an important component or agent for wastewater treatment, photo assimilation and mineralization of a variety of organic compounds (Novak 2004). According to Finn *et al.* (1983), Purple non-sulfur bacteria are able to utilize a wide range of organic compounds as photosynthetic electron donors and sources of carbon. When these compounds are metabolized under anaerobic-light conditions and excess reducing power is generated, the balance between oxidation and reduction in cells is usually maintained either by the reduction of CO_2 or by the formation of cellular reserve materials. Thus, the present study describes

properties of the isolated bacterium and its versatility on different carbon sources.

Biochemical characterization of *R. julia* exhibited positive results in most of the substrates except valerate, methionine and nicotinamide, and sodium lactate supported the highest biomass output. According to Szymona & Doudoraff (1960) *Rhodoseudomonas spheroids* species have the ability to utilize glucose, fructose mannose and glutamic acid as carbon sources during photosynthesis as same as MCCB147. Among 24 substrates, the most utilized carbon source was sodium lactate and similar result was reported in *Rhodopseudomonas gelatinosa* (Wertlieb & Vishniac 1967). Similarly Ramana *et al.* (2012) reported that *Rhodospeudomonas pseudopatustris* utilised most of the substrates same as the present study and it was considered as a high versatile organism. Out of 24 substrates one of the interesting features was the utilization of ethanol by the present isolate that could be a characteristic feature of *Rhodospeudomonas* sp. Alcohols are more reduced compounds than cell materials. Therefore, the excess reducing power seems to be generated from alcohols during the anaerobic-light metabolism (Finn *et al.,* 1983).

Utilization of nitrogen source was already reported in *Rhodopeudomonas* sp. by (Herbert *et al.,* 1978). Nitrogen is the one of the most important constituent in cell as well as cell metabolism. In the present study, importance of ammonium chloride in the medium could be substantiated and it could enhance the growth of MCCB147 significantly. Apart from that, another nitrogen source, urea, could positively influence the biomass production of MCCB147. Both the results strongly indicated that nitrogen source might

influence the growth of *R. julia* and similar observation was found in Merugu *et al.* (2012) and Ramana *et al.* (2012).

Utilization of reduced sulphur compounds was tested as growth enhancer of *R. julia* but result indicated that it could not influence the biomass production. One of the assumptions behind the result was that only cysteine influenced significant reduction in growth, and with respect to other reduced sulphur compounds, they were found to cause less variation in bio mass production compared to that of control. Under this circumstance the present study could clearly demonstrate *R. julia* prefer more sulphide as reduced sulphur compound for its photosynthetic activity. In one of the studies reported by Neutzling *et al.*, (1984), a new purple non-sulphur bacterium *Rhodospeudomonas adriatica* oxidised sulphide to elemental sulphur and was unable to assimilate other sulphur compounds and growth was possible only in the presence of sulphide. Similar such observations were reported by Srinivas *et al.* (2007) on non-sulphur bacterium *Rhodovulum imhoffii.*

Assimilation ability of nitrate, nitrite and glutamate of *Rhodospeudomonas* sp. was reported by Vivian *et al.* (1999), Castillo & Cardenas (1982.). The capacity to reduce nitrate, either for assimilatory or dissimilatory growth, is not a common feature of this group of bacteria, being restricted to several species (Hougardy *et al.*, 2000). Thus in the present study it could be found that presence of nitrate and nitrite never exhibited the enhancement of *R. julia,* andin fact, glutamate positively influenced the biomass production when compared with control. In one of the recent publications, Schott *et al.* (2010) addressed that, in the dark, some purple non-sulfur bacteria, such as *Rhodopseudomonas* sp. and *Rhodobacter* sp., could use nitrate as electron acceptor for respiratory ATP generation. However, current

organism is photoautolithotrophic and photolithohertotrophic and light is a vital factor for its growth thus there is no possibility to utilized nitrate and nitrite under dark. However, glutamate exhibited significant enhancement of biomass production of MCCB147, and in one of the observations Herbert *et al.* (1978), recorded that the photosynthetic bacteria *Rhodospeudomonas acidophila* did not grow on glutamate as sole nitrogen source and lacked glutamate dehydrogenase. But in the present study, the isolate may have the gene of glutamate dehydrogenase for glutamate metabolism, such a way that glutamate acts as nitrogen source for the biomass production of MCCB147. Similar observation was reported by Ohmori *et al.* (1974), in *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* as they were able to grow anaerobically in the light, utilizing glutamate as carbon source and electron donor.

The current isolated *R. julia* is a facultative hydrogen sulphide oxidiser and does not absolutely require H_2S as growth factor. However, in the absence of H_2S it can switch over to photolithoorganotrophic mode and it could be experimentally proved by the utilization of H_2S in the laboratory conditions. The results were quite interesting because in laboratory conditions the growth rate was more or less similar to H_2S presence or absence. Under this circumstances it could be concluded that the organism was not obligatory to hydrogen sulphide for its growth, however it utilized sulphide as elector donor when it was available for photosynthesis. Ghosh & Dam (2009) stated that, the photosynthetic electron transport and the oxidation–reduction processes of Photosynthetic Non-Sulphur Bacteria were having integrated complex mechanisms involved in photoautotrophic and photoheterotrophic growth. The photosynthetic apparatus, enzymes involved in sulphide oxidation as well as pathways of anaerobic respiration, are all influenced by the presence or absence of reduced compound in the

surrounding environment and oxygen tension. According to Weissgerber *et al.* (2014) adaptation of biological systems to changes in their environment was characterized by immediate and appropriate adjustments of physiology at every level of the cellular and molecular network and it was well studied in *Allochromatium vinosum.* In another statement from Ghosh & Dam (2009), Sulphide oxidizing genes (DSR SQR and FCSD) were found widely distributed in sulphur Bacteria even though its viability had so far been established only in facultative sulphur-oxidizing organisms. Accordingly in the present study, facultative sulphide oxidizing nature of *R. julia* was justified with the above statement.

Couple of experiments were conducted based on biochemical as well as nutritional assays and could find that MCCB 147 exhibited photolithohetrotrophic and photolithoautotrophic mode of nutrition. Similar observation was found in purple sulphur bacteria *Marichromatium indicum* by Arunasri *et al.*(2005) and Finster *et al*. (1998). According to Frigaard *et al.* (2008), the group of purple non-sulfur bacteria was by far the most diverse group of the photosynthetic purple bacteria, and their diversity was reflected by utilization of carbon sources, and by the kind of electron donors used for photosynthesis. Purple non-sulfur bacteria grow preferentially photoheterotrophically under anoxic conditions in the light. Some species even lack the ability to grow photolithoautotrophically. The study found that the present organism was able to grow photolithoautotrophically as well as photoautotrophically with sulfide as electron donor. Another important observation had been that the present organism was able to grow only in the presence of light such as*Rhodopseudomonas vinidis* (Lang & Oesterhelt 1989). Meanwhile,another *Rhodospeudomonas*sp. could survive under darkness and belonged to chemoorganotrophs (Weaver *et al.*, 1975). Apart

from that, *Rhodospeudomonas* sp. has extraordinary metabolic versatility and grows by any one of the three modes of metabolism that support life: photoautotrophic or photosynthetic (energy from light and carbon from carbon dioxide), photoheterotrophic (energy from light and carbon from organic compounds), and photolithoorganotrophs (energy from light and carbon from both carbon dioxide and organic matter) (Larimer *et al.*, 2004). Based on the above observations the present organism could be categorized under phototrophic bacteria. Another important observation was that the isolate was a facultative hydrogen sulphide oxidizer like purple sulphur bacteria *Allochromatium vinosum* and conveyed the importance of DSR operon. DSR operon play an important role in sulphide mediated photosynthesis in *A. vinosum* (Dahl *et al.,* 2005).

R. Julia was tested for growth on different salinities, and the bacterium showed the highest growth rate with 20 to 35 ppt range and beyond these limit growth was reduced at significant manner. Similar observation was made by Adessi *et al.* (2016), during cell growth of *Rhodospeudomonas palustris* in the presence of salts, where compatible solute was found accumulated and it protected the enzymatic functionality against salt stress, by the way it promoted cell biomass productions. According to Igneno *et al.* (1995), a few species of *Rhodobacter, Rhodopseudomonas,* and *Rhodospirillum* live in marine environments with high NaCl concentrations like MCCB 147.Based on the study of pH preference, this isolate preferred growth in alkaline conditions such as 7 or 8. Saharan *et al.* (2011) stated that ecological niches are positions designated to microbes where they perform individual role and interact all together to constitute ecosystem. Microorganisms derive their food via diverse behavioural adaptation in environment to put together

thriving existence similarly, the present isolate is more adapted to live in alkaline conditions, up to pH 12. According to Bryantseva *et al.* (1999)the purple non sulfur bacteria prefer to grow in alkaline environments. However, pure cultures of present isolate growth rates at pH 7-8 and pH tolerance exhibited up to 12. The preferred salinity range was 20 to 35ppt. Similarly, temperature preference ranged from 25^0C to 30^0C like photosynthetic sulphur bacteria *Chromatium tepedium.* The carotenoid pigment production and the other physiological and metabolic requirements were quite satisfactory in this temperature regime (Mandigan 1986). Apart from similar observations from Pfennig (1969), the optimum temperature of purple non sulphur bacteria *Rhodospeudomonas acidophila* was found 25 to 30^0C. Thus the study suggested that physiological conditions such as temperature, salinity and pH influenced microbial metabolism at significant level, and somehow it might affect and alter the expression of genes that might affect cell biomass productions.

Internally, MCCB147, has several intracytoplasmic membranes, circular vesicle cytomembranes, lamellar and tubular cytoplasmic membrane with inclusion bodies, poly hydroxyl butyrate, numerous vesicles and gas vacuole, and reproduction has been by budding as well. . The presence of intracytoplasmic membranes of lamellar type is typical of the genus *Rhodopseudomonas*. Similar observations have been reported by Zhang *et al.* (2002). Besides, poly hydroxyl butyrate has been found characteristic of *Rhodospeudomonas* sp. especially *R. julia* (Garrity *et al.,* 2005). Meanwhile, Dworkin *et al.*(2001) reported that the characteristics feature of *Rhodospeudomonas* is the budding mode of growth and cell division and the presence of lamellae of internal membranes lying parallel to the cytoplasmic membrane. Accordingly, it has been concluded that the

isolate *R. Julia* MCCB 147 falls under alpha 2 *proteobacteria* group in which *Rhodospeudomonas* sp. is included (Garrity *et al.,* 2005).

H_2S Utilization ability of *R. julia* in laboratory condition was analysed by methylene blue spectrophotometric method. The methylene blue method is the most commonly reported method used in literature to measure hydrogen sulfide in biological samples (Shen *et al.,* 2015). In this assay, the results were promising and it was considered as preliminary level of experiment in terms of H_2S removal efficiency by *R. julia* in the laboratory conditions. Microscopic observation found that *R. julia* was Gram negative or lipophilic in nature. Based on its lipophilic property, hydrogen sulphide can easily penetrate to the lipid bilayer of cell membranes (Shen *et al.,* 2015). Hence the isolate could be considered as "sulphide scavengers" on earth. *R. julia* exhibited 62% sulphide removal rate in laboratory condition and it was considered as obligatory sulphide oxidiser such as *Rhodospeudomonas capsulate* (Hansen 2006). Meanwhile, alpha proteobacteria *Rhodospeudomonas* sp. and its sulphide oxidizing ability were also reported by Yurkov & Veatty (1998).

On 16S rRNA sequence analysis the organism exhibited more similarity to *Afifella strain* and *R. julia.* However, based on the morphological and physiological observations it could be demonstrated more similar to *R. julia*. The nucleotide sequences determined in this study was deposited in GenBank with nomenclature as *R. julia.* The production of PHB is one of the important characteristic of *R. julia (*Garrity *et al.,* 2005) and it is clearly visible in transmission electron microscopic image of MCCB147. Based on the sequence analysis, genus *A.pfennigii* (formly known as *Rhodobium pfennigii*: Caumette *et al.,* 2007) and *Rhodospeudomonas* sp. are

coming under class Alphaproteobacteria. (Okamura *et al.,* 2009a). Meanwhile, Caumette *et al.,* (2007) & Urdiain *et al*. (2008) reported that *Afifella* was considered as a type strain of *R. julia*. Thus confirmed that these two organisms were taxonomically closer to each other and finally new group has been arisen from *Rhodospeudomonas* sp. named as *Afifella.* Meanwhile the taxonomy of the genus *Rhodospeudomonas* has been improved drastically during the past several decades along with combined use of phenotypic chemotaxonomic and phylogenetic information. This has led to the transfer of several species of *Rhodospeudomonas* to other genera. (Okamura *et al.,* 2009b).

Apart from 16S rRNA sequence analysis, other molecular markers have been analysed known as PUFM gene encoding a protein for the M subunit of the photosynthetic reaction centre in purple sulphur and purple non-sulphur bacteria (Okubo *et al.,* 2006). In photosynthetic purple and non sulphur bacteria the reaction centre consist of 3 sub unit L (Light), M (Medium) and H (Heavy).The L-and M- subunits are the integral membrane proteins. Both the sub unit L and M harbour associated pigments and provide the environment for electron transport flow in the reaction centre. Therefore, a metabolic gene, PUFM was chosen because it was unique to purple sulphur and non-sulphur phototrophic bacteria and universally distributed across the group. Thus, because the PUFM primers are based on a gene unique to organisms possessing a purple bacterial light-harvesting reaction centre, they can be used to detect any photosynthetic member of the *Proteobacteria* regardless of phylogenetic affiliation (Lancaster & Michel 1996, Barber and Andersson 1994 and Achenbach *et al.,* 2001). Another important characteristic of *R. julia* with regard to PUFM gene is the reddish pigmentation which indicated the presence of the photosynthetic pigments

carotenoids and bacteriochlorophyll "a" as discussed earlier. These pigments could be detected spectrophotometrically. The present isolate showed the characteristic reddish purple colour under anoxy-photosynthetic conditions and the presence of only bacteriochlotophyll and carotenoids pigments specifically spirilloxantin series. The photosynthetic apparatus in these organisms is controlled through a gene cluster that are composed basically of genes that encoded for bacteriochlorophyll, carotenoids pigments, light harvesting complex (PUF) and a reaction centre and molecularly, a technique frequently used to determine if the bacteria have an anoxygenic photosynthetic apparatus is the presence of a pigment binding protein encoded gene, known as PUFM gene reported by Soto-Feliciano *et al.,* 2010. Amplification of PUFM of MCCB147 indicating its reaction centre could be accomplished and Sequence analysis indicated that maximum likelihood was towards *Maricromatium* photosynthetic PUFM gene with scale of 0.02. The absorption peak at 880 nm indicated in connection with PUFM gene of *R. julia* it having LHC 1 with Bchl "a" (Zuber and Brunisholz, 1991; McLuskey *et al.* (*2*001). A second approach towards identification of light intensity in terms of absorption by *Rhodospeudomonas* lead to detection of BChl "a" but displayed extremely red-shifted QyLH1 absorption bands at wavelengths >900 nm (Tuschak *et al.,* 2004).

The enzymes involved in H_2S oxidation have been well characterized and its mechanisms of gene regulation under sulphide-dependent photosynthesis have not been elucidated (Shimizu *et al.,* 2017). In this study, we have identified a gene Guanylate cyclase (c-di-GMP) that may be regulator of sulphide-dependent gene in the present isolate *R. julia*. Molecular characterization of Guanylate cyclase (c-di-GMP) is considered to be a regulatory protein that plays an important role in the regulation of

sulphide oxidizing genes (SOX & DSR) in sulphur bacteria. The presence of SOX and DSR genes and its (c-di-GMP) mediated regulation for sulphide oxidation has already been reported in purple sulphur bacteria *Allochromatium vinosum* (Grimm *et al.*, 2011). Guanylate cyclase (c-di-GMP gene in *R. julia* and play an important role in sulphide mediated photosynthesis. Based on the study of Grimm *et al.* (2011), in the purple sulfur bacterium *Allochromatium vinosum*, thiosulfate and sulphide oxidation are strictly dependent on the presence of periplasmic SOX proteins encoded by the *SOX B, X, A, K* and *SOX Y, Z* genes. Meanwhile, it is also well documented that other genes encoded in the dissimilatory sulphite reductase (*DSR*) operon, *DSR A,B,E,F,H,C,M,K,L,J,O,P,N,R* and *S*, are essential for the oxidation of sulphur that is stored intracellularly as obligatory intermediates during the oxidation of thiosulfate and sulphide. Until recently, knowledge on the regulation of the SOX and DSR genes were not available, but now the new hypothesis leads to fill the gap. Guanylate cyclase (c-di-GMP) genes are expressed on a high constitutive level in *A. vinosum* in the presence of reduced sulphur compounds (Grimm *et al.*, 2011). However, thiosulfate and sulphide lead to an induction of SOX and DSRgene transcription by the positive regulation of Guanylate cyclase (c-di-GMP) genes. The regulation of *SOX X,A,K* is probably performed by a two-component system consisting of a multi-sensor histidine kinase and di-guanylate cyclase activity which was reported in Chao *et al.* (2013). Protein expression in the translational level of these two genes are controlled by Alvin 2166 homologous to multi-sensor histidine kinases and Alvin 2165 for di-guanylate cyclase activity and here, Alvin 2165 indicate a role of di-guanylate cyclase (di-GMP) as a second messenger for the positive regulation of the *SOX* genes in *A. vinosum*. Homologs of Alvin 2166 and

Alvin 2165 are also present in sulphur oxidizers, namely *Halorhodospira halophila,* a purple sulfur bacterium of the family *Ectothiorhodospiraceae,* and *Magnetococcus* sp. These two gene expressions are directly proportional to the availability of sulphide and thiosulfate in the medium and enhances the uptake and assimilation of thiosulphite and sulphide. This type of mechanism also has been reported in *Shewanella oneidensis* (Chao *et al.,* 2013). Thus it could be proved that *R. julia* is a facultative sulphide oxidizer, and the presence of di-guanylate cyclase (di-GMP) been positively regulated in the assimilation of sulphide for photosynthesis activity. For the above statement we need further studies in future to be confirmed of its action at metabolic level and finally elucidating sulphur oxidizing pathways from present isolate.

Besides gene regulation, another important function of di-guanylate cyclase (di-GMP) gene with regard to the aggregate nature of *R. julia*has been well described in the recent studies of Enomoto *et al.* (2014) and Castro *et al.* (2015). According to them it was a secondary messenger protein having different functions intimately associated with biofilm formation such as polysaccharide biosynthesis for cellular aggregation, production and transport of biofilm matrix components, oxygen-dependent regulation, and motility. In addition,c-di-GMP can also control virulence and morphological development.

Bioremediation has been viewed as the ecologically responsible alternative to environmentally destructive physical remediation (Prakash *et al.,* 2013). Microorganisms carry endogenous genetic, biochemical and physiological properties that make them ideal agents for several environmental conditions in connection with health and sustainability to

aquatic as well as waste water. Here an attempt has been made to develop bioremediation technology for H_2S removal by using MCCB147 from aquatic systems. For in-situ bioremediation, the microorganisms used should be potentially less expensive, faster in action and safer than conventional clean-up methods. For application, (simulated microcosm study) an appropriate protocol using *R.jullia* (MCCB147) as the bioaugmentor is under development.

The main pathways of sulfide oxidation in marine sediments involve complex interactions of chemical reaction and microbial metabolism (Gorgensen *et al.,* 2004). In microbial metabolism, several purple, non-purple and green sulphur bacteria play an important role in sulphur cycle in sediment (Holmer *et al.,* 2001). Meanwhile, dissolved hydrogen sulphide is produced during bacterial dissimilatory sulfate reduction. Since hydrogen sulphide dissolved in the pore waters, it is toxic for many macrofaunal species, the fixation of H_2S through bio oxidation of sulphides is also an important detoxifying mechanism for the macrofauna community. In the absence of oxygen, H_2S can diffuse into underlying sediments or the water column (Holmer *et al.,* 2001), can be chemically or microbially reoxidized to sulfur intermediates (elemental sulphur, thiosulphate, sulphite) or sulphate (Fossing and Jørgensen, 1990; Zhang and Millero, 1993) or react with organic matter (Sinninghe and De Leeuw, 1990). Additionally, bioturbating organisms can transport sulphide from the reduced part of the sediments, where they are subsequently re-oxidized. Thus benthic organisms might have a positive effect on the recycling of sulphur within the sediments by their bioturbating and bio-irrigating activities. Hence the MCCB 147 is considered as benthic organisms which are commonly occurred in illuminated deepest part of anoxic sediment. In the present study, data has been generated on sulphide

removal potency of MCCB147 *R. julia* from sediments by simulated microcosm studies. It was the aim to provide additional information on the influence of water quality parameters and its interaction on sulphide removal by MCCB147 during the experiment.

During primary screening in laboratory condition it could be found out that MCCB147 was a potent isolate for the bioremediation of H_2S.But simulated microcosm study conditions were quite different, there was so many factors interfering the metabolic pathways and that would make a significant difference in the H_2S utilization potency of MCCB147. In the phase 1 of study, (natural condition) sulphide could be reduced second day onwards and in the controls, concentration of sulphide increased dramatically and finally reached in static form. This phenomenon is by way of the activities of the indigenous bacterial flora (sulphate reducing bacteria) present in the control samples. But in the test samples, bioremediation of sulphide happened significantly and on the final day found 100 percentage sulphide removal. Accordingly, the sulphide removal efficiency of MCCB147 by phase two and three could be confirmed.

This work opens up new avenues for bioremediation of hydrogen sulphide in highly black sulphide rich sediment. *R. julia*MCCB147, being a facultative sulphide oxidiser it could be used as bioaugmentor in a variety of aquatic systems. According to De Gussemme *et al.* (2009) sulphide reduction is the dominant mineralization pathway in sediment which leads to microbial sulphide oxidation. His studies on enrichment of Nitrate (NR) - Sulphide Oxidizing Bacteria (SOB) have been found useful as microbial oxidants in a bio augmentation technique for rapid sulphide removal from anaerobic sewage system. In the present study enrichment (addition of

inoculum) at definite intervals in test sample for the rapid removal of sulphide was successful as 100% removal rate at each experimental phases could be observed. Similar observations could be made by Gracia-de Lomans *et al.*(2007), and on enrichment of *Thiomicrocrospia* 94% sulphide removal could be noticed in waste water treatment systems.

In the present investigation, water quality parameters and its interactions with sulphide removal could be investigated to a limited extend. According to Tugtas & Pavlostathis (2007) & Brunet & Garcia (1996) sulphide in waste water system may trigger denitrification (nitrate reduction) where sulphide acts as electron donor. In the instance also nitrate reduction could be experience from the initial to final day indicating that sulphide triggered denitrification.

Oxidation – reduction reactions are very important in fresh as well as in brackish water sediments. The movements of many elements such as carbon, iron, and sulphur involves redox reactions (Ghosh & Dam 2009). In this present study significant improvement (increase) of E*h* was noticed during the experimental period. Hence, increase in E*h* is considered a good indication of bioremediation of sulphide happening in the right way in a system. It could be concluded that presence of sulphide was inversely proportional to E*h* of a system, in such a way that presence of sulphide would lead to E*h* reduction at significant level and it could be remediated by the use of sulphide oxidiser (Lyimo *et al.,* 2005 and Brook *et al.,* 2001).

According to Anwar *et al.* (1998) concentration of S^-, HS^-, and H_2S is highly depended upon the pH of the system. At pH value of 8 and above most of the reduced sulphur exists in solution as HS^- and S^2 ions and the amount of sulphide is so less. At pH below 8, the equilibrium shifts rapidly towards the formation of unionised H_2S and is about 80% complete at pH 7(John 1960). In

the present study, in tests after sulphide oxidation or towards the end of each phases, pH could be at 8 to 8.5 compared to that of the control (pH around 7-7.5), which indicated that complete removal of H_2S happened at the end of each phase due to the activity of the bioaugmentor.

These investigations revealed that water quality and its concentrations did not influence the sulphide removal potency of *R. julia* and found 100% removal at each phases. Another important finding is that natural sunlight could enhance the removal potency of *R. julia* when compared to that in laboratory conditions (artificial light). One of the observation of Pringault *et al* (1998a) was that the higher biomass and pigment production in green sulphur bacteria *Prosthecochloris* could be observed in natural light condition than in artificial light. Thus we conclude that natural light condition favoured higher biomass and pigment production in *R. julia* in such a way that 100% removal potency could be obtained. Overall, the experiment clearly demonstrated that MCCB147 was a potent organism for the sulphide removal in a saline aquatic systems.

Figures

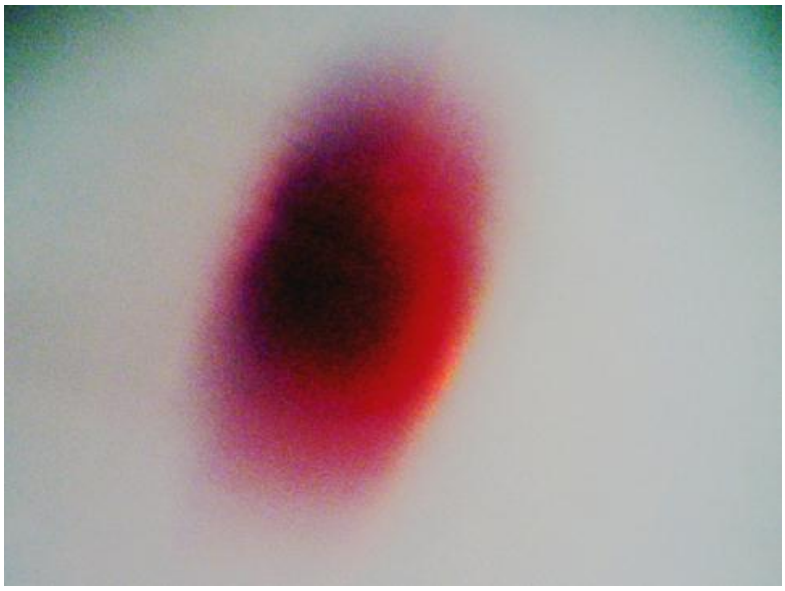


Fig.2.1. Purple colonies developed on semi solid agar medium visualized under microscope

Fig.2.2. Colonies of purple sulphur bacteria were developed in soft agar medium at the highest dilution

Fig.2.3. Growth of photosynthetic bacteria MCCB147 in modified Pfennig's medium

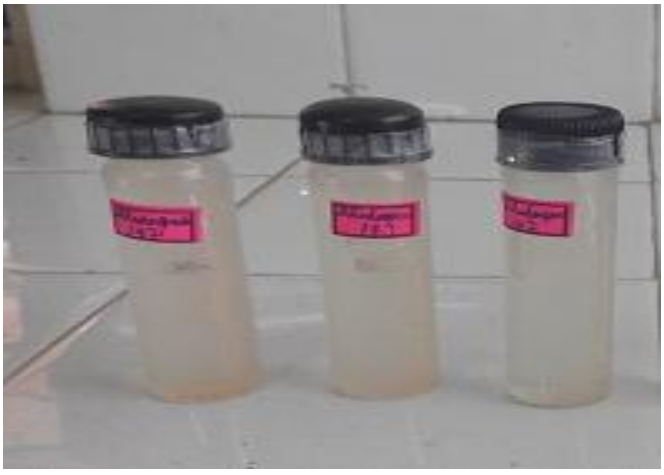


Fig.2.4. Growth of photosynthetic bacteria MCCB147 in original Pfennig's medium

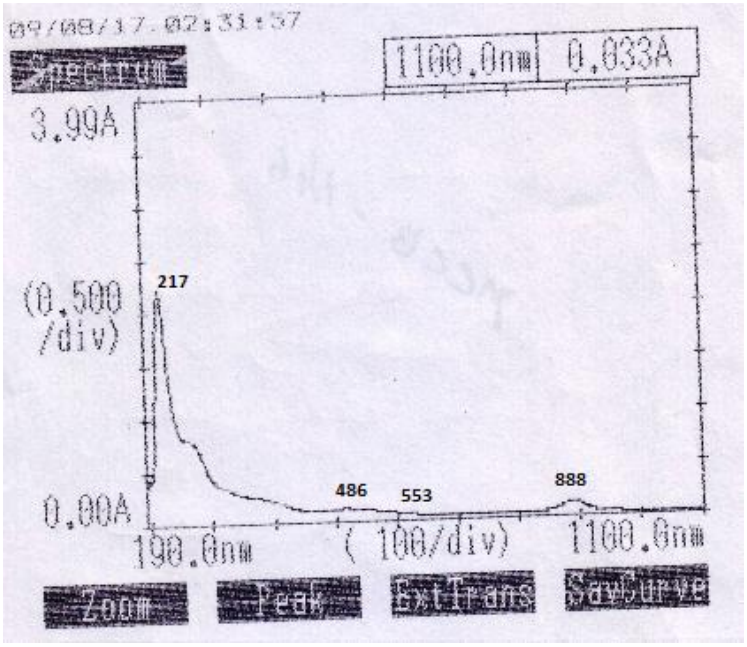


Fig.2.5 Characteristic peak obtained by sucrose mediated absorption maxima measurement of MCCB147

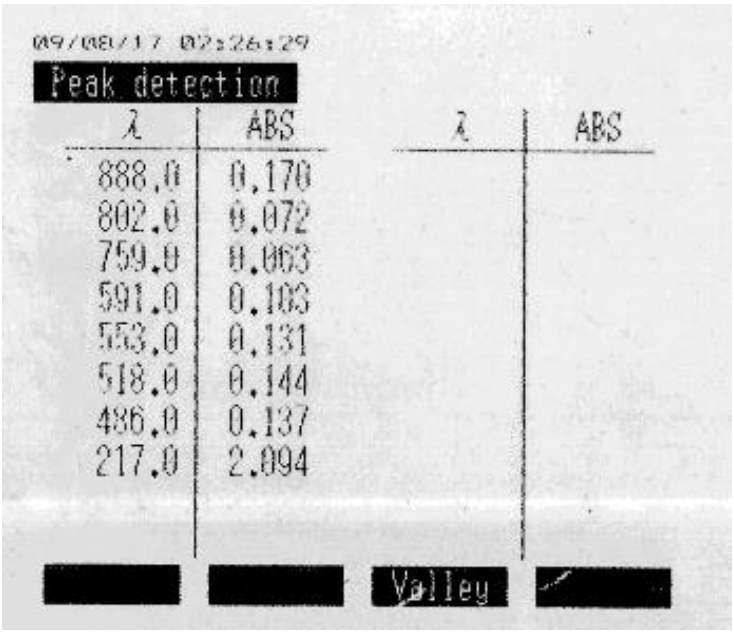


Fig.2.5.1 Characteristic peak detected in the spectrum

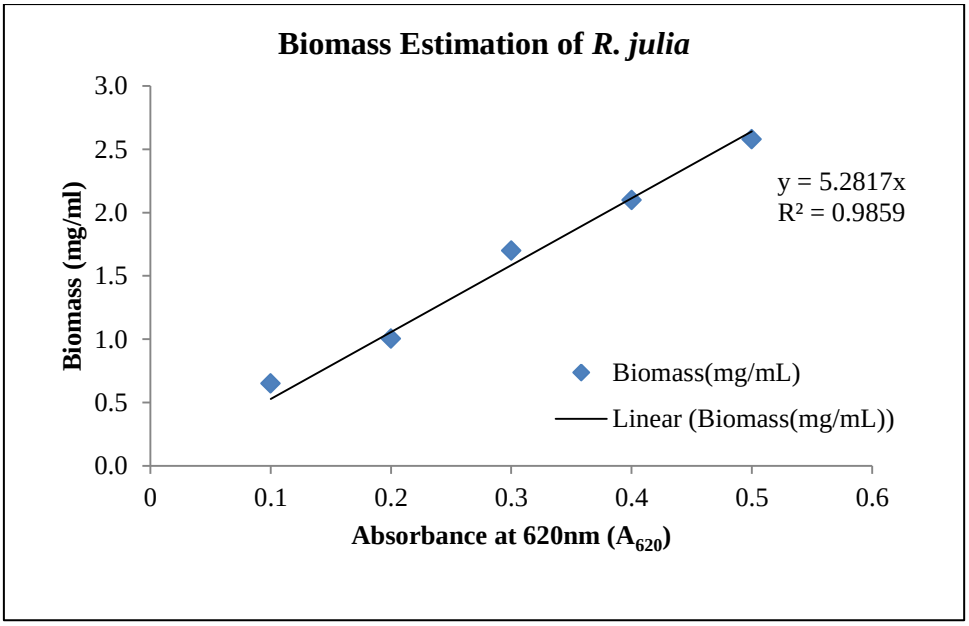


Fig.2.6 Standards graph for biomass estimation of *R.juia*

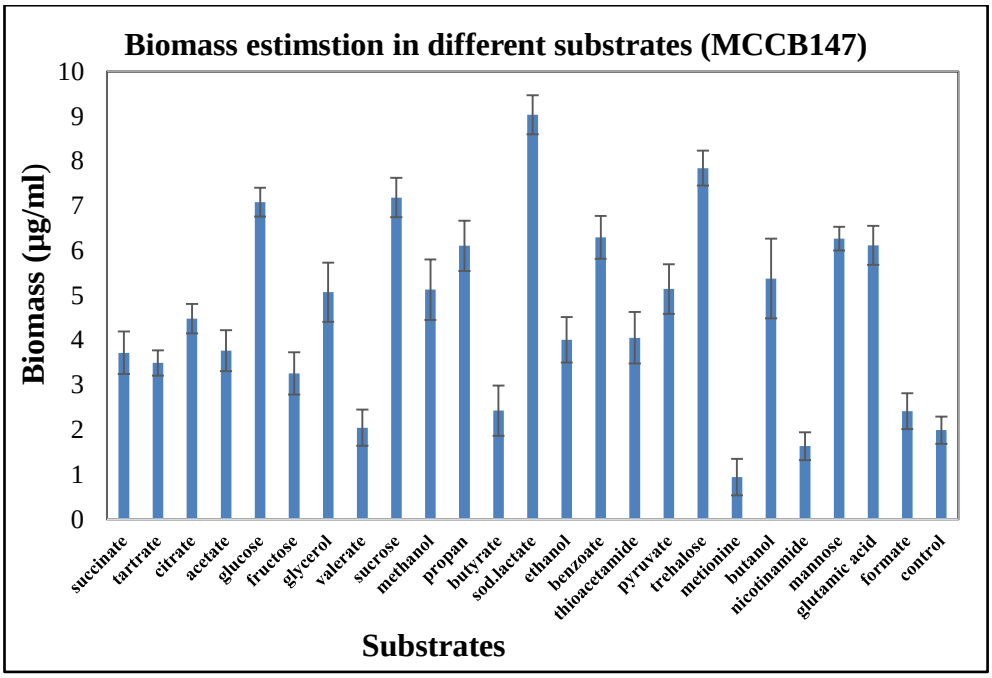


Fig.2.7. Biomass production of MCCB147 with respective substrates

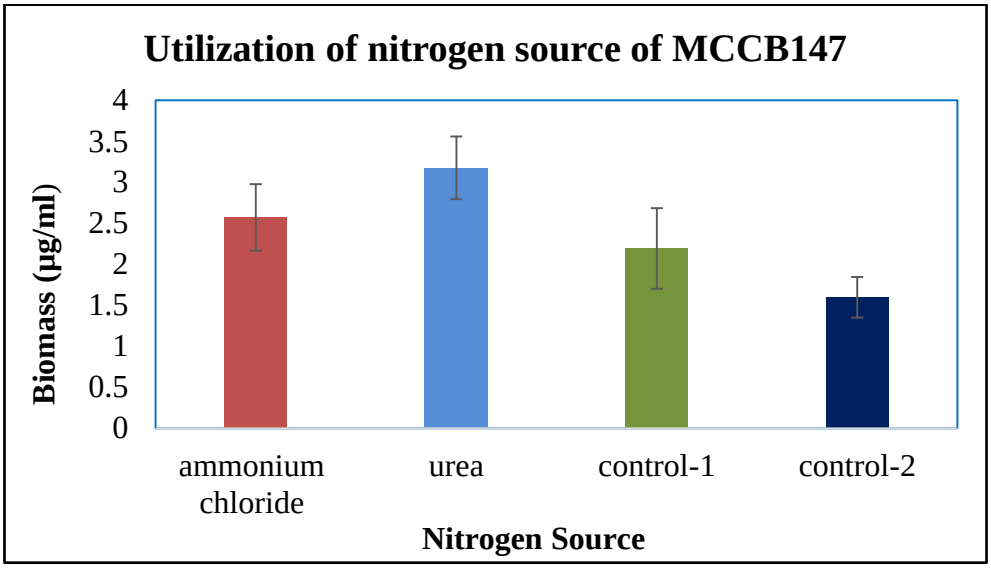


Fig.2.8. Biomass production of MCCB147 in the presence of nitrogen sources

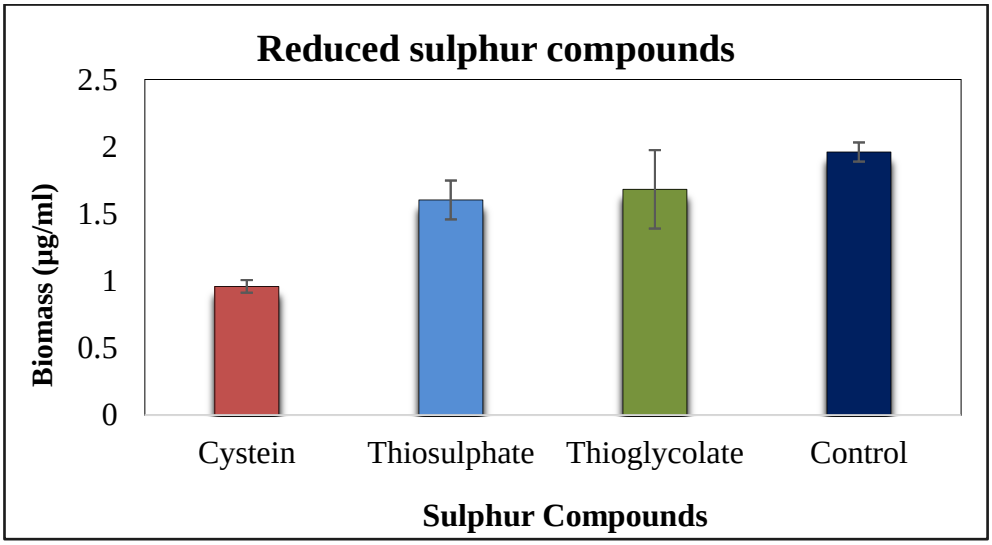


Fig.2.9 Biomass production of MCCB147 in the presence of reduced Sulphur compounds

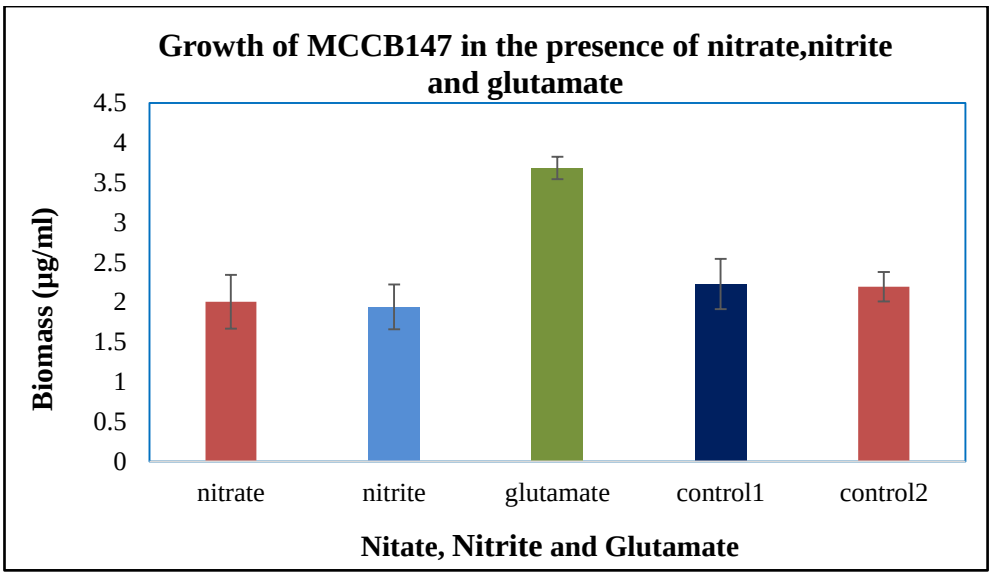


Fig.2.10 Biomass production of MCCB147 in the presence of Nitrate, Nitrite and Glutamate

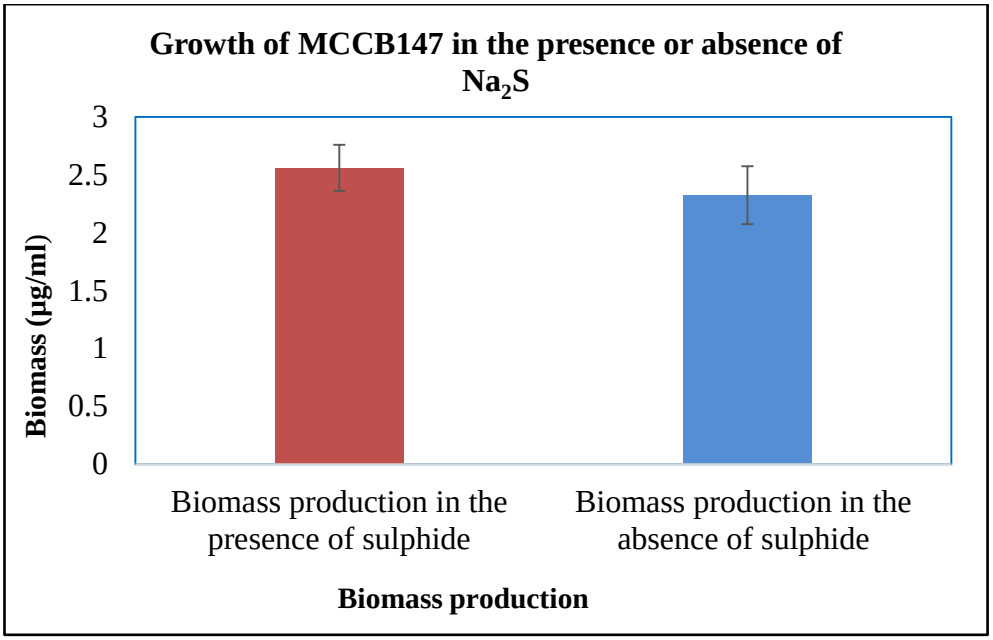


Fig.2.11 Biomass production of MCCB147 in the presence and absence of sodium sulphide

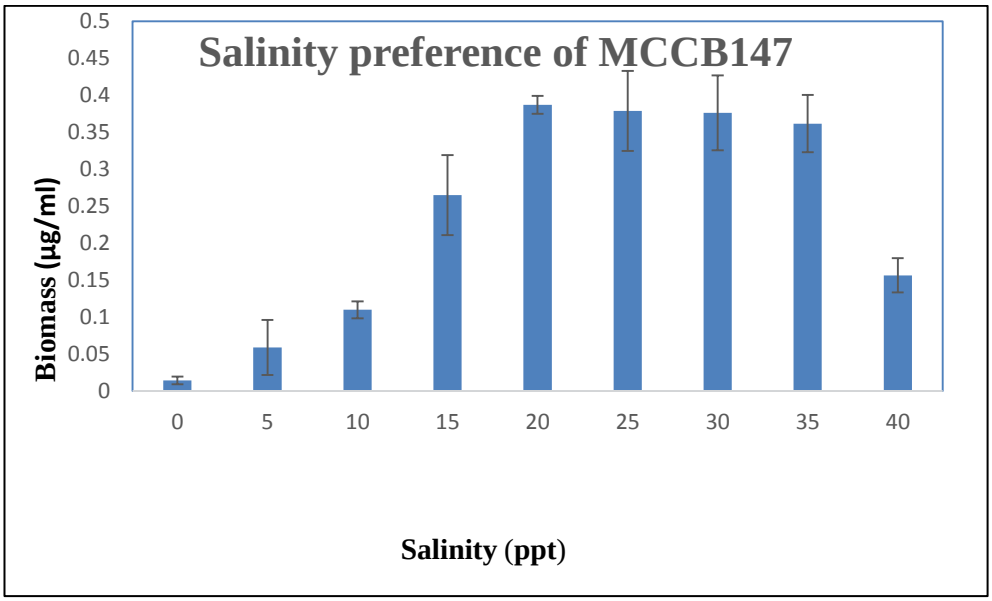


Fig.2.12 Salinity preference of MCCB147

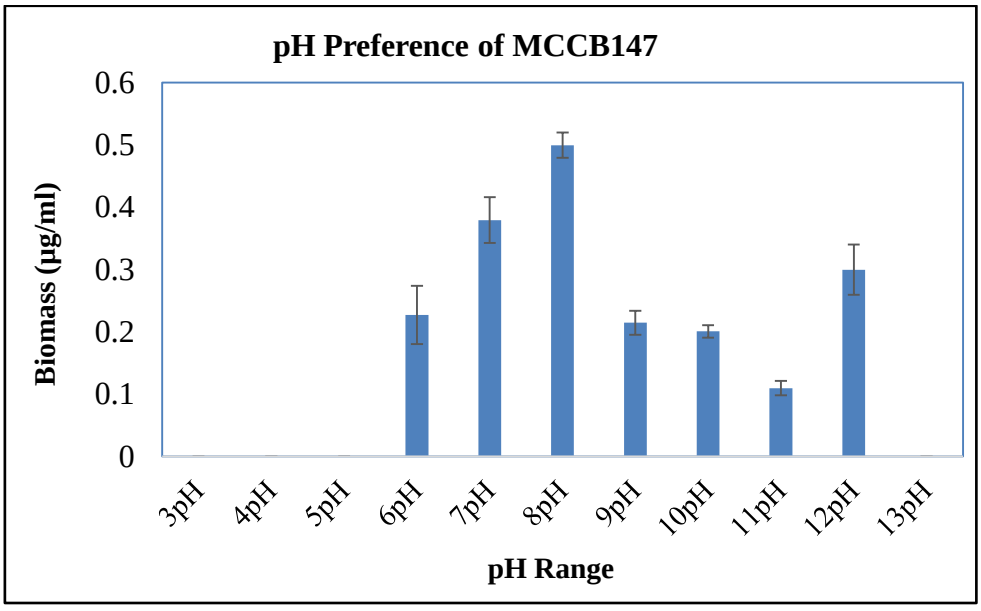


Fig.2.13 pH preference of MCCB147

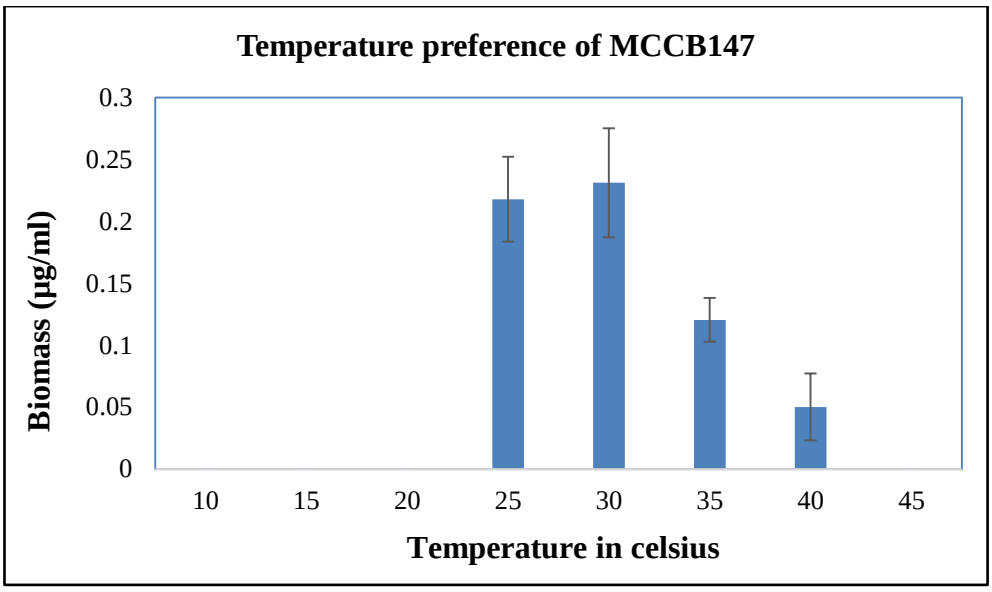


Fig 2.14 Temperature preference of MCCB147

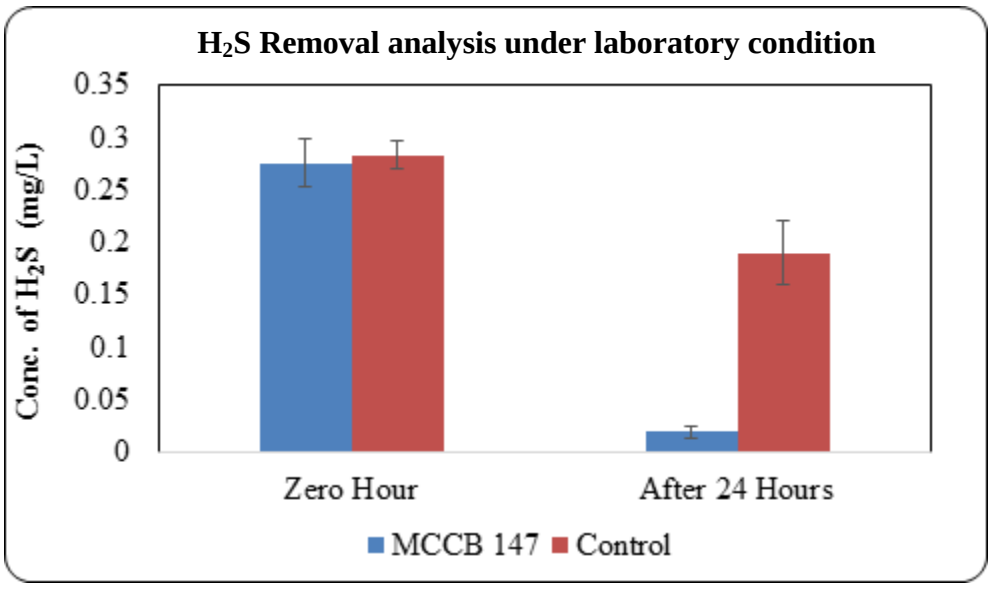


Fig.2.15 Percentage sodium sulphide removal over a period of 24 hrs incubation with MCCB147

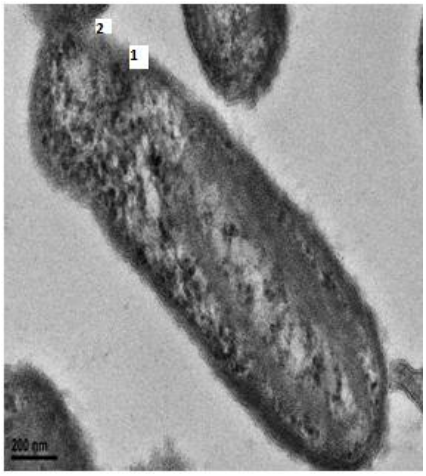


Fig.2.16 Transmission electron microscopic image of MCCB147 showing budding reproduction (1.Budding reproduction, 2.Cellular aggregation)

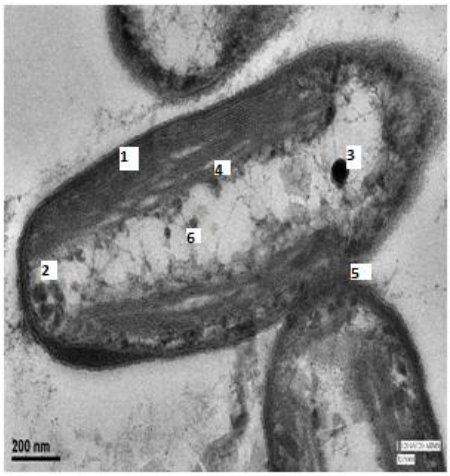


Fig.2.17 Transmission electron microscopic image of MCCB147. (1.Intracytoplasmic membrane, 2.Nuclear material, 3.Inclusion bodies 4.Tubular cytoplasmic membrane, 5.Cellular aggregation, 6.PHB like stucture

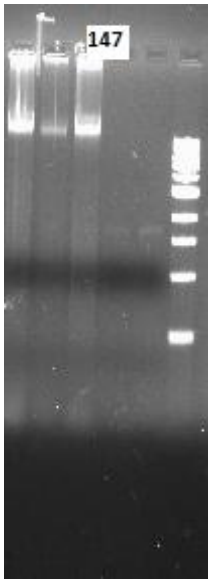


Fig.2.18 Isolated DNA of MCCB147

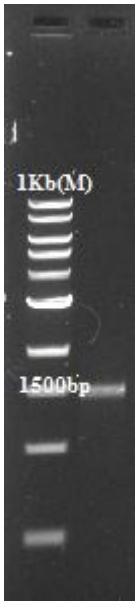


Fig.2.19 Amplification of 16S rRNA gene of MCCB147

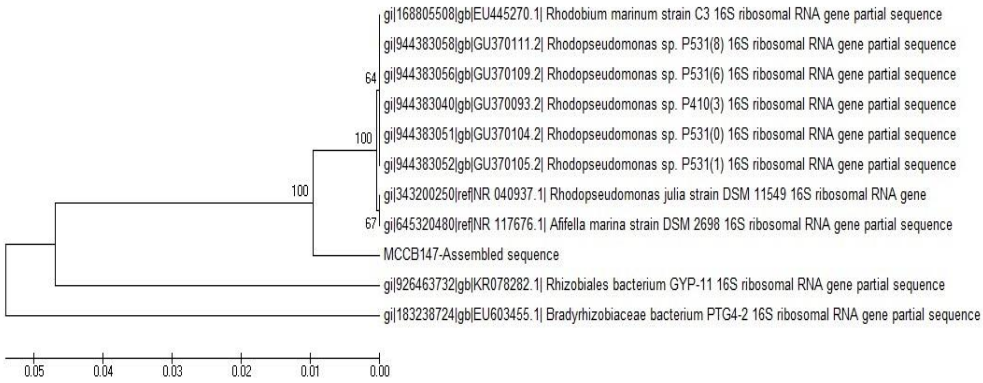


Fig.2.20 Phylogenetic tree of MCCB147

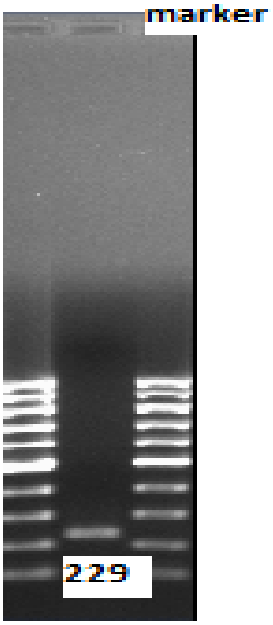


Fig. 2.21 Amplification of PUFM gene of MCCB147

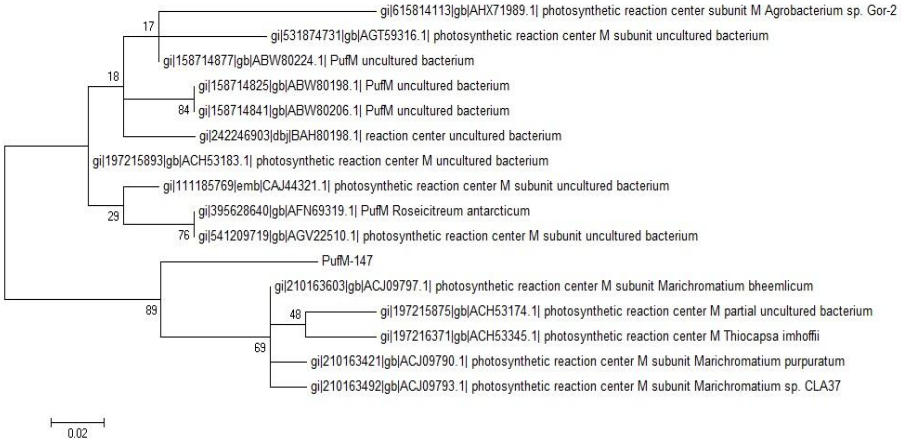


Fig.2.22 Phylogenetic similarity of PUFM gene of *Rhodospeudomonas julia* MCCB147

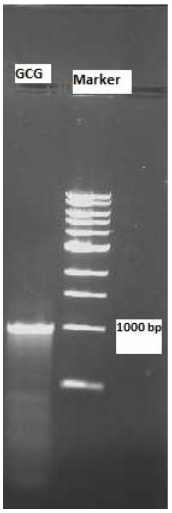


Fig.2.23 Amplification of Diguanylate cyclase gene of *Rhodospeudomonas julia*

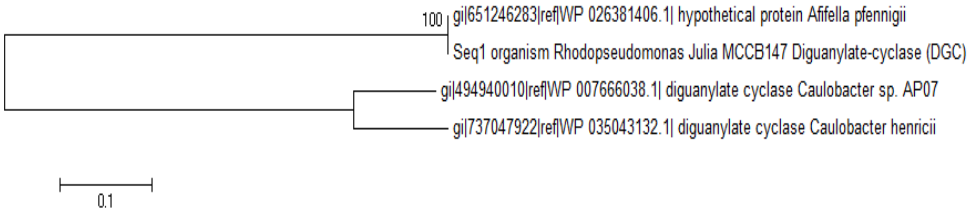
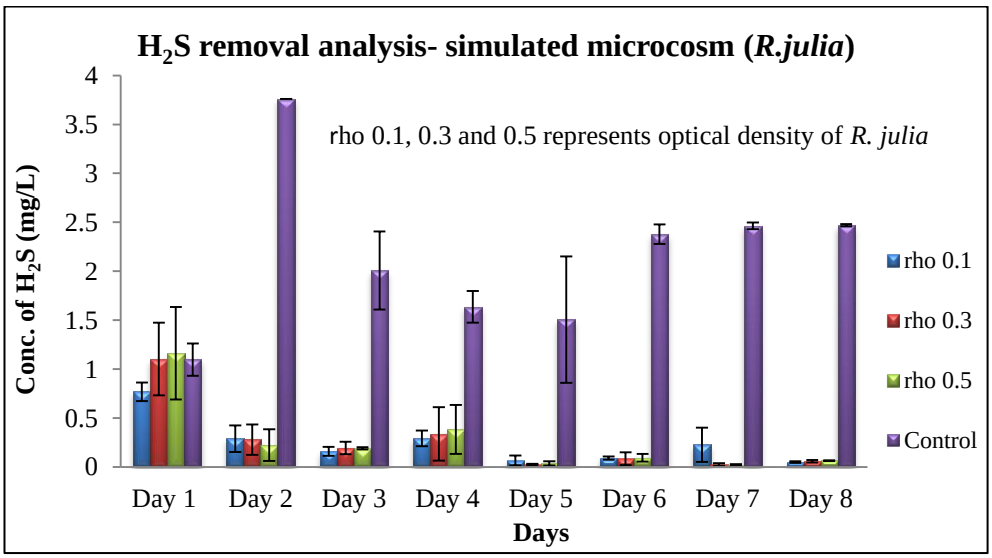


Fig.2.24 Phylogenetic tree of Diguanylate cyclase gene of *Rhodospeudomonas julia* compared with the sequence available in GenBank



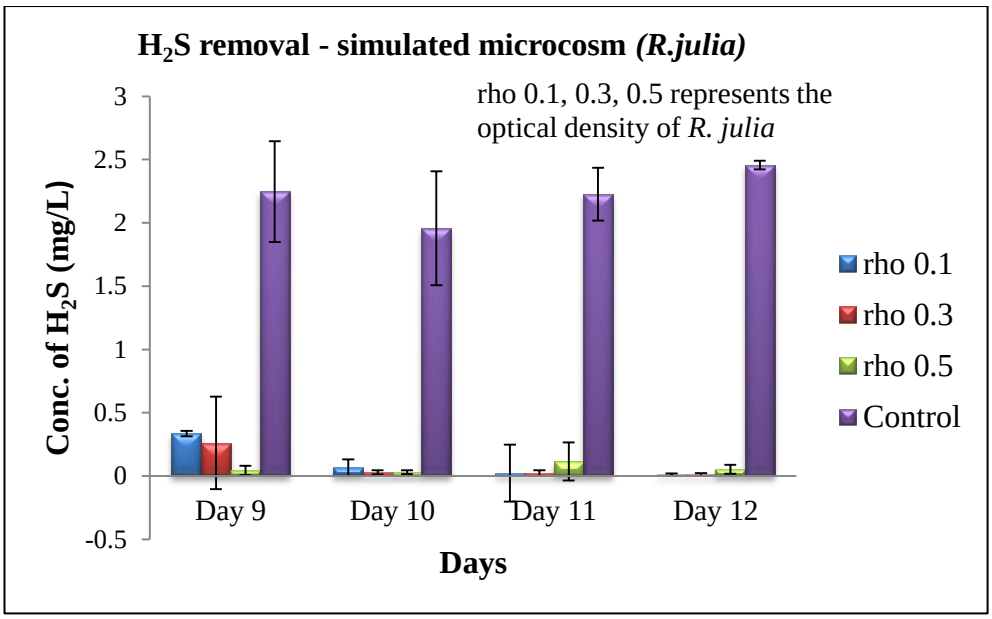


Fig.2.25 & Fig.2.26 Sulphide removal rate by *R.julia* from first phase of the experiment.

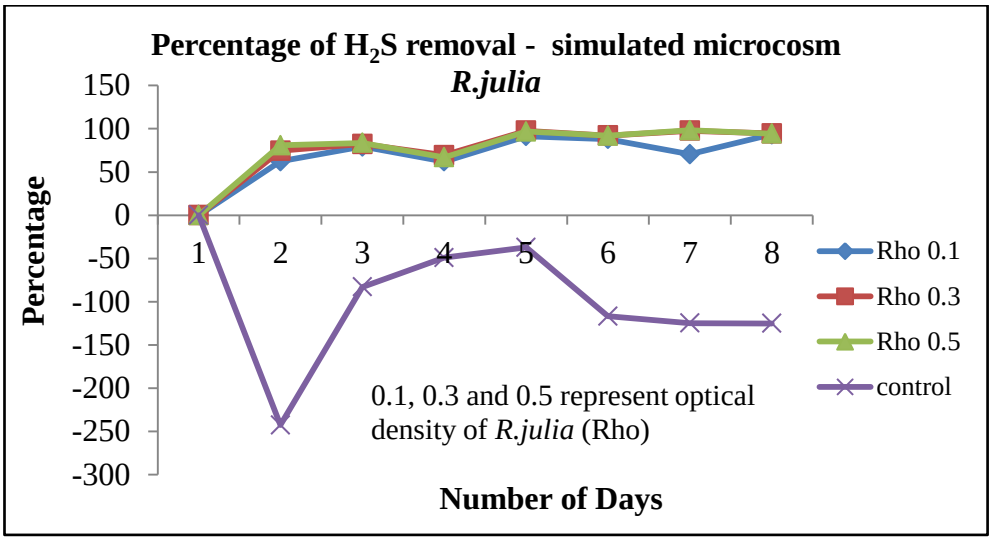


Fig.2.27 Persentage of sulphide removal rate by *R.julia* from 1st to 8th day of phase one experiment

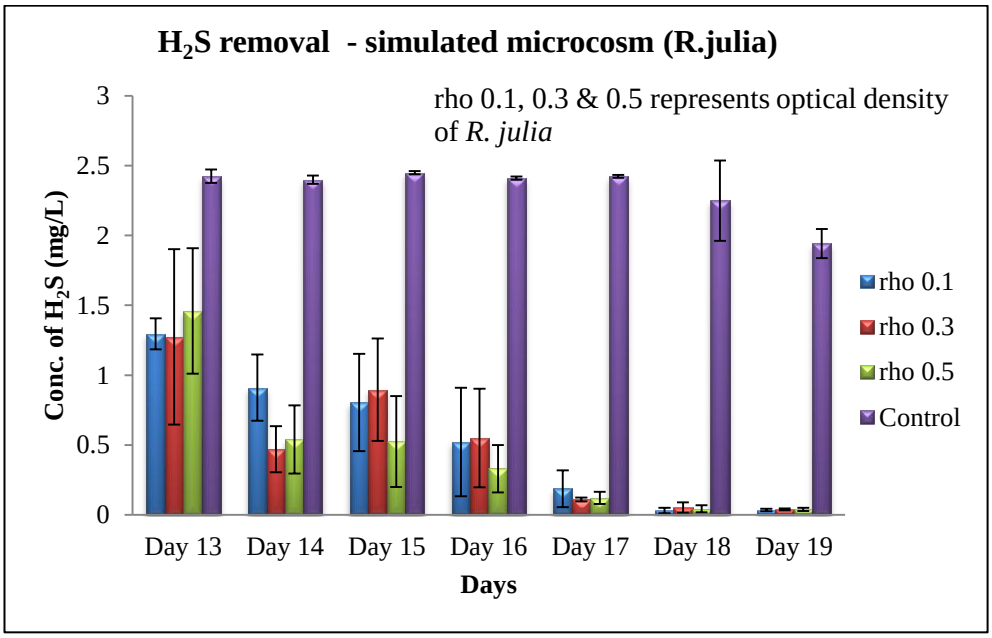


Fig.2.28 Sulphide removal by *R.julia* during the 2nd phase

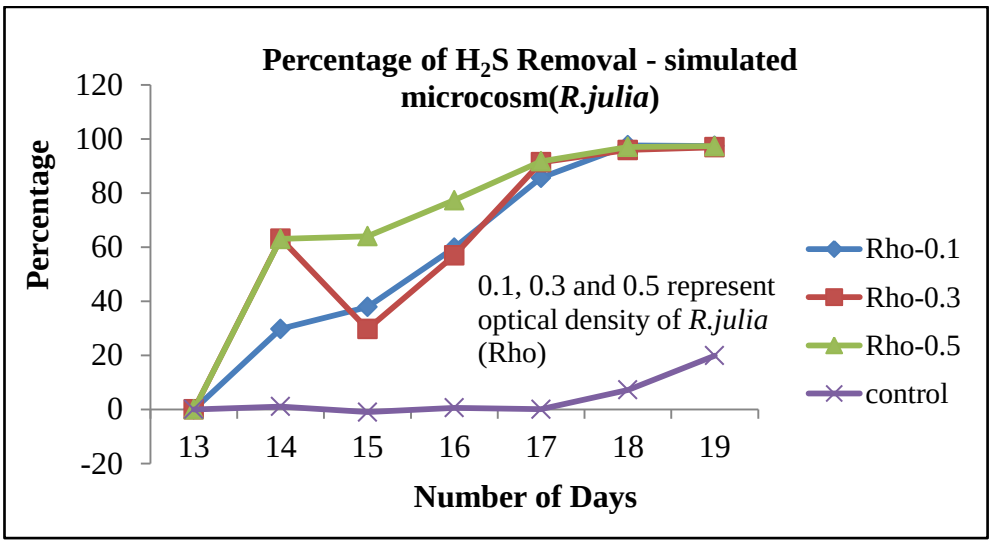


Fig.2.29 Percentage of sulphide removal by *R.julia* during the second phase of experiment

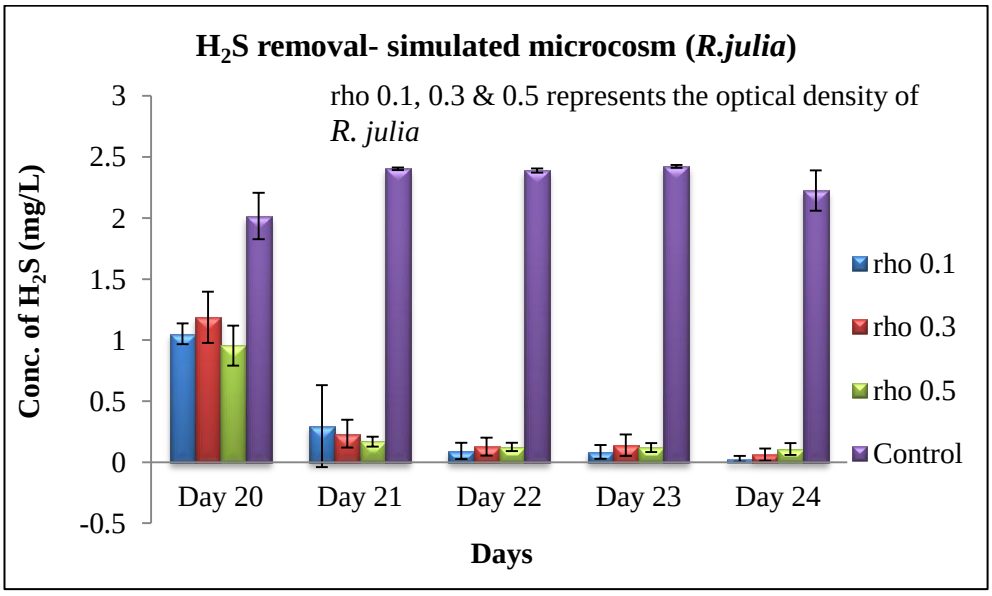


Fig.2.30 Sulphide removal by *R.julia* during the 3nd phase

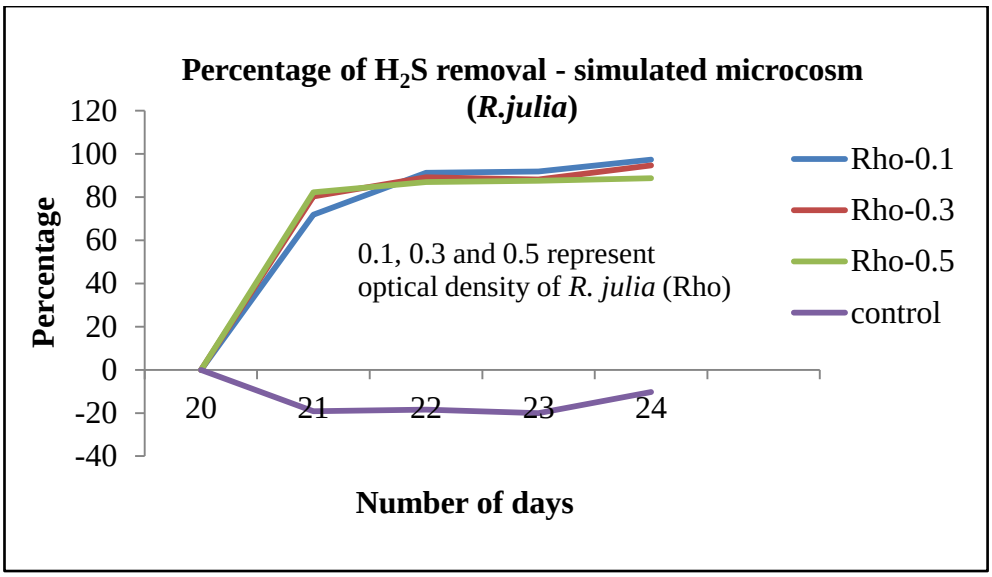


Fig.2.31 Percentage of sulphide removal by *R.julia* from 20th to 24th day of phase 3 experiment

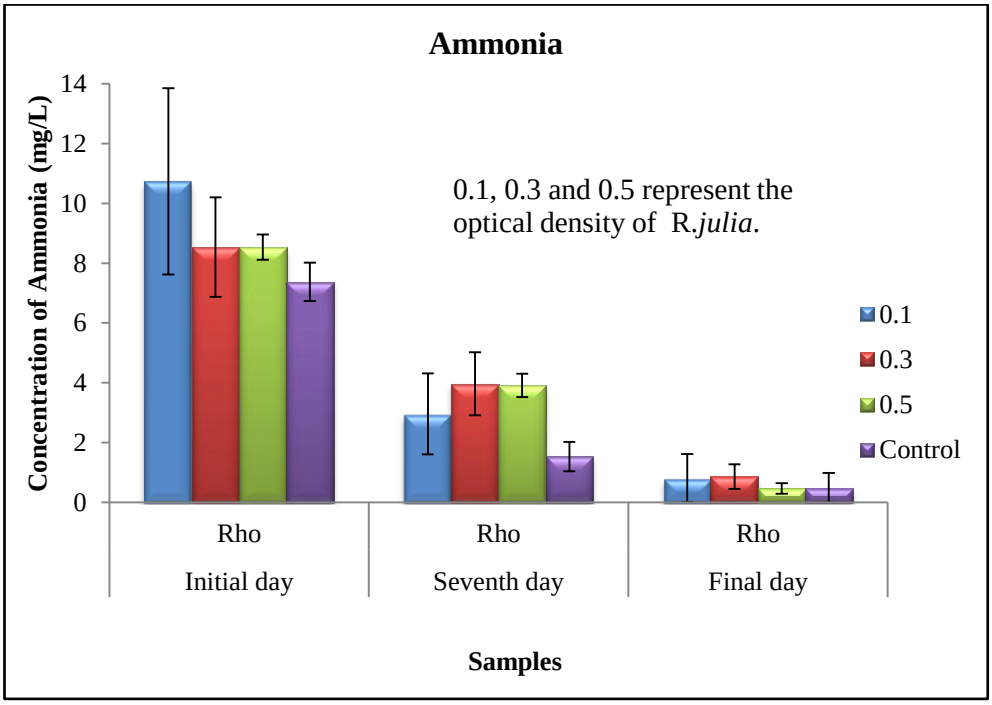


Fig.2.32 Removal of ammonia - nitrogen from the experimental system

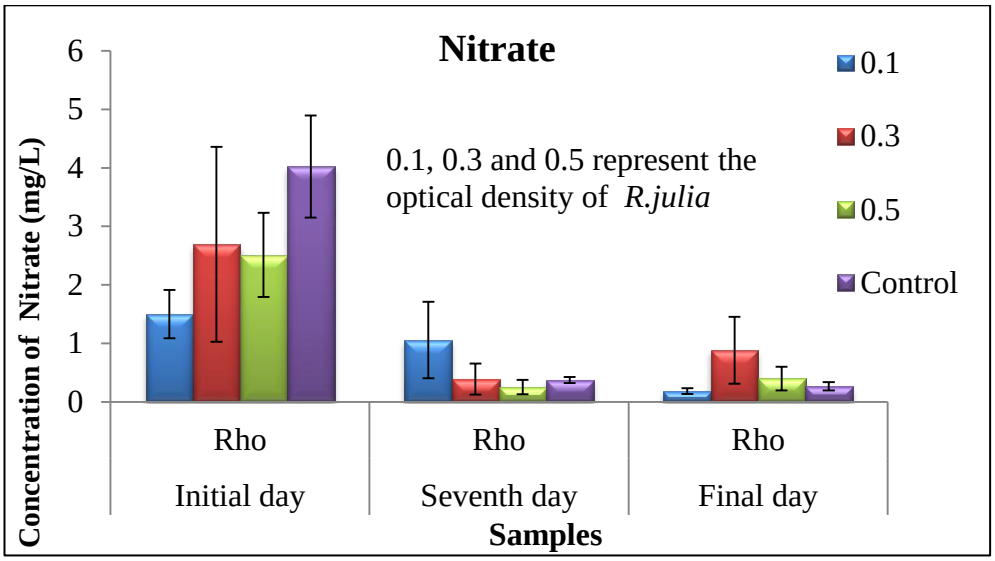


Fig.2.33. Removal of Nitrate – nitrogen from the experimental system

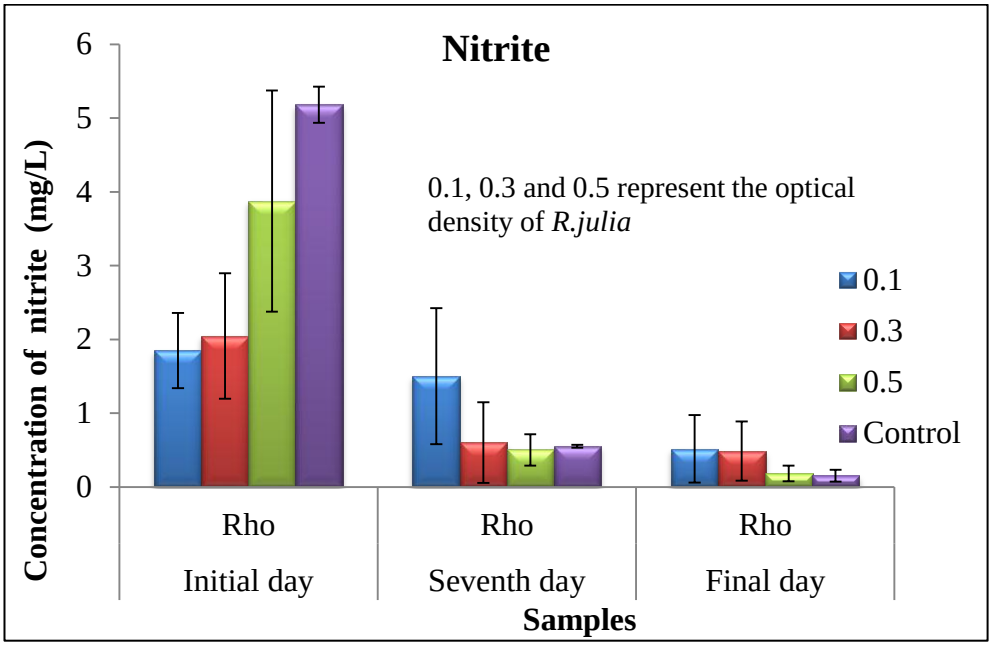


Fig.2.34 Removal of Nitrite – Nitrogen from the experimental system

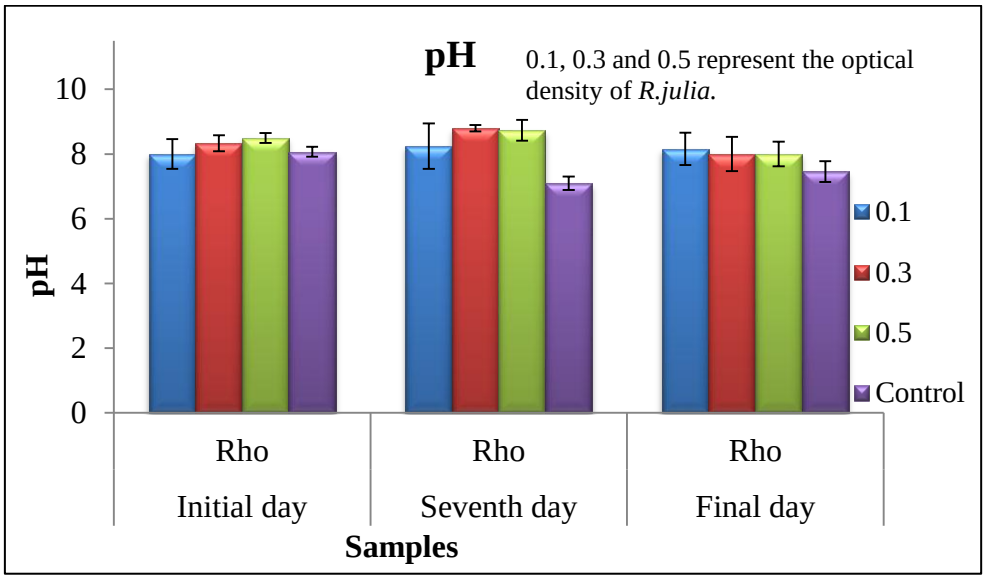


Fig.2.35 pH variation during the experiment

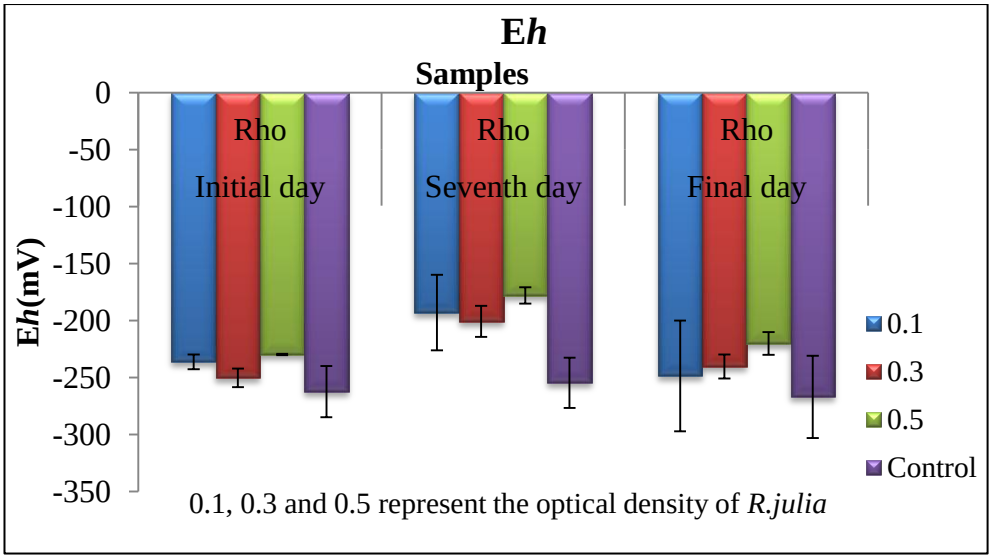


Fig.2.36 E*h* variation during the experiment

Fig.2.37 Experimental set up

Chapter 3

ISOLATION, PURIFICATION AND CHARACTERIZATION OF PHOTOSYNTHETIC BACTERIUM MCCB146 AS BIOAUGMENTOR FOR REMOVAL OF HYDROGEN SULPHIDE IN AQUATIC SYSTEM

Contents

3.1 Introduction

Anoxygenic phototrophic bacterial photosynthesis is the most important biological process on earth, which converts light energy to chemical energy Oxygenic photosynthesis is the principal metabolic process of cyanobacteria and green plants which contain chlorophylls as pigments and use water as electron donor liberating oxygen(Brayant & Frigaard 2006). Anoxygenic photosynthesis is mainly carried out by diverse purple and green bacteria which contain bacteriochlorophyll pigments and do not produce oxygen. The study of anoxygenic photosynthesis began at the end of 19th century (Gorlenko, 2004) and gained attention in microbiology in the late 1960s. Anoxygenic phototrophic microorganisms are classified into *Haloarchaea*, Filamentous green bacteria, Green sulphur bacteria, Purple sulphur bacteria, Purple non-Sulphur bacteria and *Heliobacteria*.

Generally, photoheterotrophs are not able to utilize carbon dioxide directly as the carbon source but it can utilize organic compounds for its growth These organic compounds include yeast extract, succinate, malate, alcohols, glycerol, sodium lactate, fumerate, glucose, fructose, and glutamate etc. (Wertlieb & Vishniac1967). In the selected organism, *Afiffella pfennigii* (MCCB146) is photoheterotrophic as well as photoautotrophic and it could never be grown as chemoheterotroph since light is an essential factor for the growth of MCCB146.

The present study deals with the purification and characterization of a phototrophic bacterium MCCB146 enriched from aquatic sediments. The oxic–anoxic transition zone (OATZ) in sediment and water column is characterized by opposing gradients of oxygen, reduced iron and sulphur compounds. Microorganisms that are capable of lithoautotrophic growth utilizing the energy stored in these chemical gradients by the oxidation of reduced sulphur and/or iron. These gradient microcosms provide a combination of microaerobic conditions and a low iron or sulphide concentration or anoxygenic conditions in which chemical oxidation of iron (II) and sulphide by oxygen is limited due to their low concentrations (Emerson & Moyer, 1997).

In application point of view, biological methods, using microbial activity for H_2S removal (Kim *et al.*, 1997), have drawn attention since they are more efficient and more economical than physicochemical methods if proper operational conditions are maintained. Biological oxidation is considered as an alternative to the physicochemical methods for H_2S removal. The benefits of biological methods are non-usage of catalyst and oxidants, least expenditure involved, lack of chemical sludge formation,

low energy consumption, possible reuse of the byproduct sulphate, low sulphate and thiosulphate discharge and rapidity of the process and higher removal efficiency. A number of microbial processes for H_2S removal have been proposed based on oxidation by *Thiobacillus* and other Sulphur microorganisms. *Thiobacillus*spp. has been used to oxidize H_2S to sulphate in liquid and in packed tower systems such as peat biofilters (Suk cho *et al.,* 1992). Mixed cultures of bacteria from the *Beggiatoa* and *Thiothrix* genera and the photosynthetic bacterium *Chlorobium thiosulfatophilum* have been proposed for use in the oxidation of H_2S to elemental Sulphur (Williams& Uns 1985; Cork *et al.,* 1985). Most of these are autotrophic bacteria which are often difficult to handle because of their low growth rates when compared to those of heterotrophic bacteria.

On applying phototrophic microorganisms for bioremediation of H_2S in aquatic systems, the supply of solar energy is a limiting factor for their growth (Suk cho *et al.,* 1992). The isolate MCCB146, being a facultative organism, can grow both photoautotrophically, based on H_2S mediated photosynthesis and photoheterotrophically when H_2S is absent. Thus, in the application point of view, it can be considered a potent organism for bioremediation of H_2S in aquatic system. Hence this chapter deals with the bioremediation of H_2S in aquatic system to improve water quality and health of aquatic organisms by using photosynthetic non sulphur bacteria *A. pfennigii.*

3.2 Materials and methods

3.2.1 Sampling

Samples such as pond water and sediments were collected from three different localities: Ponneri in Tamil Nadu, Kodungallur and Maradu in

Kochi. Salinity in the sampling stations varied from10-40ppt. All the samples were enriched in Pfennig's medium 1 (PF2) in a previous study by Manju, (2007).Water samples from Maradu, kochi had been taken for the present study.

3.2.2 Purification of photosynthetic sulphur bacteria by deep agar dilution method. (Pfennig *et al.*, 1981)

The procedure is same as mentioned in chapter 2, section 2.2.2

3.2.3 Absorption spectra measurement.

The procedure is same as mentioned in chapter 2, section 2.2.4

3.2.4 Disaggregation of cellular aggregate by chemical method.

Aggregation is the characteristic nature of phototrophic bacteria (Seitz *et al.*, 2006) hindering their enumeration. Therefore, disaggregation of MCCB146 was an inevitable step to carry out its biochemical characterization and enumeration. Disaggregation of cells was materialized by the addition of various chemicals as follows:

An aliquot of 1ml culture of MCCB 146 (0.1 Abs_{620}) was mixed with different volumes (100, 200, 300, 400 and 500µl) of 1M and 2M urea, 100mM EDTA, 1% v/v Tween 80, 1% w/v SDS and 1% v/v Triton X-100. These chemicals acted as detergent, disrupting the hydrophobicity between the cells (Seitz *et al*., 1993). The cells were enumerated using the haemocytometer after overnight incubation.

3.2.5 Cell Hydrophobicity Assay for calculating the percentage of Cellular Aggregation of MCCB146.

The percentage of aggregation of MCCB146 was determined by calculating the hydrophobicity between MCCB146 cells (Seitz *et al.,* 1993). An aliquot of 3 ml cell suspension was mixed with 0.5 ml of hexadecane and the gas phase was flushed with nitrogen for 1 min and the tubes were sealed with butyl-rubber stopper. After 10 minutes of pre incubation, the tubes were vortexed vigorously for 2 min, followed by phase separation. After 15 min of incubation, the absorbance (Abs_{650}) of the lower phase was recorded using spectrophotometer. Absorbance of untreated cells was maintained as control.

Calculating hydrophobicity index (H)

$$\mathbf{H= 100\ (OD_{650} - OD'_{650}) \div (OD_{650})}$$

Where

OD_{650}-optical densities of untreated cells

OD'_{650} - optical densities of lower phase

3.2.6 Optical Density Vs Biomass by packed cell volume method

The procedure is same as mentioned in chapter 2, section 2.2.7

3.2.7 Biochemical characterization - Substrate Utilization Study of MCCB146

Biochemical characterization of photosynthetic non sulphur bacteria, MCCB146 was performed by using different carbon sources (Shabeb *et al.,* 2008). The substrate utilization capability of MCCB146 was tested in

modified pfennig's medium. The following substrates were used for the present study.

succinate (5mM), tartrate (5mM), citrate (5mM), acetate (5mM), glucose (2mM), fructose (5mM), glycerol (5mM), valerate (5mM), sucrose (5mM), methanol (5mM), propane (5mM), butyrate (5mM), sodium lactate (5mM), ethanol (5mM), benzoate (0.5mM), thioacetamide (2mM), pyruvate (5mM), trehalose (5mM), methionine (5mM), butanol (5mM), nicotinamide (2mM), mannose (5mM), glutamic acid (5mM), formate (5mM).

An aliquot of 1 ml MCCB146 (0.1 OD absorbance) was inoculated into modified Pfennig's medium with respective carbon source mentioned above. Tubes without substrates were maintained as control and triplicates were kept for all the tubes. After 15 days of incubation under light and dark condition, optical density was measured at 620 nm using spectrophotometer. (Shimadzu UV-1601, Shimadzu Corporation, Tokyo Japan). The obtained data was statistically analysed by ANOVA.

3.2.8 Utilization of Nitrogen Source

Ammonium chloride (5mM) and Urea (5mM)

Inoculum of MCCB146 (1ml) having absorbance of 0.1 OD was added to 30 ml screw capped tube containing modified Pfennig's medium with 5mM concentration of ammonium chloride and urea separately. Pfennig's medium without nitrogen source (ammonium chloride) kept as control-1 and modified pfennig's medium as such considered as control-2. All the tubes were incubated under light and dark condition. After 15 days of incubation, the growth were measured using spectrophotometer at 620

nm (Shimadzu UV-1601, Shimadzu Corporation, Tokyo Japan). The obtained data was statistically analysed by ANOVA.

3.2.9 Utilization of other reduced sulphur compounds

The utilization capability of MCCB 146 was tested with reduced sulphur compounds such as cysteine (5mM), thiosulphate (5mM) and thioglycolates (5mM). The 1 ml culture with 0.1 OD absorbance was inoculated into 30 ml screw capped tube containing modified Pfennig's medium, incorporated with 5mM concentration of cysteine, thiosulphate and thioglycolates independently. Modified pfennig's medium alone was kept as control. All the tubes were incubated under light and dark condition. After 15 days of incubation, growth was measured using spectrophotometer at 620 nm (Shimadzu UV-1601, Shimadzu Corporation,Tokyo Japan). The data were statistically analysed by using one way ANOVA.

3.2.10 Growth of photosynthetic purple sulphur bacteria in the presence of nitrate, nitrite and glutamate

The isolate, 1 ml MCCB 146 having 0.1 OD absorbance was inoculated into 30 ml screw capped tubes containing modified pfennig's medium separately provided with 5mM concentration of nitrate, nitrite, and glutamate. Modified Pfennig's medium was used as control-1 and the pfennig medium without ammonium chloride as control-2. The experiment was conducted as described previously. The data were statistically analysed by using one way ANOVA.

3.2.11 Growth of MCCB146 in the absence of hydrogen sulphide.

An aliquot of 1 ml culture of MCCB146 having absorbance 0.1 was inoculated into 30 ml screw capped tube containing the basal medium

without sodium sulphide. Pfennig's medium incorporated with 11 µg/ml sodium sulphide was maintained as the control. Then the experiment was performed as mentioned previously. The data were statistically analysed by using one way ANOVA.

3.2.12 Growth of MCCB146 under different mode of nutrition

The procedure is same as mentioned in chapter 2, section 2.2.13

3.2.13 Optimization of physical conditions of growth for MCCB146

Optimization of growth condition of MCCB146 was carried out in 30 ml screw capped tubes with modified Pfennig's medium.

3.2.13.1 Optimization of Sodium Chloride

Pfennig's medium (30 ml) was prepared at different salinities such as 0, 5, 10, 15, 20, 25, 30 and 35ppt by providing adequate NaCl (gL^{-1}). The pH was adjusted to 7.2. The screw capped tubes were inoculated with 1ml MCCB146 cell suspension having absorbance of 0.1 and incubated at room temperature for 15 days in a photo bioreactor having 1000 lux unit. After incubation, growth was measured at 620 nm using UV-Vis spectrophotometer (Shimadzu UV-1601, Shimadzu Corporation, and Tokyo Japan) and calculated the biomass. The obtained data were statistically analysed by way one way ANOVA.

3.2.13.2 Optimization of pH

Modified Pfennig's medium, as mentioned earlier, was prepared at 25ppt salinity having different pH, ranging from 3- 13, using either 1N HCL and 1N NaOH in 30 ml screw capped tubes and inoculated with 1 ml cell

suspension of MCCB 146 having absorbance 0.1.The data were statistically analysed by one way ANOVA.

3.2.13.3 Optimization of temperature

Pfennig's medium was prepared in saline water (25ppt) having pH adjusted to 7.2 and inoculated with an aliquot 1 ml MCCB 146 having absorbance of 0.1. All tubes were incubated in a temperature controlled illuminated light shaker incubated (Scigenics Biotech orbital shaker, LETTD-1L) at different temperatures such as 5, 15, 20, 25, 30, 35, 40 and 45 $^{\circ}$C for 15 days. After incubation, growth was measured using spectrophotometer at (Abs) 620nm. The data were analysed statistically by way of one way ANOVA.

3.2.14 Determination of the ability of MCCB 146 to utilize Hydrogen Sulphide by way of Methylene blue spectrophotometric method.

The procedure is same as mentioned in chapter 2, section 2.2.15

3.2.15 Transmission Electron Microscopy of Photosynthetic Bacteria MCCB146

The procedure is same as mentioned in chapter 2, section 2.2.16

3.2.16 Molecular characterization of MCCB146

3.2.16.1 DNA extraction

All chemicals and reagents used for this study, unless specifically stated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

To extract DNA, a sample of 2 ml of bacterial cell suspension (20 days old bacterial cell suspension grown in Pfennig's medium) was

centrifuged at 15,000*g* for 10 min at 4 ^{0}C. (Eppendorf, India, PVT Limited), The pellet was collected and re-suspended in 500µl of TNE buffer (10mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.15 mM NaCl) and centrifuged again at 15,000*g* for 10 min at 4 ^{0}C. Subsequently, the pellets were re-suspended in 500 µl lysis buffer (0.05 mM Tris-HCl, pH 8.0, 0.05 mM EDTA, 0.1 mM NaCl, 2%, SDS, 0.2 % PVP and 0.1% mercaptoethanol) (Lee *et al*., 2003) and 10µl proteinase K (20mg/mL) added and incubated initially for 1 hr at 37 ^{0}C and 2 hrs at 55 ^{0}C. Further extraction was carried out by following phenol-chloroform method (Sambrook and Russell, 2001). The sample was deproteinated by adding equal volume of phenol (Tris- equilibrated, pH 8.0), chloroform and isoamyl alcohol mixture (25:24:1). The phenol and the aqueous layers were separated by centrifugation at 15,000*g* for 15 min at 4 ^{0}C. The aqueous phase was carefully pipetted out into a fresh tube and the process was repeated once more. Subsequently, an equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, mixed by gentle inversion and centrifuged at 15,000*g* for 15 min at 4 ^{0}C to separate the aqueous phase, which was then transferred to fresh tube. The DNA was precipitated by incubation at -20 ^{0}C overnight after adding equal volume of chilled absolute ethanol.

The precipitated DNA was collected by centrifugation at 15,000*g* for 15 min at 4 ^{0}C and the pellet washed with 70% ice cold ethanol. Centrifugation was repeated once more and the supernatant decanted and the tubes left open until the pellet got dried. The DNA pellet was dissolved in 100µl MilliQ (Millipore) grade water. The isolated DNA was quantified spectrophotometrically (Abs_{260}) and the purity of DNA assessed by

calculating the ratio of absorbance at 260 nm and 280 nm (Abs_{260}/Abs_{280}). Electrophoresis was done using 1% agarose gel.

Concentration of DNA (µg/µL) = Abs_{260}×50×dilution factor.

3.2.16.2 16S rRNA amplification of photosynthetic MCCB146

Amplification of 1500bp bacterial rRNA gene fragment was performed using 16S rRNA gene, 16S -1F (5' GAG TTT GAT CCT GGC TCA 3') and 16S-1R (5' ACG GCT ACC TTG TTA CGA CTT 3') primer systems were used (Reddy *et al*., 2000). PCR amplifications from pure cultures were performed in a total volume of 25µl containing 1X PCR buffer (10mM KCl, 10mM $(NH_4)_2SO4$, 2mM $MgSO_4$, 0.1% Triton X-100, 20mM TrisHCl, pH 8.8), 500µM each deoxyribonucleoside triphosphate, 1µl of both forward and reverse primer (7.5 pmol/µl), 1µl of template DNA (100ng/µl) and 1U taq polymerase (New England Biolabs) were added. PCR was performed (Thermal cycler: Eppendorf Mastercycler Personal) with the following thermal profile: initial denaturation at 95 ^{0}C for 5 min followed by 34 cycles of denaturation at 94 ^{0}C for 20sec, annealing at 58 ^{0}C for 30sec and extension at 68 ^{0}C for 2 min followed by final extension at 68 ^{0}C for 10 min.

Cloning to pGEMT easy vector.

Fresh PCR product of 16S rRNA of MCCB146 was used for cloning in to the pGEMT easy vector (Promega,USA). The ligation mix (10µl) comprised 5µl ligation buffer (2X), 0.5µl the vector (50 ng/µl), 3µl of PCR product and 1µl of T4 DNA ligase (3 U/µl). The ligation mix was incubated overnight at 4 ^{0}C. The entire ligation transform mix was transformed in to *Escherichia coli* DH5α competent cells prepared using calcium chloride method. The ligation mix was briefly centrifuged and added to 1 ml micro

centrifuge tube previously placed in ice to which 50µl of competent cells were added and incubated on ice for 20 min. A heat shock at 42 ^{0}C was given for 90sec, immediately the tubes were placed back on ice for 2 min and then 600µl of SOC media was added and incubated for 2 hrs at 37 ^{0}C with shaking at 250rpm. The transformation mixture (100-200µl) was spread on Luria-Bertani (LB) agar plates supplemented with ampicillin (100µg/ml), IPTG (100 mM) and X-gal (80µg/ml). The plates were kept for 37 ^{0}C overnight incubation. Blue/white screening helped to distinguish the positive clones. The white colonies were picked and streaked on LB-Amp+-X-gal+IPTG+ plates and incubated at 37 ^{0}C overnight.

Colony PCR of the white colonies were done using the vector primers T7 (5' TAATACGAC TCACTATAGGG) and SP6 (5' GATTTAG GTGACACTATAG) to confirm the insert. White colonies (template) selected from the transformed plate were inoculated into the PCR mix (25µl) containing 2.5µl 10x PCR buffer, 2.5µl of 2.5mM dNTPs, 1µl of 10 pmol/µl of T7 and SP6 primers, 0.5 U Taq polymerase, and the remaining volume was made up with MilliQ. The thermal cycling conditions were as follows: 1 × 95 ^{0}C for 5 min; 35 × (94 ^{0}C for 15sec, 57 ^{0}C for 20sec, 72 ^{0}C for 60sec); 1 × 72 ^{0}C for 10 min following which the temperature was brought down to 4 ^{0}C. The amplicons were observed in 1% agarose gel and size of the product was determined by comparing with molecular weight marker (1Kb) and documented in a gel documentation system.

Plasmid extraction and purification

Plasmid extraction and purification were done using 'GenElute HP' plasmid miniprep kit (Sigma). Cells were harvested by centrifuging 2 ml of overnight grown recombinant *E.coli* culture at 16000 x g for 20 min. The

pellet was re-suspended in 200µl resuspension solution with RNase. The suspended cells were lysed by adding 200µl of the lysis buffer. This suspension was immediately mixed using gentle inversion until the mixture became clear and viscous. The cell debris was precipitated by adding 350µl of the neutralization buffer. The tube was gently inverted and the cell debris was pelleted by centrifuging at 16000 x g for 10 min. Column was prepared by inserting a Gen Elute HP Miniprep Binding column into a micro centrifuge tube. Transferred the column to a fresh collection tube and added 100µl of elution solution (10 mM Tris-HCl) to the column and centrifuged at 16000 g for 1 min. The DNA present in the eluate (plasmid DNA) was stored at -20 ^{0}C.

Nucleotide sequencing was done in SciGenom Labs Pvt. Ltd., Kochi, and Kerala, India. The primers used were T7 and SP6. Sequenced DNA data was compiled and analyzed. The sequence obtained was first screened for vector regions using 'VecScreen' system accessible from the National Centre for Biotechnology Information (NCBI). The Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al*., 1990) was used to search the GenBank database for homologous sequences (http:// www.ncbi.nlm.nih.gov/). The sequences were multiple aligned using the program Clustal W (Thompson *et al*., 1994). The aligned 16S rRNA gene sequences were used to construct a phylogenetic tree (Saitou and Nei, 1987) using the MEGA 6 package (Tamura *et al*., 2007).

3.2.16.3 Specific primers of photosynthetic sulphur bacterial PUFM gene encoding the "M" subunit of the photosynthetic reaction centre of MCCB146

Oligonucleotide primers for PUFM genes got synthesized from Sigma Aldrich, USA. Primer sets were selected to target specific photosynthetic unit forming gene PUFM gene encoding the M subunit of the photosynthetic reaction centre, which is universally distributed among purple sulphur bacteria (Achenbach *et al.,* 2001). PUFM gene was used for the identification of isolate in the species level.

Genomic DNA of MCCB146 was amplified using the primer PUFM.55F CGCACCTGGACTGGAC and PUFM.750R CCCATGGTCCA GCGCCAGAA. (Achenbach *et al.,* 2001). The PCR mix included 15µl deionized water, 2.5µl 10X Thermopol buffer (New England Biolabs, USA), 1µl 10 mM dNTPs, 2µl Taq polymerase (New England Biolabs, USA), 1µl 10 pmol primers and 120ng DNA template in a final volume of 25µl. The amplification of PUFM gene was carried out in a thermal cycler (Eppendorf, Germany) The PCR condition was in the following manner, 94 ^{0}C for 3 min followed by 30 cycles of 94 ^{0}C for 1 min and annealing temperature was 55 ^{0}C for 1 min then 72 ^{0}C for 1 min and final extension was 72^{0}C for 10 min. Amplified product was obtained at 229 bp and was separated by cutting the gel and purified using sigma gel extraction kit (Gen elute^{TM} Sigma Aldrich) and was documented (BIO-RAD-molecular imager Gel DOC^{TM}U.S.A) and sequenced at Scigenom Lab Pvt.,Kochi, Kerala, India.

Gel purification of PCR product.

Gel purification was carried out using (Gen elute^{TM} Gel Extraction kit, U.S.A, Sigma Aldrich). For purifying the 16S rRNA gene PCR product,

the agarose gel containing the DNA fragments of appropriate size was excised and mixed with 3 gel volume (approximately 450µl) of gel solubilization solution and incubated at 60 ^{0}C for 10 min with repeated vortexing in every 2 min. After incubation, added 1 gel volume (approximately 150µl) of 100% isopropanol, mixed gently until it became homogenous. The solubilizing gel solution was loaded in the binding column that was pretreated with column preparation solution, centrifuged at 12,000 x g for 1 min. Added 700 µl wash solution and centrifuged for 1 min at 12000 x g, repeated the centrifugation, and residual wash solution was removed. The binding column was transferred in to a fresh collection tube (2 ml MCT) and added 50µl of preheated (at 65 ^{0}C) 10 mM Tris-HCl (pH 9.0), centrifuged at 12000 x g for 1 min, stored at -20 °C. The concentration of DNA was measured spectrophotometrically at 260/280nm in a UV-VIS spectrometer (U2800, Hitachi, Japan). The purified PCR product of 229 bp PUFM gene was sequenced (Scigenome Lab Pvt.,Kochi,Kerala,India).

3.2.17 In-vitro (Simulated microcosm) analysis of bioremediation potential of photosynthetic bacteria MCCB146 in aquatic system

Preparation of MCCB146 biomass

MCCB146 biomass was generated in the laboratory condition using modified pfennig's medium with pH 7.3. A loopful of MCCB 146 culture was aseptically transferred in to the medium and incubated at room temperature for 15 days under light/dark condition. After the incubation period, cell biomass were divided in to three portions on the basis of optical density as 0.1 OD, O.3 OD and 0.5 OD (absorbance at 620 nm). The experiment comprised three phases.

First phase of experiment

Black sediment (Refer chapter 2, Fig.2.37) was brought from the pond tank, Arookkuty, Cherthala district, Kerala. The sample was allowed to settle in a FRP tank for a couple of week. Thereafter the initial hydrogen sulphide gas in the sediment sample was measured by Methylene blue spectrophotometry.

Weighed approximately 150g of black sediment into 3.5 L capacity plastic bottles containing 1.5 L of sea water with 25 ppt salinity. The water quality parameters such as ammonia, E*h*, pH, nitrate and nitrite (Grasshoff *et al.*, 1999) of the samples were analyzed before inoculation. The cell suspension (10 ml) of MCCB146 (25ppt) at three absorbance (0.1, 0.3, 0.5) were applied into the subsequent bottles provided with 1200 lux dim light as measured by lux meter (PCE-EM886). The bottles without inoculum were taken as control and triplicates were kept for all the bottles. They were tightly closed using polythene transparent cover for providing anaerobic conditions and incubated for 4 days for the acclimatization of the culture with the environment. After incubation, 5ml of 3.25g/100 ml concentration of Sodium Sulphide was added to all the tubes and calculated the initial concentration (0 hrs) of H_2S by Methylene Blue Spectrophotometric method. Further, the removal rate of H_2S of test samples was checked from 1st to 8^{th} day of the experiment by comparing with the control.

2^{nd} phase of experiment

The water quality parameters were again checked before starting the second phase. Adequate volume of hydrogen sulphide and cell suspension of MCCB 146 could be added to the test bottles depending on the sulphide

removal efficiency. Experiment could be continued same as first phase for a week for checking the removal potency.

3rd phase of experiments

Before starting the third phase, water quality parameters were again measured. Depending on the removal efficiency, hydrogen sulphide could be added accordingly. Then the experiment proceeded for one more week as the other two phases.

Procedure of water quality parameters are described in chapter 2.2.19

3.3 Results

3.3.1 Purification of photosynthetic Sulphur bacteria MCCB 146

Couple of months were taken for the purification of Sulphur bacteria from PF-2 consortium. Among different colonies developed in each tubes, (Fig.3.1) one of them was picked from lower dilution by Pasture pipette and purity was confirmed microscopically. The purified culture has been deposited at the culture collection of National Centre for Aquatic Animal Health, Cochin University Science and Technology and numbered as MCCB146.

Modified Pfennig's medium could support better growth as compared to Pfennig's medium (Pfennig *et al*., 1981). The pigment production and growth rate were clearly visible on modified pfennig's medium as compared to those in other media. Hence, modified pfennig's medium was finally used for further analysis because of the fastest productivity as compared with the normal medium. (Fig 3.2 & Fig. 3.3)

3.3.2 Absorption spectra measurement of MCCB 146.

The peaks were obtained in the absorption spectra of MCCB146 at 889,216, 553, and 487 nm. (Fig.3.4 & 3.5) According to prokaryote 1991, (Refer chapter 2, Table.2.8) the peaks corresponded to bacterial chlorophyll "a" and bacterial chlorophyll "b".

3.3.3 Disaggregation of the cellular aggregate by chemical method

As far as disaggregation is concerned, none of the chemicals used could disrupt the cellular aggregation of MCCB 146 and hence the aggregates were clearly visible under hemocytometer. The result clearly showed that cellular aggregation was the main characteristic nature of photosynthetic bacteria MCCB 146.

3.3.4 Cell Hydrophobicity Assay for calculating the percentage of Cellular Aggregation.

The hydrophobicity index of MCCB 146 was calculated as 63%. Cell hydrophobicity is directly proportional to cellular aggregation (Seits *et al*., 1993).

3.3.5 Optical Density Vs Biomass by packed cell volume method.

Determination of biomass as absorbance with respective wave length was essential for calculating the initial cell count during an experiment. The culture having increased cell hydrophobicity index was very difficult to be dealt with to find out the initial cell count. Therefore, enumeration of MCCB 146 was carried out by determining the packed cell volume. When the culture was at exponential phase, its respective absorbance was used for

the present study, and its weight was calculated gravimetrically and standard graph was plotted and found 6.8 as the factor value. (Fig.3.6)

3.3.6 Biochemical characterization-Substrate Utilization Study MCCB 146.

On analyzing the mode of substrate utilization MCCB 146 was found to utilized all substrates except methionine and valerate and hence it could be considered highly versatile. The isolate exhibited higher growth with substrates such as sodium lactate, trehalose, glucose, glycerol, pyruvate and sucrose medium compared to control (Fig. 3.7) ($P < 0.05$).

3.3.7 Nitrogen Source

The isolate, MCCB 146 exhibited effective growth by utilizing both nitrogen sources as compared to control. Out of the two nitrogen sources, utilization of urea by MCCB 146 was found to be higher than that of ammonium chloride (Fig.3.8) The biomass generation of MCCB 146 with different Nitrogen source was found to be highly significant($P < 0.05$).

3.3.8 Utilization of other reduced sulphur compounds

The isolate exhibited increased biomass production in the presence of one of the reduced Sulphur compounds, cysteine. While the other two compounds thiosulphate and thioglycolate had no profound effect on the isolate. (Fig.3.9) However, statistically, the biomass generation of MCCB 146 with reduced sulphur compounds was found to be insignificant ($P > 0.05$).

3.3.9 Growth of photosynthetic bacteria MCCB146 in the presence of nitrate, nitrite and glutamate

Nitrate was found to have prominent effect on the growth of MCCB 146 compared to that in the control, and the other two substrates such as nitrite and glutamate never enhanced the growth of the isolate (Fig.3.10) ($P < 0.05$).

3.3.10 Growth of MCCB 146 photosynthetic purple sulphur bacteria without hydrogen sulphide

The isolate was found to have effective growth even in the absence of sulphide suggesting it as facultative hydrogen sulphide oxidizer (Fig.3.11) ($P < 0.05$).

3.3.11 Growth of MCCB 146under different modes of nutrition

The isolate was found to grow well under photolithoheterotrophic, photolithoautrotrophic and photoorganoheterotrophic conditions and not under chemolithotrophic conditions provided. Hence, it could be concluded that light was essential for growth and survival of MCCB 146.

3.3.12 Biomass production of MCCB 146 at varying temperature, pH and salinity

3.3.12.1 Sodium Chloride

Statistical analysis on biomass generation of MCCB146 at different salinities were found to be highly significant ($P < 0.05$). The observed biomass values was significantly low for 0ppt and 5ppt salinities. A significant ($P < 0.05$) preference towards 10ppt salinity is highly commendable. (Fig. 3.12). However it could thrive well in Pfennig's medium prepared at salt concentrations of 15 - 35 ppt salinities also with a

mean value of 0.29 ± 0.03 µg per ml. Meanwhile its ability to grow at 40ppt salinity emphasizes its salt tolerance.

3.3.12.2 pH

Statistical analysis, biomass generation of MCCB 146 with different pH range, was statistically significant ($P < 0.05$). Biomass generated was significantly high ($P < 0.05$) for pH 8 followed by neutral pH 7 (Fig.3.13). Apart from optimum pH 8 significant growth observed at pH 9 and 10 in comparison to high alkaline and acidic conditions provided is suggestive of that neutral to moderate alkaline conditions enhances growth MCCB 146 in terms of biomass generation.

3.3.12.3 Temperature

The optimum temperature for MCCB 146 was 30 ^{0}C and the least preferred being 35 ^{0}C (Fig 3.14). Half of the biomass of optimum temperature could be attained at 25 ^{0}C whereas the other temperatures did not support the growth of the organism under the laboratory conditions ($P > 0.05$).

3.3.13 Determination of Hydrogen Sulphide utilization ability of MCCB146 by way of Methylene blue spectrophotometric method

The Hydrogen sulphide removal efficiency of MCCB 146 was analyzed in laboratory condition by methylene blue spectrophotometric method. Significant reduction of hydrogen sulphide was observed (75%) for the test sample as compared to that in the control within 24hrs (Fig. 3.15) ($P < 0.05$).

3.3.14 Transmission Electron Microscopy of Photosynthetic bacteria MCCB 146

Transmission Electron Micrograph of MCCB146 as shown in Fig.3.16 demonstrates cellular aggregation having stack like intracytoplasmic membranes, circular vesicles, cytomembranes, lamellar and tubular cytoplasmic membrane with inclusion bodies, numerous vesicles and gas vacuoles.

3.3.15 Molecular characterization

3.3.15.1 DNA extraction, 16SrRNA gene amplification and sequencing.

DNA was isolated (Fig.3.17) and amplified using universal primers and the PCR product of 1500 bp (Fig.3.18) was cloned in to pGEMT easy vector and transformed in to *Escherichia coli* (JM109) and confirmed the insert by colony PCR using T7 and SP6 vector primers which produced a product of 1500 bp. Plasmid from the transformed organism was extracted and partially sequenced using T7 and sp6 primers. The sequences of clones containing 16S rRNA region when matched with the GenBank database, showed 99% similarity with *Afifella pfennigii*. The phylogenetic tree constructed by using MEGA 6 (Tamura *et al*., 2007) is shown in Fig.3.19. The nucleotide sequences determined in this study were deposited in GenBank data base of NCBI (www.ncbi.nlm.nih.gov) and assigned the accession number KM921803.

3.3.15.2 Functional gene (PUFM) amplification and sequencing

The functional gene of the isolate, PUFM gene was amplified with a product size of 229 bp (Fig 3.20). The sequence was submitted in Genbank and assigned the accession number KX784508. It showed 43% similarity

with photosynthetic gene of *A. pfennigii*. The phylogenetic tree was constructed with the aligned sequence of functional gene and similar sequences from Genbank database (Fig .3.21).

3.3.15.3 In-vitro analysis (Simulated microcosm study) of bioremediation potential of photosynthetic bacteria MCCB 146 in aquatic system

1st phase of experiment

Initially the concentration of hydrogen sulphide in the sediment system was found to be negligible. Hence appropriate volume of hydrogen sulphide was provided in both the test and control systems. Within 24hrs of incubation, the concentration of hydrogen sulphide was found to get elevated in control system as compared to that of the first day. This might be due to the generation of hydrogen sulphide by the indigenous action of sulphate reducing bacteria prevailed in the sediment because of the anaerobic conditions. Meanwhile, hydrogen sulphide concentration got diminished within 24hrs in the case of test systems. Thus as the experiment progressed, hydrogen sulphide was found to get decreased and finally reached equilibrium during the final day of the first phase (Fig. 3.22). This indicated the efficient hydrogen sulphide removing potency of the isolate. The assay was continued for further 5 days without changing the conditions (Fig.3.23) in order to determine the viability of MCCB146 in terms of H_2S removal efficiency. Accordingly, 100% removal efficiency (Fig.3.24) was observed for the isolate and hence it could be considered as an efficient bioaugmentor for the sulphide removal from aquatic systems. Removal efficiency was significant test samples during first 8 days ($P < 0.05$).and last 5 days ($P < 0.05$).

2nd phase of experiment

During the initial stage of the second phase, the concentration of H_2S in the test system was zero, which might be due to the sulphide removal potency of the isolate. Hence adequate volume of Na_2S was provided in the test system for further analysis and the removal rate was found significantly higher (Fig.3.25 & Fig.3.26) ($P < 0.05$).

3rd phase of experiment

During the third phase of experiment, the concentration of H_2S remained zero in the tests and was found elevated in the control. Hence appropriate volume of sodium sulphide was provided to the tests to proceed with the experiment. An efficient sulphide removal was observed in all test samples and the sulphide concentration remained the same in the control. ($P < 0.05$). (Fig.3.27 & 3.28). In all test samples irrespective of the inoculum size removal efficiency in all the three phases was considerably higher.

3.3.16 Water quality – Physico -chemical analysis

On analyzing the water quality parameters, ammonia, nitrite and nitrate were found removed accordingly in both the controls and tests as the days progressed. Ammonia, nitrite and nitrate removal might be due to the action of nitrifiers and denitrifiers already predominant in the sediment. That might be the reason for their disappearance in the controls as well. (Fig. 3.29) ($P < 0.05$) & (Fig.3.30) ($P < 0.05$).

As shown in Fig.3.31 concentration of nitrate in the test and control systems was higher and gradually declined to zero level on the 7th and final day. ($P < 0.05$).

As presented in Fig. 3.32, during the initial, 7th and final day of the experiment, pH in test sample was more or less the same (8-8.5). But in the control system from 7th day onwards there was the declining tendency of pH compared to that of the tests ($P > 0.05$).

Meanwhile, Redox potential (E*h*) during the initial, 7th and final day of the experiment, in both the test and control systems, did not show significant variation. (Fig. 3.33) ($P > 0.05$). However, in the general sense, E*h* of the control system remained throughout the experiment lower compared to that of the tests, and among the tests, the one supplemented with *A. pfennigi* having absorbance 0.5 experienced the highest E*h*. (Final day)

3.4 Discussion

Bioremediation uses biological agents, mainly microorganism i.e. Yeast, Fungi or Bacteria to clean up contaminated soil and water (Kumar 2012).Perhaps organisms selected for treating particular pollutants should be highly active and potent. In the present study, a potential isolate, MCCB146 was successfully isolated from the water sample, Ernakulum and successfully demonstrated their bioremediation potential in aquatic system. The isolate could be identified as *A.pfennigii* formerly known as *Rhodobium pfennigii* belong to phylum proteobacteia, class *alphaproteobacteria,* order *Rhizobiales* and family *Rhodobiaceace and* genus *Afifella.* It was recently discovered and reclassified by Caumette *et al.* (2007). Detailed information about the organism was not reported. According to Brettar *et al.* (2007) members of *Alphaproteobacteria* is more difficult to be cultivate as compared to *Gamaproteobacteria* due to their high degree of adaptation to specific environmental conditions. Purification of MCCB 146 by deep agar

dilution method on Pfennig's medium took several months to get completed of its slow growth rate. Pfennig's medium was also modified for the enhancement of growth to reduce the generation time by incorporating 0.14% yeast extract (Caumette *et al.,* 2007). It was also observed that MCCB 146 had the ability to grow under photolithohetrotrophic condition like *Rhodospeudomonas julia*. According to Renuka *et al.* (1987) enhanced biomass production was observed in the modified Pfennig's medium with 8Mm malate and it has been used for the bioremediation of sulphide from waste water system. Apart from that, Vethanayagam (1991) used modified pfennig's medium by incorporating organic compound for the isolation of photosynthetic sulphur bacteria from mangrove mud.

Pigment analysis of the bacteria is of major concern in determining their biological characteristics. The absorption peak of MCCB 146 was obtained at the region corresponding to chl "a" and chl "b". Besides, an unusual high peak was observed at 889nm, which is the characteristic feature of *A. pfennigii* (Caumette *et al.,* 2007). Similar study was found in Hiraishi *et al.,* 1995, photosynthetic cultures exhibited absorption maxima at 377, 468, 500, 530, 591, 802, and 870 nm, indicating bacterio-chlorophyll "a" and carotenoids of the spirilloxanthin series. Apart from that pink colour formation in the culture period also aided the information regarding the presence of spirilloxanthin pigment in *A. pfennigii*. Another observation found by Soon *et al*.(2014) was that pink to reddish coloration of the bacterial culture under anaerobic-light conditions was one of the important characteristics of Photosynthetic Non Sulphur Bacteria. This coloration is the result of biosynthesis of reddish pigments of the spirilloxanthin series.

The major carotenoid pigment present in MCCB 146 was of spirilloxanthin series by identifying the peak at 553 nm (Mehrabi *et al.,* 2001). The peaks of typical carotenoids of normal spirilloxanthin series are usually found to be at 483, 512 and 548-557 nm, with the highest peak at 512 nm (Caumette *et al.*, 2007).

Aggregation is the characteristic feature of purple non sulphur bacteria. Aggregation, however, could also confer better a resilience of organisms to environmental stresses such as the presence of oxygen that could occur in zones with overlapping oxygenic and anoxygenic photosynthesis or reduced sulphide availability in the upper part of the bacterial layer. (Peduzzi *et al.,* 2003). Release of extracellular nucleic acid by organism *A. marina* induced the formation of strengthening linkages between the cells in aggregates that acted as intercellular connector (Quorum sensing) to stabilize the aggregate matrix and also facilitated the gene exchange among cells (Soon *et al.,* 2014). Apart from that, extracellular nucleic acids and extracellular polymeric substances play important roles in the establishment of cell aggregation. Under hostile condition, high level of extracellular polymeric substances produced by the microbes help them to adapt to stressful environments by maintaining stable environment within the cell aggregates. The other reason for the aggregate formation of *Amoebobacter purpureus* was attributed to the hydrophobic effect involving surface proteins and it was considered as defensive mechanism against virus attack, pH change and light intensity (Seitz *et al.,* 1993). In the present study, *A. pfennigii* exhibited this kind of aggregation throughout the culture period. The enumeration and quantification of cells could be hindered by the aggregation. Several detergents, chemicals and physical method like sonication were used for disintegrating the cells, but

satisfactory results could not be obtained. Hence packed cell volume was used to determine dry weight of the cells so as to proceed with further experiments. Hydrophobicity was found the real reason behind cellular aggregation (Seitz *et al.*, 1993). Accordingly, hydrophobicity index of MCCB 146 was calculated as 63%.

Generally photosynthetic sulphur bacteria are able to use a wide variety of organic acids, alcohols, sugars and aromatic compounds as carbon sources and act as electron donors during photoheterotrophic growth. Their metabolic and physiological diversity make them an important component of wastewater treatment, photo assimilating and mineralizing a variety of organic compounds (Novak 2004) Fukuda *et al.* (2012) reported that *Rhodoium* never was found to utilize broad spectrum substrates for biomass generation. But in the present study among the twenty four substrates tested, biomass production was enhanced by utilizing all the substrates except the two, methionine and valerate. There were also reports that *A. pfennigii* never had the capacity to utilize carbohydrates and alcohols (Caumette *et al.*, 2007). Kappell *et al.* (2014) stated that *A.afifella* was a photosynthetic purple, non-sulphur bacteria that has not be associated with hydrocarbon degradation. But fortunately the studied isolate utilized glucose, trehalose fructose and alcohols for its biomass generation. The organism also had the capability to utilize nitrogen source such as urea and ammonia. Hence the isolate could be considered as a high versatile organism like *R. julia* (MCCB 147).

According to Caumette *et al.* (2007) *A. pfennigii* used ammonium chloride as nitrogen source for growth thereby supporting the present study. *A. pfennigii* and *Rhodobium gokarnense* were never reported to utilize urea

for biomass generation in previous studies (Srinivas *et al.,* 2007a, b). But,the studied isolate utilized urea for its biomass generation. Statistically, biomass generation and utilization of nitrogen source were significantly related ($P < 0.05$).

The isolate was found utilize only cysteine among the reduced sulphur compounds tested. Since cysteine can be easily transported across cytomembranes by using less energy, the isolate might have preferred cysteine for biomass generation. Apart from, Cysteine desulfhydrase (CDS) degrades cysteine to pyruvate, ammonia, and sulphide (Oguri *et al.,* 2012), hence cysteine could act as sulphide batteries for sulphide mediated photosynthesis. Besides, good biomass yield was observed in the presence of cysteine indicating that reduced sulfur compounds were required for sulphur assimilation in photosynthetic sulphur bacteria (Arunasri *et al.,* 2005). Caumette *et al.* (2007) and Srinivas *et al.* (2007a, b) demonstrated that Genus *Afifella* (*A. pfennigii*, *R. gokarnense*) were not utilizing sulphide as electron donor under photolithoautotrophic conditions. Nevertheless, the isolate MCCB 146 utilized sulphide as electron donor under photolithoautotrophic conditions and utilization of cysteine aided the process.

Biomass generation of MCCB 146 was found to be observed in the presence of nitrate and not with nitrite and glutamate. Intracellular NO_3^- accumulation at concentrations of 130 to 160 mM has also beenreported in large, vacuolated, filamentous sulphur bacteria, *Beggiatoa* spp. (Preisler *et al.,* 2007) and also observed the respiratory conversion of NO_3^- to NH_4^+ driven by oxidation of hydrogen sulphide. This indicated the correlation between sulphide, nitrate and ammonia metabolism occurring in the cell in-vivo. Furthermore, a new genus of nitrate accumulating Sulphur bacteria,

Thiomargarita, also found to oxidise sulphide in the presence of NO_3^- (Gusseme *et al.*, 2009). Hence it is clear that in all the above cases, the organism might have been utilizing Sulphide as the electron donor and nitrate as the electron acceptor under anaerobic conditions (Sayama 2001). This indicated that nitrate mediated sulphide oxidation might be possible with the isolate, MCCB 146. Detailed description of *A. pfennigii* was recently explained by Caumette *et al.* (2007) and Urdiain *et al.* (2008), in which it was stated that the organism was not able to use nitrate as nitrogen source. But on the contrary, it was found that MCCB 146, *A. pfennigii*, was a potent organism to utilize nitrate and sulphide for its biomass generation.

Significantly, the isolate is a facultative hydrogen sulphide oxidiser and does not required H_2S as the sole hydrogen donor for photosynthesis. Hence, in the absence of H_2S, it can switched over to photolithohetrotrophic mode of nutrition. It was proved by the effective growth of MCCB 146 on medium with or without the presence of hydrogen sulphide. Anoxygenic photolithotrophic bacteria carry out the light-dependent, bacteriochlorophyll mediated, energy transfer processes with reduced Sulphur compounds as electron donors and such organisms are reported to belonging to purple sulphur bacteria, green sulphur bacteria and a few some non-sulphur bacteria such as *Rhodospeudomonas and Rhodovulum* (Ghosh & Dam 2009). Besides, the aerobic anoxygenic bacteriochlorophyll-containing bacteria, the obligatory anaerobic anoxygenic phototrophic *Heliobacteria*, and the aerobic/anaerobic anoxygenic filamentous phototrophic green non-Sulphur bacteria belonging to *Chloroflexaceae* also have the capabilities to oxidize Sulphur compounds under a variety of metabolic conditions (Dahl, 2008). The physiology and biochemistry of Sulphur oxidation by the last three groups of bacteria were very poorly understood. These phototrophic bacteria

are incapable of using water as electron donor. Instead they require more reduced chemicals such as elemental Sulphur and sulphide (and sometimes hydrogen too) to serve this purpose. This kind of photosynthetic metabolism thus clearly differs from that encountered in cyanobacteria, algae and green plants and the phylogenetic and biomolecular fossil data suggest that the ubiquity of sulphide oxidizing genes could be due to horizontal transfer, and coupled sulphate reduction/sulphide oxidation pathways, from anoxygeneic phototrophic ancestor (Ghosh & Dam 2009). In such a way that MCCB146 could possibly survive under photolithohetrotrophic, photolithoautrotrophic and photoorganoheterotrophic type of nutritional conditions since it belongs to alphaproteobacteria which has high degree of adaptation to specific environmental conditions (Brettar *et al.,* 2007). The prokaryotes could oxidize sulphide and sulphur compounds under alkaline, neutral and acidic conditions (Friendrich *et al.,* 2001). Two major pathways proposed for oxidation of sulphur compounds: SOX pathways and S_4 mediated pathways which function as an "operon" concept. In this point of view, the studied isolate could be classified under facultative sulphide oxidizers. Hence, even in the absence of sulphide, it can survive photolithoorganotrophically. This is the first report *A. pfennigii* as facultative sulphide oxidizer based on the study of Caumette *et al.* (2007) and Urdiain *et al.* (2008).

The isolate MCCB 146 was found to be well adapted to the marine environment. The microscopic bacterial determination emphasized the influence of salinity on the bacterial diversity, with higher diversity found in low salinity, mainly for purple non-sulphur bacteria (Mao Che *et al.,* 2001). The isolate was found to grow in different ranges of salinities such as 10-40 ppt. This revealed the salt adaptivity of organism. However higher growth was found at 10 ppt salinity. These types of organisms have the ability to

produce osmotically active compatible solutes or osmotica, which regulate the osmotic imbalance in bacteria (Oren 2008). Apart from that, cell membrane regulates salinity tolerance by changing membrane lipid composition which has a pronounced effect on lipid phase behaviour. Similar kind of behaviour was found in sulphur bacteria *Ectothiorhodospira* also (Galinski & Truper 1994).

Salt (NaCl) was obligatory as reported by Srinivas *et al.* (2007) the salt obligatory property of *Rhodobium gokarnense* (Genus *Afifella)* which could tolerate 0.5–10.0 % salt. Similar capability was fond to be present with the studied isolate *A. pfennigii* and it was a halotorent organism like photosynthetic sulphur bacteria *Thiorhodospira sibirica* (Bryantseva *et al.,* 1999).

The maximun biomass generation was noticed at pH 7-8 and it mostly preferred to grow in slightly alkaline conditions and never beyond at pH 10. Similar observation has been reported by Caumette *et al.,* (2007) also. Sanchez *et al.* (2001) and Makzum *et al.*(2016) also reported that high rate of sulphide oxidation occured at alkaline conditions pH >9 which strengthened the present study.

The isolate was found to be heat sensitive since enhanced biomass production was found at 25 ^{0}C to 30 ^{0}C only and not at higher tepmeratures. This is also supported by Caumette *et al.* (2007) and Urdiain *et al.*(2008) in which *R. pfennigii* (*A. pfennigii)* exhibited higher growth at 25 – 30 ^{0}C.

The methylene blue method is the most commonly reported method to measure hydrogen sulphide in biological samples (Shen *et al.,* 2015). The preliminary study of hydrogen removal efficiency of the isolate by methylene blue spectrophotometric method revealed 75% efficiency of the isolate. Even though previous studies reported that the organism was

sulphide tolerant and not found to utilize sulphide as electron donor under photolithohetrotrophic condition (Caumette *et al.,* 2007, Urdiain *et al.,* 2008), the isolate, MCCB 146 was found to be a a true facultative sulphide oxidative organism.

Considering the morphological structure, MCCB146 was found to have several intracytoplasmic membranes, circular vesicle cytomembranes, lamellar and tubular cytoplasmic membrane with inclusion bodies (Stolz 2007) the characteristic feature of aerobic or micro aerobic photosynthetic bacteria. Apart from, numerous vesicles, large gas vacuoles were also visible and cellular aggregation was clearly observed. Formation of intra-cytoplasmic membrane (ICM) is the peculiarity of purple as well as non-sulphur bacteria and is contiguous with the cytoplasmic membrane (CM); and may form vesicles, tubules, or thylakoid-like sheets (Yurkov & Beatty 1998). In most anaerobic or micro aerobic phototrophic bacteria, light and oxygen tension regulate the formation of the ICM, such that ICM formation is induced when the oxygen tension is lowered and the most extensive ICM development occurs during anaerobic growth. Characteristic feature of photosynthetic sulphur bacteria like lamellar and vesicular cytoplasmic membrane was earlier reported (Okubo *et al.,* 2006; Arunasri *et al.,* 2005;Bryantseva IA *et al*., 2015).

Based on molecular characterization, the organism showed 99% similarity to *A. pfennigii* at 16S rRNA gene level identification. By analysing the phylogenetic tree, MCCB 146 seemed to be closely related to *A. pfennigii* with 99% 16S rRNA sequence similarity and more distant from the type strain *Rhodospeudomonas* sp thereby confirming it as *A. pfennigii* (Caumette *et al.,* 2007, AR2102).

Apart from 16S rRNA sequence analysis, functional gene marker, PUFM gene encoding a protein for the M subunit of the photosynthetic reaction centre in purple sulphur and purple non-Sulphur bacteria(Okubo *et al.,* 2006; Tank *et al.,* 2009, Thusak *et al.,* 2004 and Soto-Feliciano *et al.,* 2010) also has been used for the identification. PUFM was selected since it is unique to purple sulphur or non-sulphur phototrophic bacteria and universally distributed across these groups (Achenbach *et al.,* 2001). Since PUFM gene primers were designed to amplify the genes unique to organisms possessing a purple bacterial light-harvesting reaction centre, they can be used to detect any photosynthetic member of the Proteobacteria regardless of phylogenetic affiliation. Based on the phylogenetic tree, PUFM gene sequence of MCCB 146 was positioned in the cluster with photosynthetic reaction centres of purple sulphur and non-sulphur bacteria and more specifically with that of *A. pfennigii* thereby confirming their identification.

Positive amplification of PUFM gene and absorption peak obtained at 880 nm confirmed the possibility of the occurrence of reaction centre LHC 1 with Bchl "a" (Zuber and Brunisholz, 1991; Tuschak *et al.,* 2004; Soto-Feliciano *et al.,* 2010). Another important characteristic of *A. pfennigii* in terms of PUFM gene is the reddish pigmentation which indicated the presence of the photosynthetic pigments carotenoids and bacteriochlorophyll "a" which were discussed earlier. These pigments could be successfully detected in the isolate.

A short Description of *Afifella pfennigii* obtained from the present study is given here: (gen. n. pfennigii of Pfennig, named after Norbert Pfennig, a German Microbiologist).

Straight to ovoid rods, Gram-negative, Colour of cell suspension is pink. Stacks of lamellar photosynthetic membranes occur. Contains BChl "a" and "b" as photosynthetic pigment and spirilloxanthin as the major carotenoid. Absorption maxima of living cell suspensions at 216, 487, 553, and infrared absorption peak at 889 nm. Electron donors for photo-organotrophic growth are sodium lactate, trehalose, glucose, glycerol, pyruvate, sucrose, cysteine, ammonium chloride, urea, and utilization of nitrate. Methionine and valerate were never utilized. Photolithotrophic growth occurs with sodium bicarbonate; chemoorganotrophic growth not observed, photolithohetrotrophic growth in the presence of pyruvate and photoorganohetrotrophic growth in the presence of cystien. Thiamine or yeast extract is required for growth. Cellular aggregation is visible. Optimal pH is 7–8 (range pH 7–10). Optimal temperature is 25–30 ^{0}C. Salinity range is 10-–35 ppt (w/v) NaCl (optimum 10 ppt NaCl). 16SrRNA and PUFM sequence more similar to *A. pfennigii* Habitat: microbial mats exposed to light in coastal marine environments or brackish ponds. Present strain, MCCB 146, was isolated from a shrimp aquaculture brackish water pond located at Maradu, Kochi, and Kerala, India.

The oxidation of sulphide, which is usually generated by the action of sulfate reducing bacteria, is the key process in biogeochemistry of aquatic sediment while the pathways related to sulphur cycle were least understood (Jorgensen 1990). Meanwhile dissimilatory nitrate reduction, ammonia and sulphite production were frequently observed in sulphide rich sediments

which indicated that sulphur and nitrogen cycle were strongly coupled and found to be more complex (Brunet & Garcia 1996). Presence of ammonia, nitrite and nitrate in sediment might vary with the ecological conditions. But in the present study, water quality parameters such as ammonia, nitrite, and nitrate were found to be higher in the first phase (without providing sulphide and inoculum). Similar kind of observation was reported by Brunet & Garcia (1996) and Gao *et al.* (2012). However, during the later phase of the experiment, they were found to be brought down in both control and test samples. This might be due to the natural phenomenon of nitrification and denitrification processes occurred in sediment. Jensen *et al.* (1994) reported that nitrification and denitrification were coupled pathways and never could be expected the steady microprofile of ammonia nitrite and nitrate in sediment. Meanwhile, these parameters could not make any significant difference in sulphide removal efficiency of *A. pfennigii*.

Redox potential was initially very low but found to be slightly increased depending on the removal of H_2S at the end of each phases in test samples as compared to the control. The spatial and temporal variation in redox potential depends on the energy rich compounds and the microbial community (Lyimo *et al.*, 2005). In the present study, slight enhancement of E*h* which occurred might be due to the sulphide oxidation by the action of the bioaugmentors. Similarly, slight variation in pH also was observed in test samples. Due to the hydrogen sulphide oxidation in test samples, acidic condition was not formed (Anwar *et al.*, 1998), but in the case of control, as hydrogen sulphide being not oxidised, alkaline conditions got slightly reduced.

Biochemical characterisation confirmed total hydrogen sulphide removal potency of the isolate, MCCB146. Various previous studies reported the bioremediation potential of this kind of sulphide oxidising bacteria for the waste water treatment (Ferrera *et al.*, 2004; Okubo *et al*., 2006; Syed *et al.*, 2006). Primary level screening and further bioassay revealed that MCCB 146 was a potent isolate for the bioremediation of H_2S. Caumette *et al.* (2007) stated that the organism *A. pfennigii* was found to be sulphide tolerent only and never utilised sulphide as a electron donor during anoxygenic photosynthesis. But on the contrary, the present study confirmed that MCCB 146 could utilize sulphide in simulated microcosm study and thereby confirmed its potency for sulphide oxidation in sediment water system. This is the first report of its kind.

Figures

Fig.3.1 Colonies of purple photosynthetic bacteria (MCCB 146) were developed in soft agar medium at highest dilution tube same as MCCB147.

Fig.3.2 and Fig.3.3 Indicated the growth of MCCB 146 in modified as well as un modified pfennig's medium . Severe aggreagations were observed at the bottom of tube and it was considered as the charecteristic nature of MCCB 146.

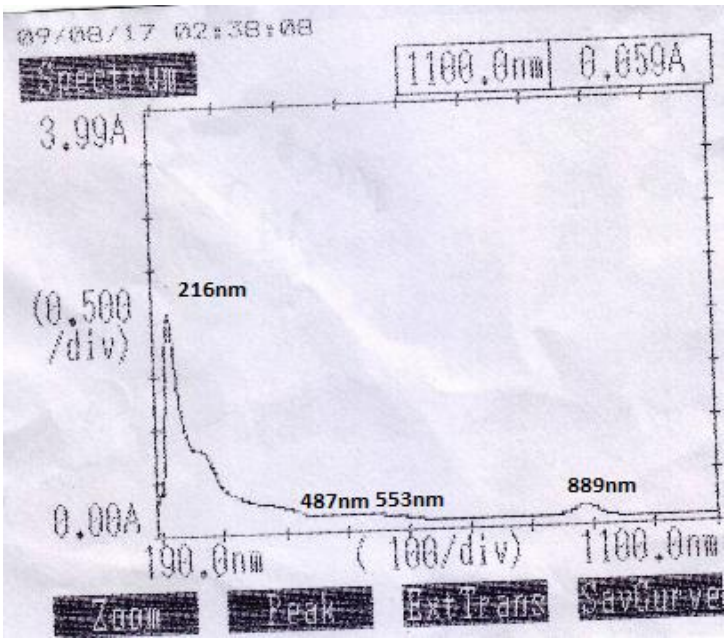


Fig No 3.4. The characteristic peak obtained by sucrose mediated absorption maxima of MCCB 146

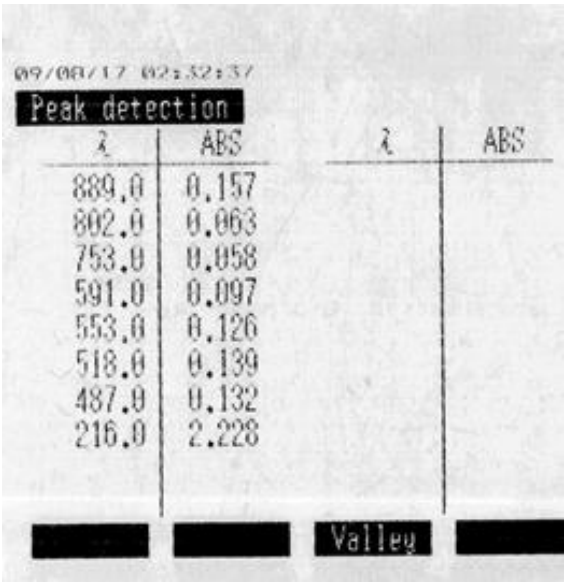


Fig.3.5 Peak detected in the spectrum

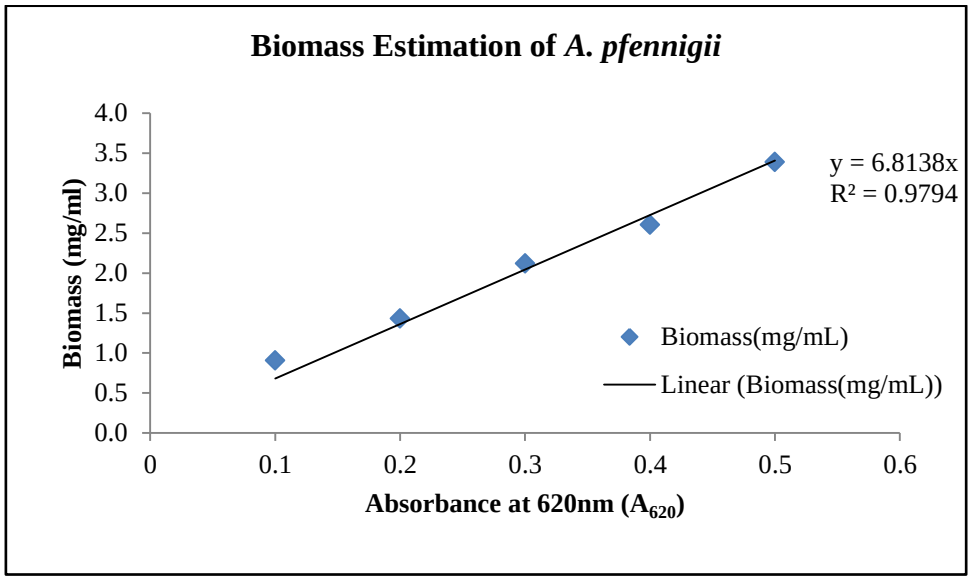


Fig.3.6. Standards graph for biomass estimation of *A.pfennigii*

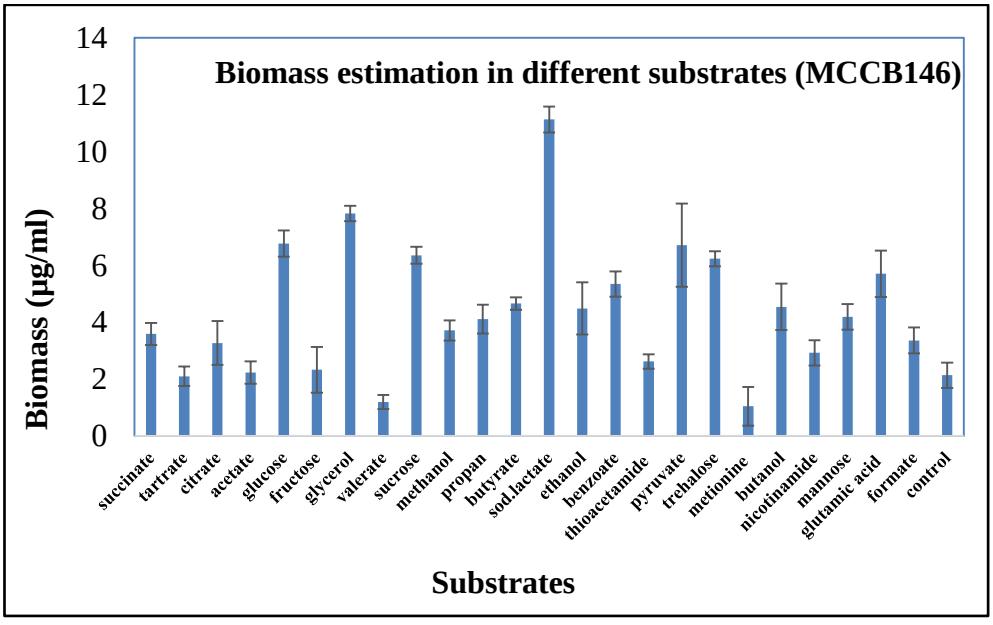


Fig.3.7 Biomass production of MCCB 146 with respective substrates

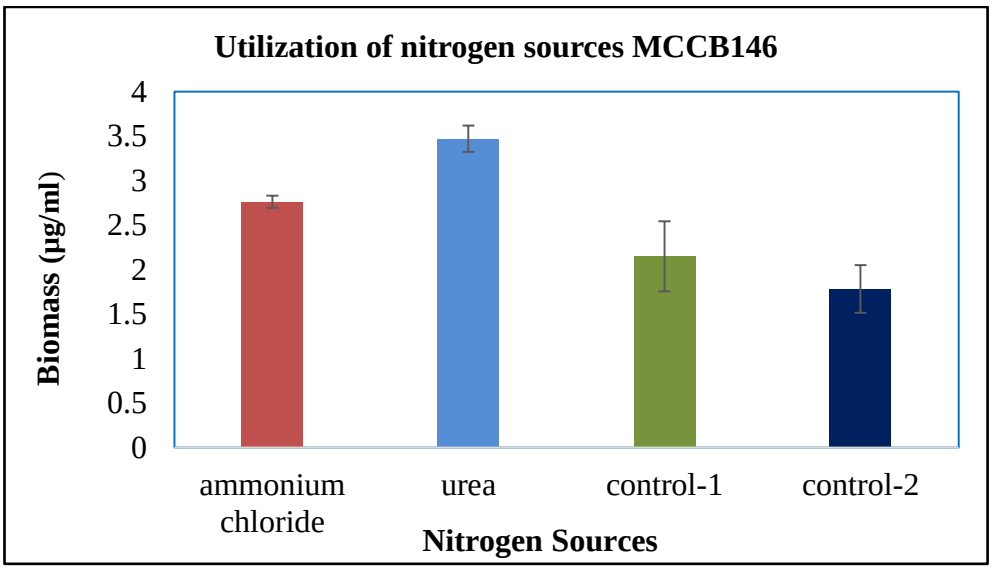


Fig.3.8. Biomass production of MCCB 146 in the presence of nitrogen sources

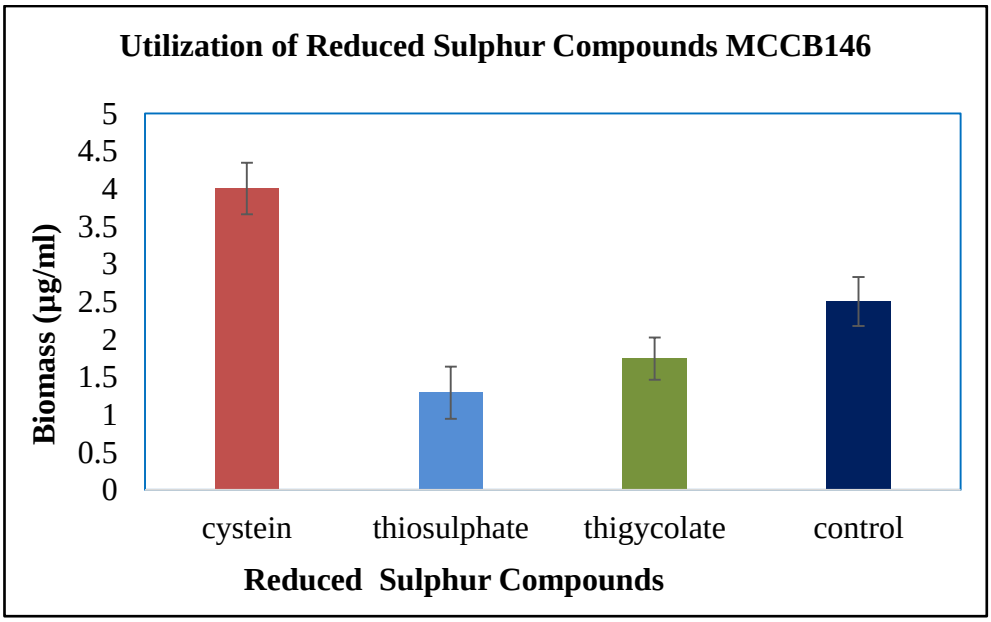


Fig.3.9. The biomass production of MCCB 146 in the presence of reduced Sulphur compounds

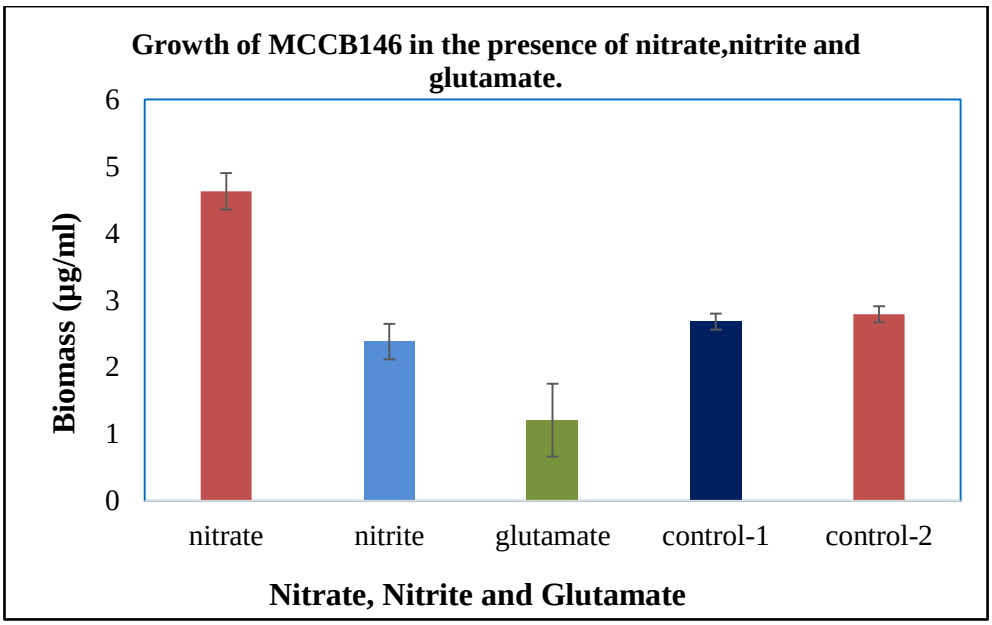


Fig 3.10 The biomass production of MCCB 146 in the presence of Nitrate, Nitrite, and Glutamate

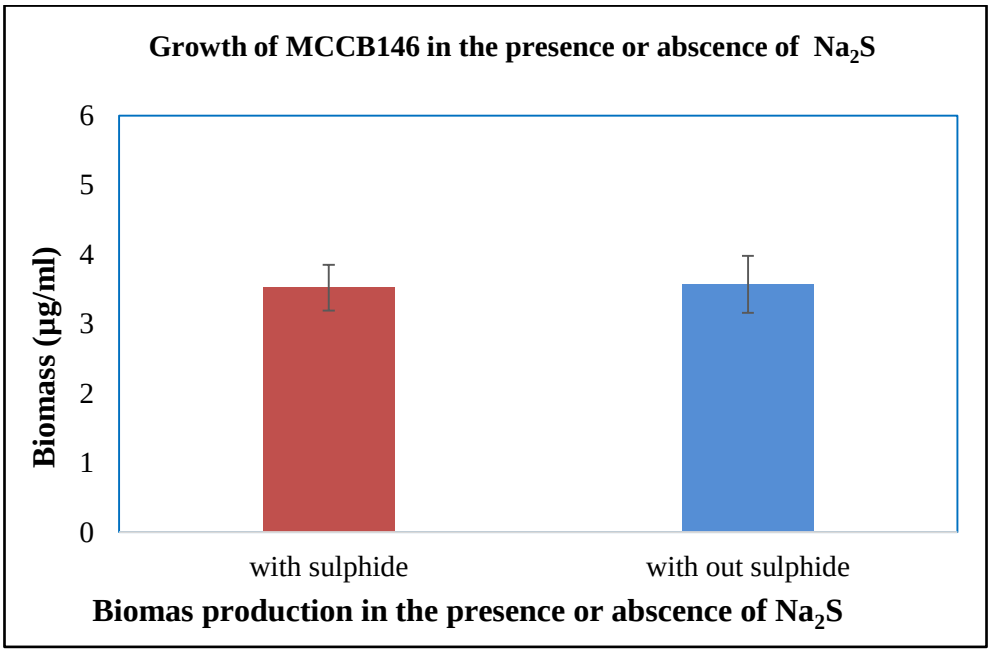


Fig.3.11 The biomass production of MCCB 146 in the presence and absence of sodium sulphide.

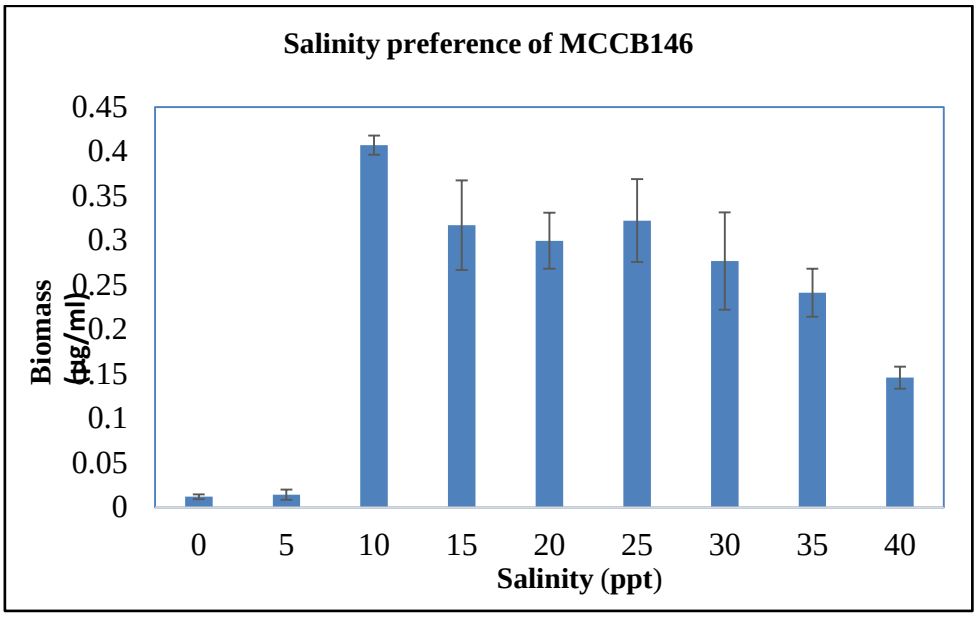


Fig 3.12. Salinity preference of MCCB 146

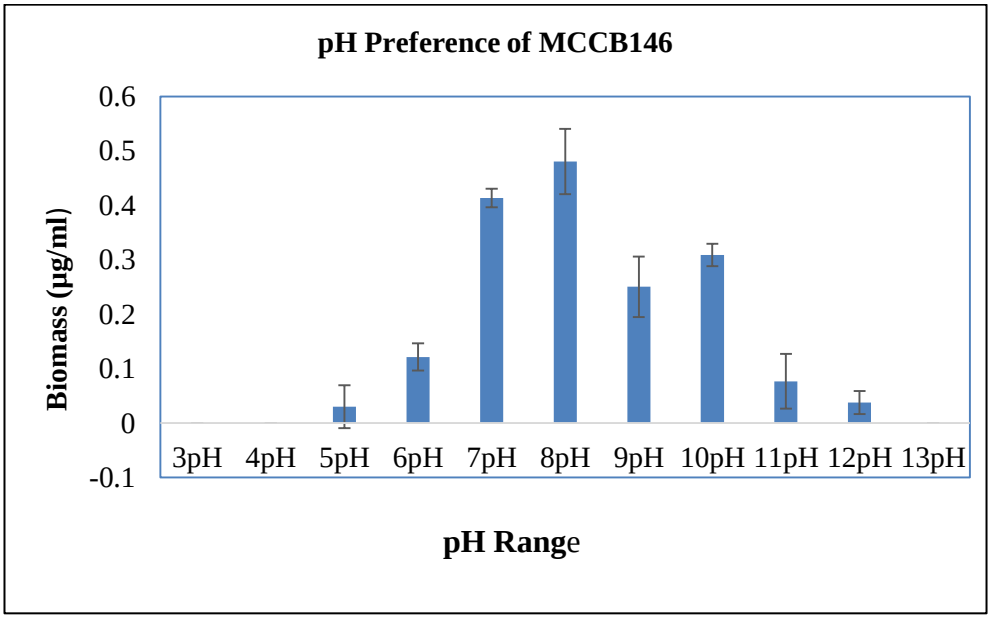


Fig 3.13 pH preference of MCCB 146

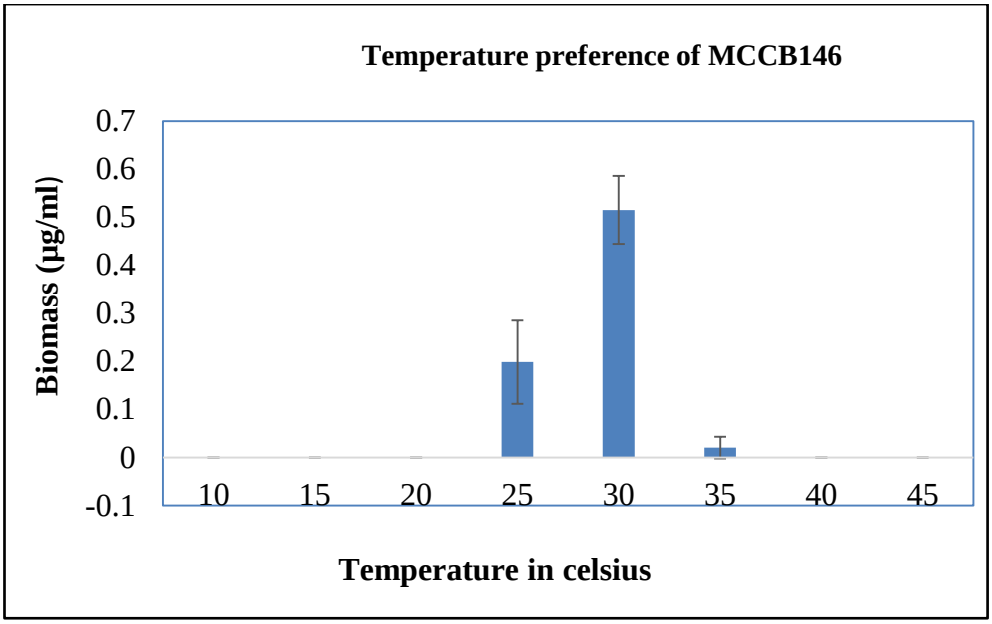


Fig 3.14 Temperature preference of MCCB 146

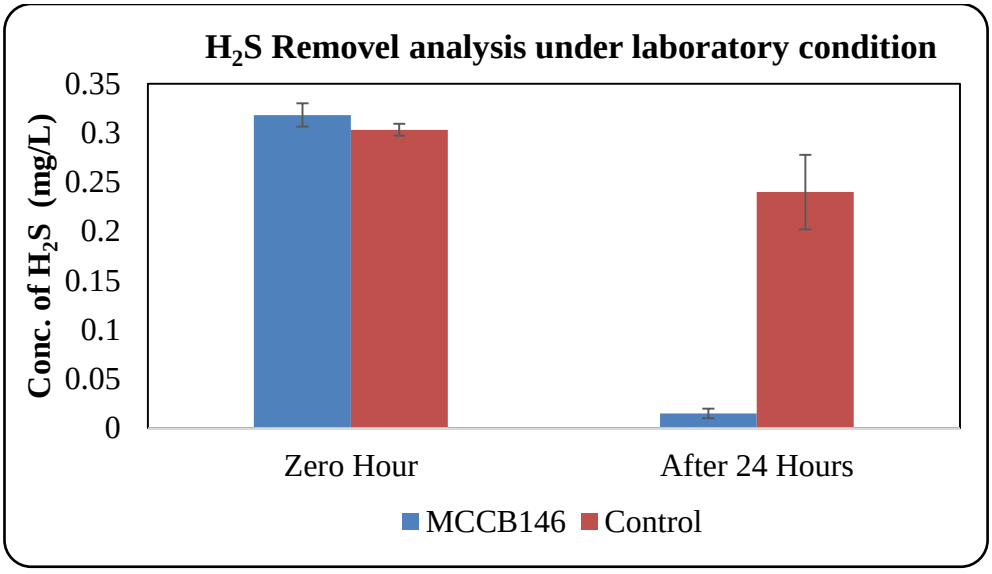


Fig 3.15 Hydrogen sulphide removal by MCCB 146 after 24 hrs incubation

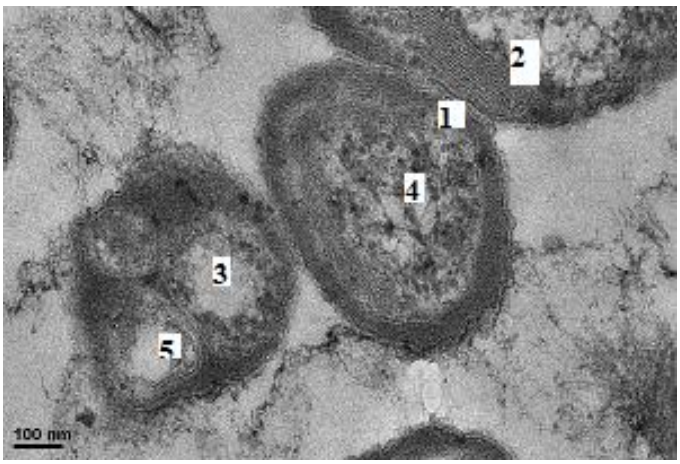


Fig.3.16 Transmission Electron Microscopic image of MCCB 146. 1. Cellular aggregation, 2. Cells with stack like intra-cytoplasmic membranes, 3. Gas vacuoles, 4. Inclusion bodies, 5. Vesicles

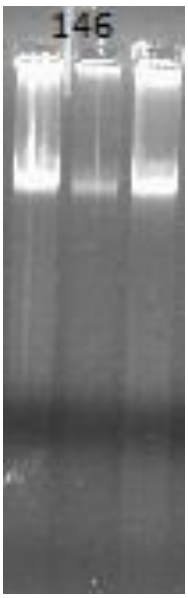


Fig.3.17 Genomic DNA of MCCB 146

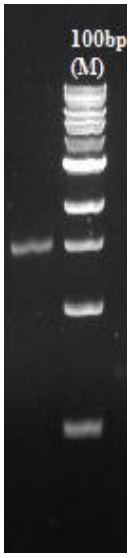


Fig.3.18 16S rRNA gene of MCCB 146 (*A. pfennigii*)

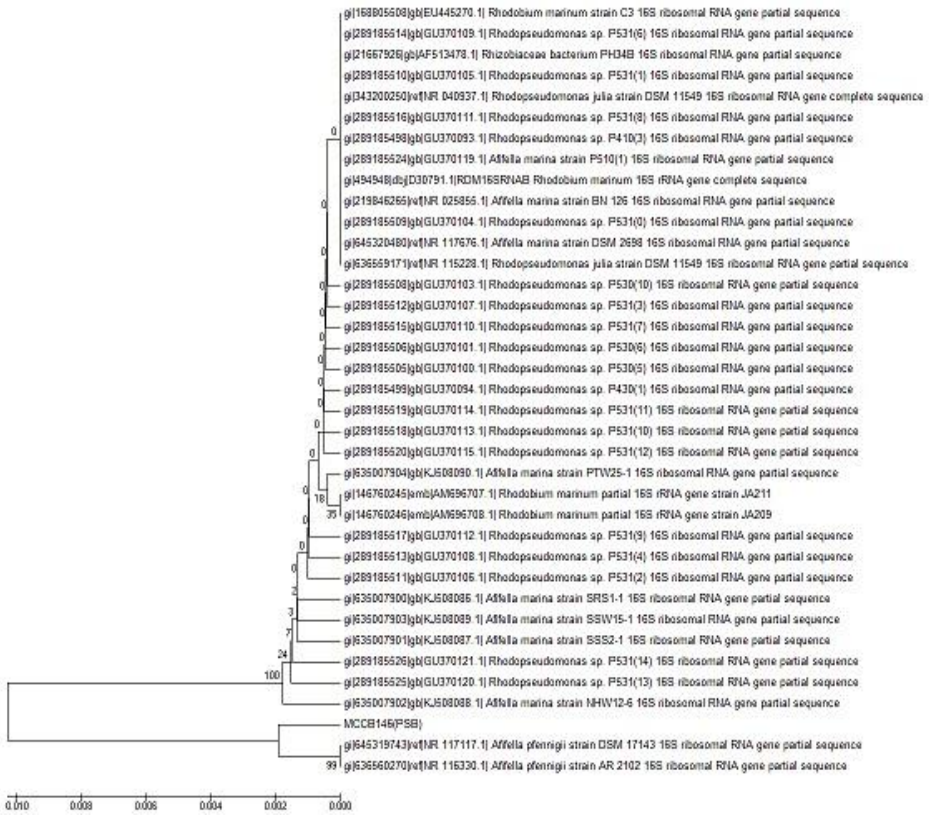


Fig. 3.19 Phylogenetic tree of photosynthetic bacteria MCCB 146 (*A. pfennigii)*

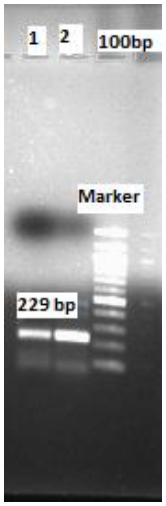


Fig 3.20 PUFM gene of MCCB 146 (*A. pfennigii*)

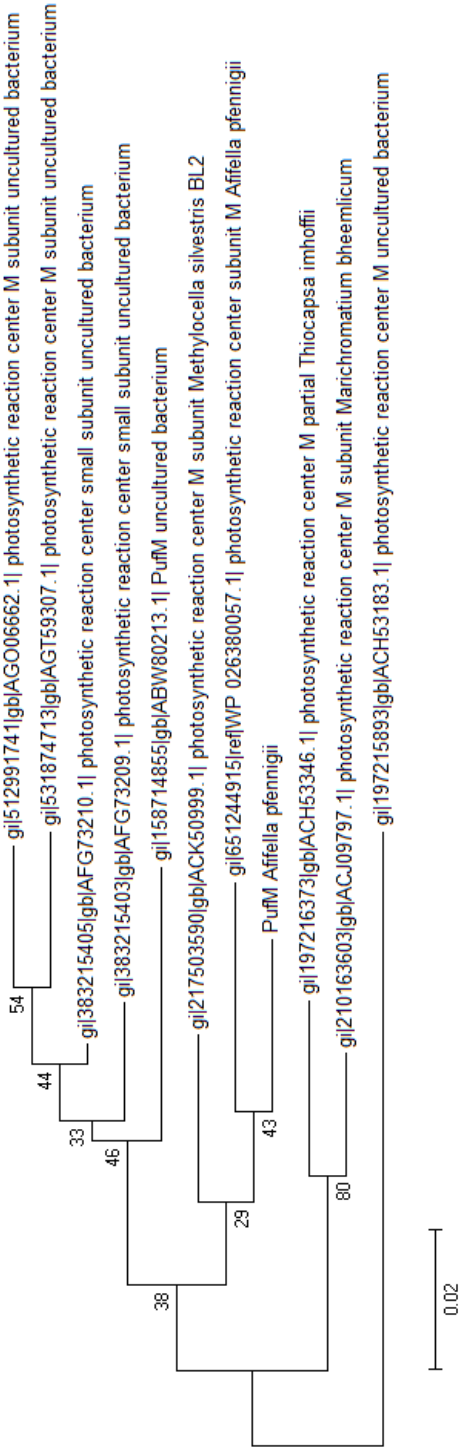
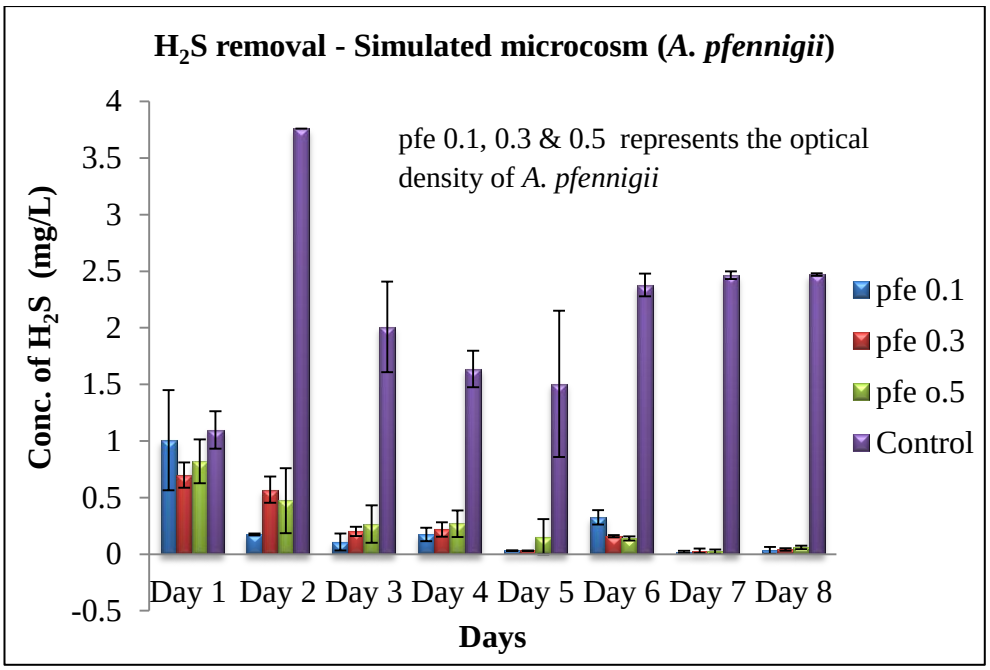


Fig 3.21 The phylogenetic tree of PUFM gene of MCCB146. (*A. pfennigii*)



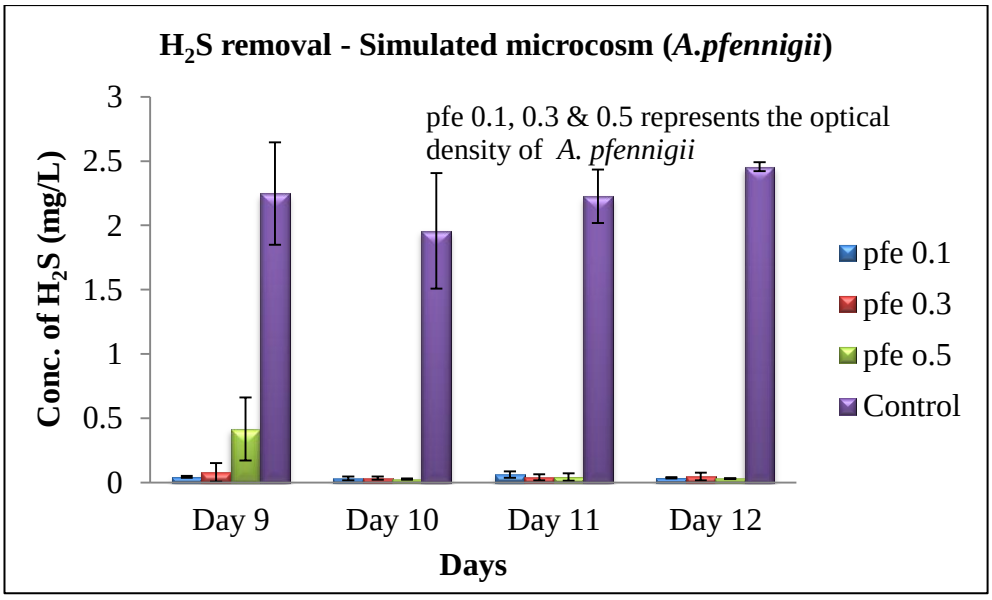


Fig.3.22 and Fig.3.23 Sulphide removal by *A.pfennigii* during in the first phase of the experiment

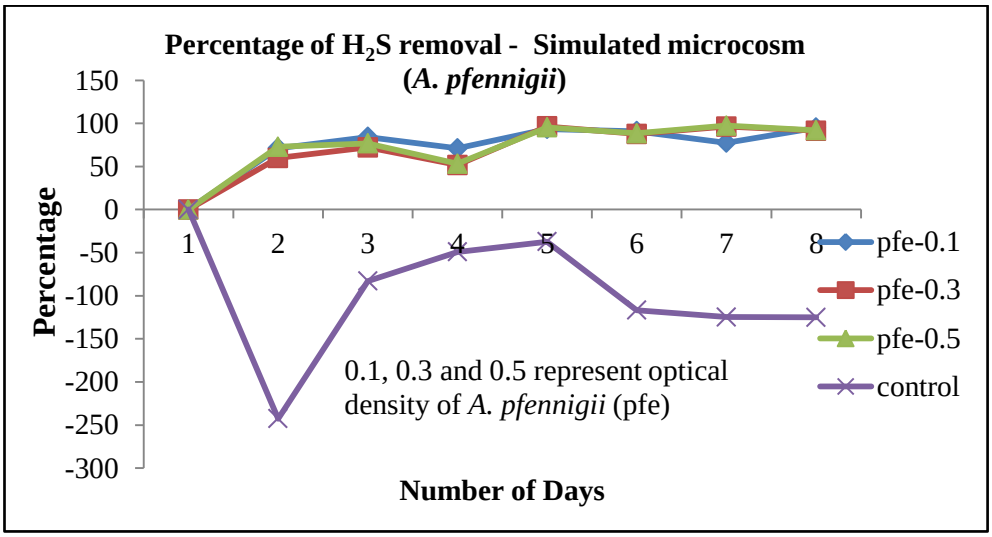


Fig.3.24 Percentage sulphide removal by *A.pfennigii* during in the phase one experiment

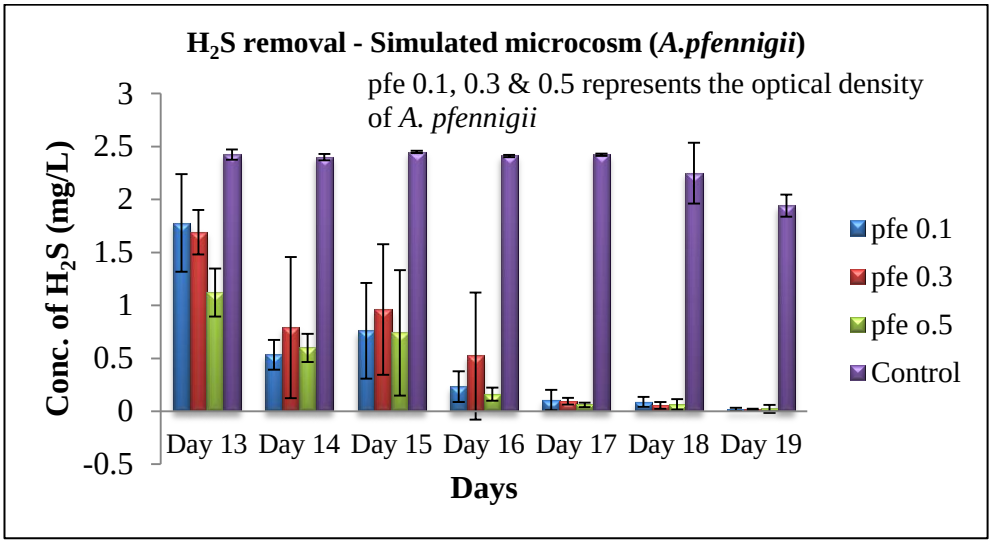


Fig 3.25 Sulphide removal by *A.pfennigii* during in the 2nd phase experiment

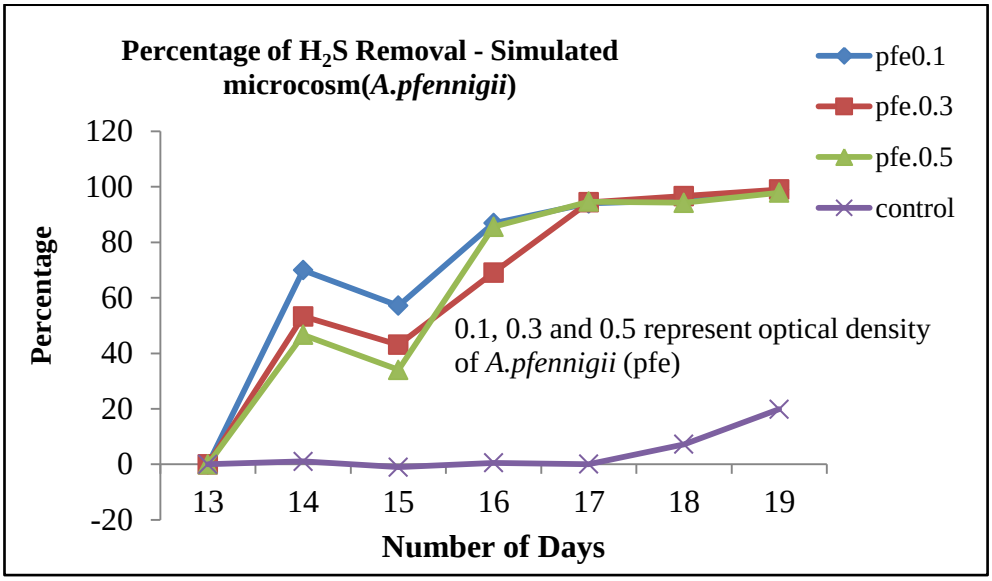


Fig.3.26 Percentage of sulphide removal by *A.pfennigii* during in the phase 2 experiment

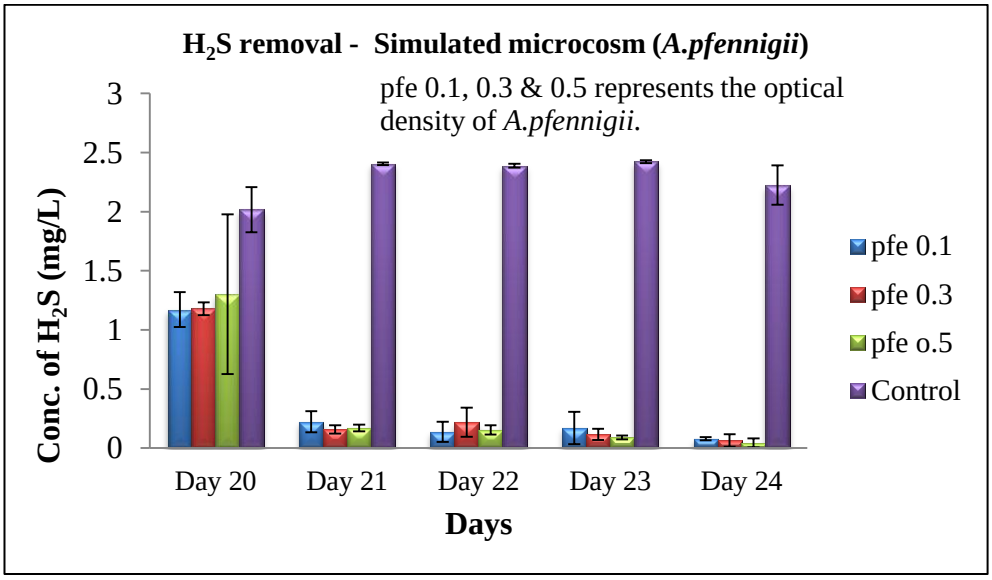


Fig 3.27 Sulphide removal by *A.pfennigii* during in the 3[ed] phase of experiment

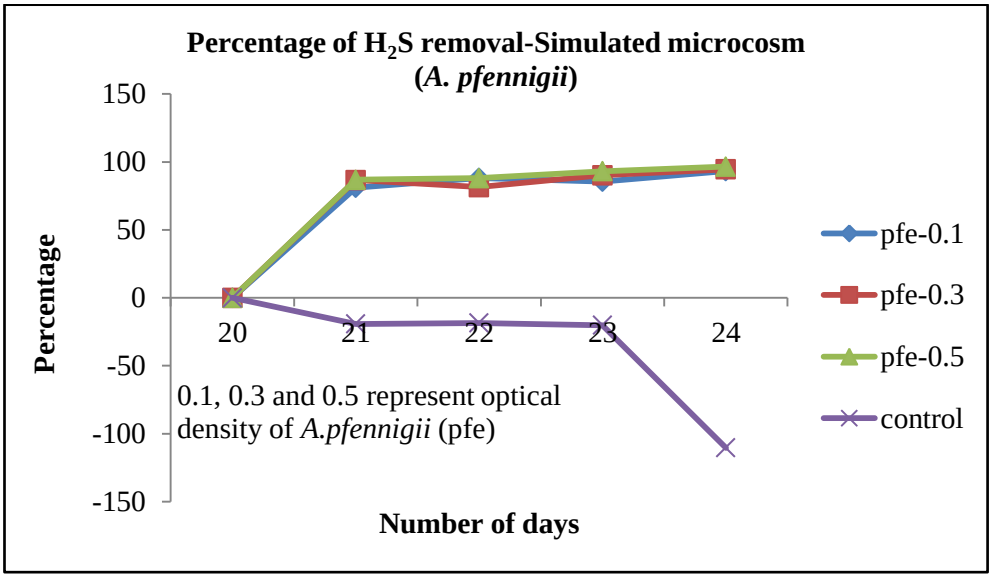


Fig.3.28 Percentage of sulphide removal by *A.pfennigii* during in the 20th to 24th day of phase 3 experiment

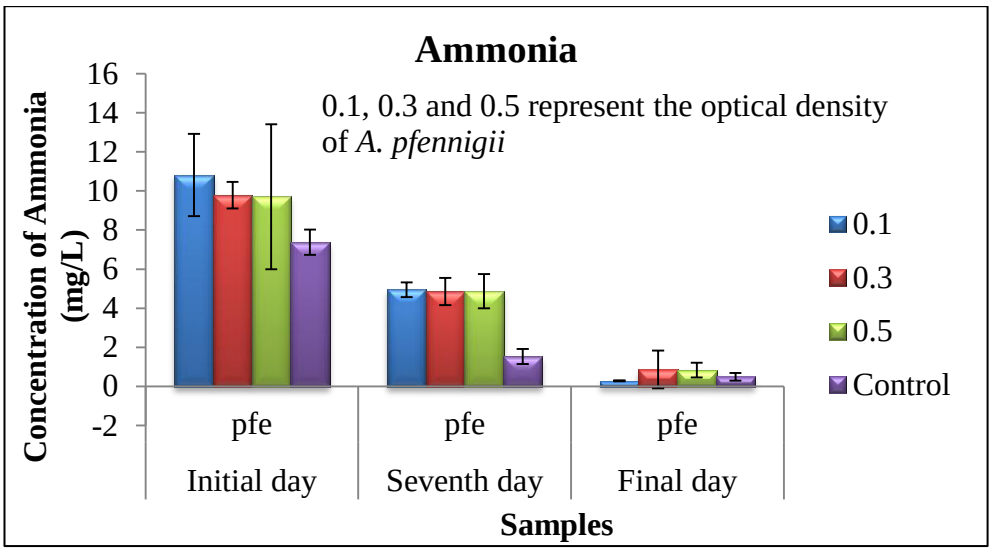


Fig. 3.29. Concentration of ammonia during in the experiment

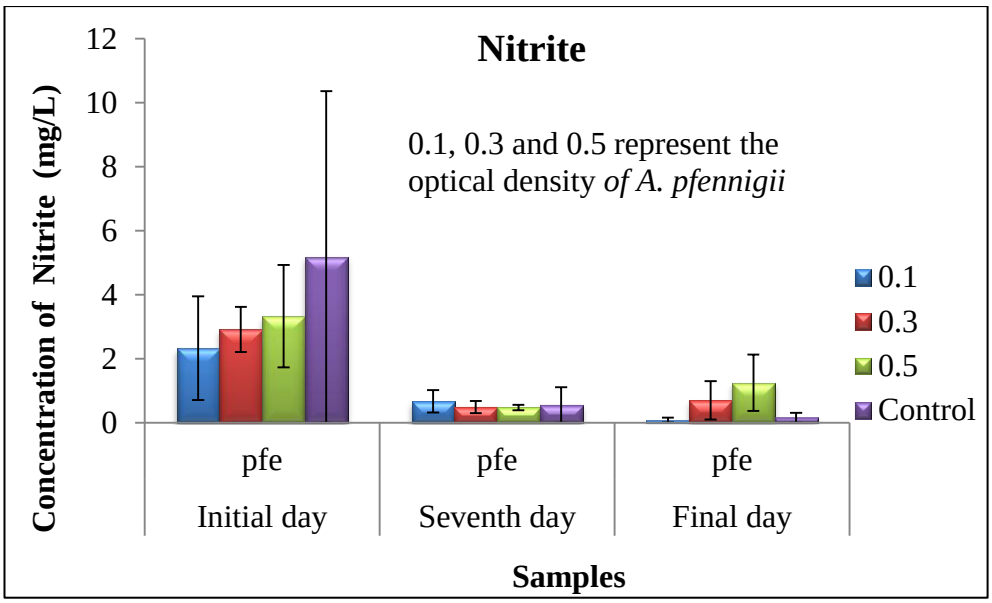


Fig.3.30. Concentration of Nitrite during in the experiment

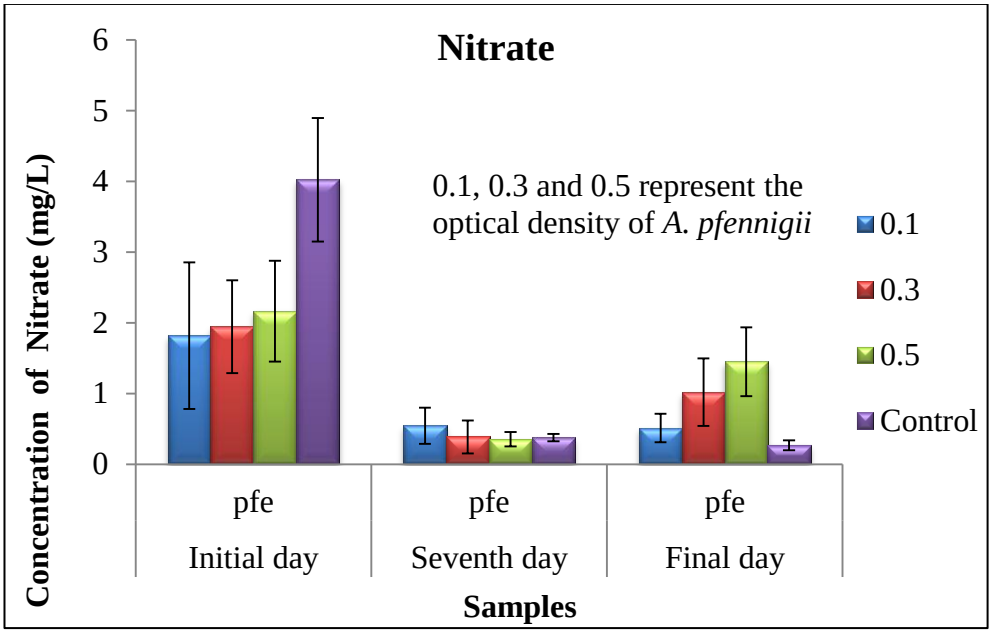


Fig.3.31. Concentration of Nitrate during in the experiment

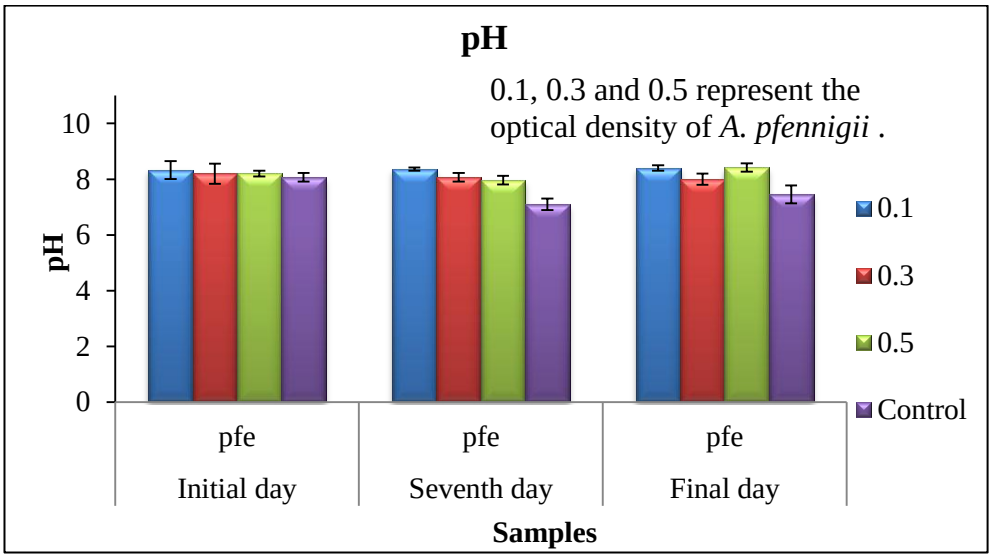


Fig.3.32. pH during in the experiment

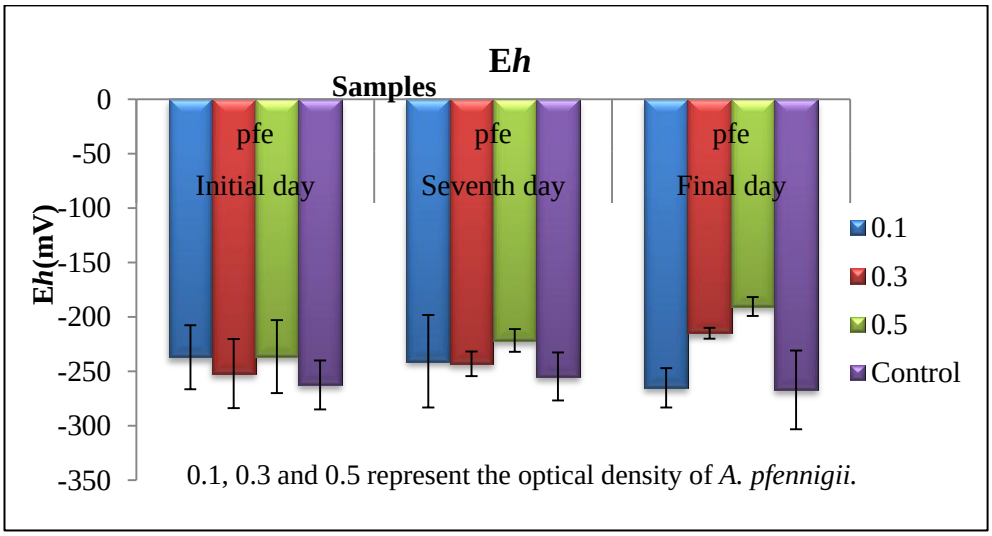


Fig.3.33. E*h* during in the experiment

Chapter 4

ISOLATION, PURIFICATION AND CHARACTERIZATION OF PHOTOSYNTHETIC BACTERIUM MCCB234, AS BIOAUGMENTOR FOR BIOREMEDIATING HYDROGEN SULPHIDE IN AQUATIC SYSTEM

Contents

4.1 Introduction

Cyanobacteria are a unique assemblage of organisms which occupy and predominates a vast array of habitats and have several general characteristics (Wilmotte, 1991, AbdAllah, 2006, Haande *et al.,* 2011). Perhaps they are very susceptible to sudden physical and chemical alterations of light, salinity, temperature and nutrient composition.

The application of cyanobacteria showed immense potential in wastewater and industrial effluent treatment, bioremediation of aquatic and terrestrial habitats, chemical industries, bio fertilizers, food, feed, fuel, etc. (Cairns and Dickson, 1971). In this context, a method for the removal of hydrogen sulphide by using filamentous photosynthetic sulphur bacteria MCCB 234 *Geitlerinema* sp. from aquatic system has been developed.

Mechanism of anoxygenic and oxygenic sulphide dependant photosynthesis in cyanobacteria

Sulphide is toxic to most oxygenic photosynthetic organisms including cyanobacteria because it irreversibly blocks photosystem II (PSII) (Cohen *et al.,* 1986; Garcia-Pichel & Castenholz, 1990). Previous studies have determined that there are four photosynthetic responses of cyanobacteria to sulphide (Cohen *et al.,* 1986). The most common response, exhibited by most cyanobacteria, is sulphide-sensitive oxygenic photosynthesis, in which sulphide exposure results in the total and permanent inhibition of photosynthesis by blocking electron transport associated with PSII (Cohen *et al.,* 1986). This inhibition may be a result of sulphide targeting the donor side of PSII (Oren and Shilo, 1979; Miller & Bebout, 2004) or cytochromes on the acceptor side of PSII (Cohen *et al.,* 1986). Less common are three strategies by which cyanobacteria can tolerate sulphide. A few cyanobacteria conduct sulphide-resistant oxygenic photosynthesis, which allows the continuation of oxygenic photosynthesis during exposure to sulphide. Cyanobacteria that can utilize this strategy includes *Synechococcus lividus*, and *Oscillatoria terebriformis* (reclassified as a member of the genus *Geitlerinema;* Castenholz *et al.,* 2001), which were all isolated from sulphide-rich hot spring outflows (Castenholz, 1977; Richardson & Castenholz, 1987). The second strategy is the partial inhibition of oxygenic photosynthesis combined with stimulation of an oxygenic photosynthesis (Cohen *et al.,* 1986), in which sulphide is an electron donor to photosystem I (PSI). The ability to conduct simultaneous oxygenic and anoxygenic photosynthesis allows for efficient CO_2 assimilation by cyanobacteria in environments that exhibit dual transitions

between oxic and anoxic conditions. Cyanobacteria that utilize this strategy include *Microcoleus chthonoplastes* and *O. amphigranulata* (Castenholz, 1984; Cohen *et al.,* 1986; Garcia-Pichel & Castenholz, 1990). The third strategy is the total inhibition of PSII (oxygenic photo synthesis) and stimulation of PSI to allow anoxygenic photosynthesis using sulphide as an electron donor (Miller & Bebout, 2004). An example of a cyanobacteria that exhibits the third strategy was found as *O. limnetica* (Cohen, 1984), which has been reclassified as *Geitlerinema* PCC9228 (Boone *et al.,* 2001).

Significance of sulphide oxidizing bacteria in aquatic systems

Aquatic vertebrates, invertebrates, aquatic plants and other microorganisms are also susceptible to environmental H_2S toxicity. Environmental and industrial H_2S pollution contributes to elevated ambient levels H_2S which could be both toxic to the individual as well as disrupt any physiological regulatory roles of the molecule. Some fish, such as rainbow trout, are susceptible to pathology conditions (reduce oxygen carrying capacity) from H_2S during virtually every life stage (Smith *et al.,* 1976) while other species of fish (i.e. carp) are more tolerant of elevated ambient H_2S levels (Volkel and Berenbrink, 2000). H_2S appears to have similar pathological cellular targets (e.g. Cytochrome c oxidase) and is toxic at similar concentrations to purified enzymes in fish as it is in mammals (Affonso *et al.,* 2002; Torrans and Clemens, 1982). Invertebrates and aquatic plants will also affect the concentration of hydrogen sulphide at biochemical as well as physiological levels as described in chapter one.

Hydrogen sulphide remediation is very necessary for the development of natural sustainability and health of aquatic systems. Generally, cyanobacteria have been used for the enhancement of soil

fertilization via nitrogen fixation in various aquatic systems (Valiente *et al.*, 2000). This is the new area of research mainly focuses on the bioremediation of H_2S by the action of cyanobacteria *Geitlerinema* sp. from various aquatic sediment systems.

The genus *Geitlerinema* (named in honour of Prof. Dr. Lothar Geitler) was first described by Anagnostidis (1989). Taxonomically, this genus has to be placed within the family *Pseudanabaenaceae* (subfamily *Pseudanabaenoideae*), within the order *Oscillatoriales*, in the class of the *Cyanophyceae*. The genus *Geitlerinema* currently comprises 33 species, but none of them has been validly published under the Bacteriological Code of Nomenclature.

Members of this genus are significant microbial mat-formers; especially found in the sulphide-rich microbial mat. (Meyers *et al.*, 2007). Meyers and co-workers found different species of *Geitlerinema* and *Leptolyngbya* strains prefer to grow in low light conditions and to carry out a sulphide-resistant oxygenic photosynthesis, which is relatively uncommon among cyanobacteria. This ability of *Geitlerinema* sp. is considered beneficial and can be developed as a novel technique for the bioremediation of sulphide using MCCB 234.

The present study mainly deals with purification and characterization of MCCB234, and an attempt has been made to develop it as bioaugmentor or bioremediator for the removal of sulphide from aquatic system.

4.2 Materials and methods

4.2.1 Purification of photosynthetic bacteria MCCB 234 from enrichment culture (GSB2)

The water samples were collected from sulphide-rich areas of Minicoy Island, Lakshadweep, India. Samples were enriched in Pfennig's medium prepared in 20 ppt salinity regimes and named as GSB2 (Manju,2007)

Refer Chapter 2: Section 2.2.2, Purification of photosynthetic sulphur bacteria by deep agar dilution method (Pfennig *et al.*, 1981).

4.2.2 Determination of Chlorophyll of MCCB 234 using Sucrose-mediated Absorption Spectra Measurement

Refer Chapter 2: Section 2.2.4.

4.2.3 Growth of MCCB 234 under different laboratory conditions based on the different nutritional parameters

Cyanobacteria can grow in different environmental conditions as described earlier. This experiment was truly focused on the growth condition of MCCB234 under laboratory conditions. Pfennig's medium was used as a basal medium for the experiment. In this experiment, four media combinations were prepared (Table 4.1, 4.2 and 4.3) in 20 ppt seawater as well as in distilled water. Three sets of culture conditions such as continuous light incubation, light/dark incubation and dark incubation were provided. Similarly, a few changes were made in media compositions to evaluate the growth under different cultural environment. A loopful of MCCB 234 was inoculated on each set of plates and one set plates each

were incubated under continuous light (1200 lux) (Table.4.5), light (1200 lux)/dark (Table.4.6), and dark **(**Table.4.7) conditions. (All the experiment were done in triplicate) After 25 days of incubation, the growth was monitored in terms of appearance of filamentous colonies on plates and visually documented.

Table.4.1 Continuous light

Sl.No	Media compositions
1	Pfennig's medium with distilled water + trace element solution+ Sodium Sulphide.
2	Pfennig's medium with sea water + trace element solution+ Sodium Sulphide.
3	Pfennig's medium with distilled water –trace element solution+ Sodium Sulphide.
4	Pfennig's medium with sea water – trace element solution+ Sodium Sulphide.

Table.4.2 Light and dark incubation

Sl.No	Media compositions
1	Pfennig's medium with distilled water+ trace element solution+ Sodium Sulphide.
2	Pfennig's medium with sea water+ trace element solution+ Sodium Sulphide.
3	Pfennig's medium with distilled water – trace element solution + Sodium Sulphide.
4	Pfennig's medium with sea water – trace element solution + Sodium Sulphide.

Table.4.3 Under dark incubation

Sl.No	Media compositions
1	Pfennig's medium with distilled water+ trace element solution+ Sodium Sulphide.
2	Pfennig's medium with sea water+ trace element solution+ Sodium Sulphide.
3	Pfennig's medium with distilled water – trace element solution + Sodium Sulphide.
4	Pfennig's medium with sea water – trace element solution + Sodium Sulphide.

(+ indicates included and -indicates exclude the compound in the medium)

4.2.4 Observation of growth of MCCB 234 without sodium sulphide and different nutritional conditions

The study of ecological conditions of MCCB 234 is important to understand its ability to grow in extreme environments and their role in the biogeochemical cycle. Thus, to understand their ability to grow in the absence of H_2S, various combination of Pfennig's medium without Na_2S was employed.

MCCB234 was inoculated in purified agar plates which was prepared based on various combinations of Pfennig's medium without Na_2S as described below (Table. 4.4). All plates were incubated at 28 °C for 25 days under light (1200 lux)/dark condition. After 25 days of incubation, the growth was observed visually on the plates and documented. (All the experiment were done in triplicate)

Table. 4.4 Culture medium and conditions

Sl.No	Media compositions
1	Pfennig's medium with distilled water + trace element solution
2	Pfennig's medium with distilled water – trace element solution
3	Pfennig's medium with sea water + trace element solution
4	Pfennig's medium with sea water – trace element solution
5	Pfennig's medium with distilled water + trace element solution –Acetate.
6	Pfennig's medium with distilled water – trace element solution + Acetate.
7	Pfennig's medium with sea water + trace element solution – Acetate.
8	Pfennig's medium with sea water – trace element solution + Acetate

(+ indicates included and -indicates excluded the specific compound in the medium)

4.2.5 Subculture of MCCB 234 into BG-11 medium for better growth

In the laboratory condition, the growth observed for MCCB 234 revealed that BG-11 medium (Rippka *et al*., 1979) prepared in 20 ppt has enhanced the growth of the organism when compared to that in Pfennig's medium. Accordingly, BG-11 was selected for further studies. Media composition is described below.

BG-11 Medium - composition

$NaNO_3$	1.5g
K_2HPO_4	0.04g
$MgSO_4 \cdot 7H_2O$	0.075g
$CaCl_2 \cdot 2H_2O$	0.036g
Citric acid	0.006g
Ferric ammonium citrate	0.006g
EDTA (disodium salt)	0.001g
Na_2CO_3	0.02g
Trace metal mix A5	1.00 ml
Agar (if needed)	10.00g
Distilled water	1.00 L

The pH should be 8-8.3, after sterilization and added 10ml/L Na_2S (3.25g/ml) in the medium.

Table. 4.5: Trace metal mix A5

H_3BO_3	2.86g
$MnCl_2.4H_2O$	1.81g
$ZnSO_4$	0.22g
$NaMoO_4.2H_2O$	0.39g
$CuSO_4$	0.079g
$Co(NO_3)_2.6H_2O$	49.4 mg
Distilled water	1.00 L

A loopful of inoculum was taken from agar plate and inoculated into plates with BG -11 medium in 20 ppt and the final pH was adjusted between 8 and 8.3. The plates were incubated under light and dark condition as described earlier. Repeated subculturing on BG-11 medium was conducted for the maintenance of the culture under laboratory conditions.

4.2.6 Optimization of physical conditions of MCCB 234 by one factor at a time method.

Optimization of growth condition was carried out in 30 ml screw cap tubes with the BG-11 medium.

4.2.6.1 Optimization of Salinity

The BG-11 medium was prepared at 30 ml aliquots at different salinities such as 0, 5, 10, 15, 20, 25, 30, 35 and 40 ppt by the addition of NaCl (g L^{-1}) to distilled water. pH was adjusted to 7.2 with sterile 1N HCl and 1N NaOH. The tubes were inoculated with 0.1ml of filamentous cell suspension having an absorbance of 0.1 at 730 nm (Zha *et al.*, 2012) and incubated at RT for 15 days in a photobioreactor having 1200 lux unit.

Growth was measured as absorbance at 730 nm using a UV-Vis spectrophotometer (Shimadzu UV-1601, Shimadzu Corporation, and Tokyo Japan). Growth was measured in terms of biomass and standardization of biomass by 'packed cell volume method' as described in Chapter 2 and 3 was undertaken. Data were statistically analysed using ANOVA.

4.2.6.2 Optimization of pH

BG-11 medium was prepared in 30 ml aliquots in 20 ppt sea water pH was adjusted to 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 using 1N HCl and 1N NaOH, inoculated 0.1 Abs_{730} culture into each respective bottles and incubated at RT for 15 days in a photobioreactor and growth was measured as described earlier. Data were statistically analysed using ANOVA.

4.2.6.3 Optimization of temperature

The BG-11 medium was prepared in 30 ml aliquots in 20 ppt seawater and pH adjusted to 7.2 with sterile 1N HCl and 1N NaOH and inoculated with the culture having 0.1 Abs_{730} as described previously. The tubes were incubated at different temperatures such as 5, 15, 20, 25, 30, 35, 40 and 45 ^{0}C in a temperature controlled illuminated orbital shaker (Scigenics Biotech) for 15 days. Data were statistically analysed using ANOVA.

4.2.7 Scanning Electron Microscopy of MCCB234

Karnoskey's method

The cells of filamentous photosynthetic sulphur bacteria MCCB 234 was centrifuged at 8000 rpm for 15 minutes, washed with PBS and again centrifuged at 8000 rpm for 5 minutes (Eppendorf, India, PVT Limited). These steps were repeated thrice. After centrifugation, 1 ml Karnoskey"s fixative was added and incubated at 4 ^{0}C overnight for prefixing. After

overnight incubation, it was washed with sodium cacodylate buffer. Centrifuged at 8000 rpm for 15 min (Eppendorf-Centrifuge 5418R) and discarded the supernatant and post-fixed with added 1 ml of 1% osmium tetroxide and 3 ml of sodium cacodylatebuffer stored at 4 ^{0}C for 3 hrs. Scanning electron microscopic analysis of samples was carried out at Sophisticated Test and Instrumentation Centre, CUSAT, Kochi.

4.2.8 Determination of hydrogen sulphide utilization of MCCB 234 by way of methylene blue spectrophotometric method (under laboratory condition).

Sodium sulphide was used as the substrate for the following analysis (Fonselius *et al.*, 1983).

When MCCB234 attained growth over a period of 15 days incubation in a photobioreactor under light/dark regime in the absence of sodium sulphide in the BG-11 medium, added 100 µl sodium sulphide (3.25g/100ml) to 30 ml culture. Mixed well and added 1 ml culture medium to 50 µl N-Dimethyl-P-Phenylene Diamine Dihydrochloride (4g/L) and 50 µl of the Ferric Chloride solution (16 g/L). The tube without culture was maintained as control. After the addition of reagents, the blue colour developed within a minute was read at 670 nm in a spectrophotometer (Shimadzu UV-1601, Shimadzu Corporation, Tokyo, Japan). After 24 hr incubation, the final reading was taken and calculated the percentage removal rate of the dye. Data were statistically analysed by way of ANOVA.

4.2.9 Molecular characterization of MCCB234

4.2.9.1 DNA isolation and amplification of 16S rRNA Sequence

(All chemicals and reagents used for this study, unless specifically stated otherwise, were purchased from Sigma-Aldrich, St. Louis, MO, USA).

Refer Chapter 2, section 2.17.1

4.2.9.2 Amplification of Internal Transcribed Sequence (ITS)

The ITS is the most widely sequenced region in bacterial DNA for taxonomic purposes. ITS (Internal Transcribed Spacer) refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. According to Laloui *et al.* (2002), ITS diversity was observed in different organism within the same Genus. Genomic DNA was amplified with ITS primer sequence ITS F 322(5'-TGTACACACCGCCCGTC-3') ITS R 340 (5'-CTCTGTGTGCCTAGGT ATCC-3') by DNA thermal cycler (Eppendorf, Germany). The product size was expected 700 bp. PCR mix included 15 µl deionized water, 2.5 µl 10X Thermopol buffer (New England Biolabs, USA), 1 µl 10 mM dNTPs, 2 µl Taq polymerase (New England Biolabs, USA), 1 µl 10 pmol primers and 120 ng DNA template in a final volume of 25 µl. The PCR condition was in the following manner, 94 ^{0}C for 5minitue followed by 30 cycles of 94 ^{0}C for 5 min and 55 ^{0}C for 1 min then 72 ^{0}C for 1 min and final extension was 72 ^{0}C for 10minutes. Amplified product was obtained at 700 bp and was separated by cutting the gel and purifying using Sigma gel extraction kit (Gen elute^{TM}, Sigma-Aldrich) and was documented (BIO-RAD-molecular Imager Gel DOC^{TM}, U.S.A) and sequenced at Scigenome Lab Pvt., Kochi, Kerala, India.

Gel purification of PCR product.

Gel purification was carried out using (Gen elute^{TM} Gel Extraction kit U.S.A Sigma Aldrich). For purifying the gene product, agarose gel that contained the DNA fragments of appropriate size was excised and taken in a 1.5ml tube, weighed and added 3 gel volume (approximately 450 µl) of gel solubilization solution and incubated at 60 ^{0}C for 10 minutes with repeated vortexing in every 2 min. After incubation, added 1 gel volume (approximately 150 µl) of 100% isopropanol, mixed gently until it became homogenous. This solubilizing gel solution was loaded in the binding column that was pretreated with column preparation solution, centrifuged at 12,000x *g* for 1 min. Added 700 µl wash solution and centrifuged for 1 min at 12000 x *g*, repeated the centrifugation, and residual wash solution was removed. The binding column was transferred into a fresh collection tube (2 ml MCT) and added 50 µl of preheated (at 65 ^{0}C) 10 mM Tris-HCl (pH 9.0), centrifuged at 12000 x *g* for 1 min, stored at $^{-}$20 ^{0}C. The concentration of DNA was measured spectrophotometrically at 260/280nm in a UV-VIS spectrometer (U2800, Hitachi, Japan). The purified PCR product of 229bp PUFM gene was sequenced at Scigenome Lab Pvt., Kochi, Kerala, India.

4.2.9.3 Amplification of cpcBA-Intergenic Spacer Sequence for the molecular identification of MCCB 234

The cpcBA-IGS, code for phycocyanin in cyanobacteria, including the highly variable Intergenic spacer (IGS) region which covers the terminal end of the cpcB gene and the proximal end of the cpcA gene (Neilan *et al.*, 1995), has exhibited high levels of sequence variation and is therefore a potentially effective molecular marker for differentiation of cyanobacterial strains (Neilan *et al.*, 1995; Bolch *et al.*, 1996).

Genomic DNA was amplified with cpcBA-IGS primer sequence F-TAGTGTAAACGACGGCCAGTTGYYTKCGCGACATGGA and R-TAGCAG GGAAACAGCTATGACGTGGTGTARGGGAAYTT by DNA thermal cycler (Eppendorf, Germany). The product size was expected 600 bp. PCR mix included 15 µl deionized water, 2.5 µl 10X Thermopol buffer (New England Biolabs, USA), 1 µl 10 mM dNTPs, 2 µl Taq polymerase (New England Biolabs, USA), 1 µl 10 pmol primers and 120 ng DNA template in a final volume of 25 µl. The PCR condition was in the following manner, 98 ^{0}C 10 sec, followed by 34 cycles of 98 ^{0}C for 10 sec, 54 ^{0}C for 30 sec, 68 ^{0}C for 1 min, final extension at 72 ^{0}C for 10 min. Amplified product 600 bp was documented (BIO-RAD-molecular Imager Gel DOC^{TM}U.S.A) and sequenced at Scigenome Lab Pvt., Kochi, Kerala, India.

4.2.9.4 Amplification of Rbc-LX gene from MCCB 234 photosynthetic bacteria.

Ribulose -1, 5 bisphosphate Carboxylase Oxygenase or Rubisco is one of the most abundant enzyme responsible for catalyzing CO_2 assimilation to organic carbon via Calvin cycle. It was found in many proteobacteria, cyanobacteria, algae and higher plants. Rubisco consists of hexadecamor consisting of eight large subunits and eight small subunits, assembling in to an L_8 S_8 holoenzyme.

As an evolutionary concept, Rbc-LX is suitable for the study of phylogeny and is often used as a phylogenic investigation (Mondal *et al.*, 2013). Apart from 16SrRNA, ITS and cpcBA-IGS, phylogenetic identification of MCCB234, Rbc-LX gene was used for confirming the isolate of MCCB234 more scientifically. Information of Rbc-LX primer sequence was obtained from Rajaniemi *et al.*, 2005.

Genomic DNA was amplified using Forward CX (GGCGCAGGTA AGAAAGGGTTTCGTA and Reverse CW (CGTAGCTTCCGGTGGTAT CCACGT) primers of Rbc-LX by thermal cycler (Eppendorf, Germany). The product size was expected 900 bp. PCR mix included 15 µl deionized water, 2.5 µl 10X Thermopol buffer (New England Biolabs, USA), 1 µl 10 mM dNTPs, 2 µl Taq polymerase (New England Biolabs, USA), 1 µl 10 pmol primers and 120 ng DNA template in a final volume of 25 µl. The PCR condition was in the following manner, 98 ^{0}C 10 sec, followed by 34 cycles of 98 ^{0}C for 10 sec, 54 ^{0}C for 30sec, and 68 ^{0}C for 1 min, and final extension at 72 ^{0}C for 10 minutes. Amplified product 900bp was documented (BIO-RAD-molecular Imager Gel DOC^{TM}U.S.A) and sequenced at Scigenome Lab Pvt., Kochi, Kerala, India.

16SrRNA, ITS and cpcBA-IGS, and Rbc-LX genes sequence similarity and phylogenetic analysis.

The sequence obtained was matched with Genbank database using the BLAST search algorithm (Altschul *et al.,* 1990). All the gene sequence of MCCB 234 were multiple aligned using the ClustalW algorithms (Thompson *et al.,* 1994). Phylogenetic tree of all the genes were constructed by the bootstrap method (Saitou and Nei, 1987) using the software MEGA 6 (Tamura *et al*., 2007)

4.2.9.5 Amplification of sulphide oxidizing genes SOX from MCCB234.

SOX gene is a periplasmic protein and considered as a key gene which catalyses the oxidation of sulphur compounds such as sulphide, thiosulphide sulphite-dependent cytochrome "C" reduction *in vitro* (Appia-Ayme *et al.,*2001).Genomic DNA amplified with SOX primer 5'forward AAAATCTAGACCAATACCGTGAAAGTCACCATCGGCGGCT-3' and

5'Reverse AAAAGGATCCAGATCTCGCGGCCCTTCTCCCAGG TCGA CT-3 by thermal cycler (Eppendorf, Germany) (Appia-Ayme *et al.*, 2001). The product size was expected to be 300 bp. PCR mix included 15 µl deionized water, 2.5 µl 10X Thermopol buffer (New England Biolabs, USA), 1 µl 10 mM dNTPs, 2 µl Taq polymerase (New England Biolabs, USA), 1 µl 10 pmol primers and 120 ng DNA template in a final volume of 25 µl. The PCR condition was in the following manner, 98 ^{0}C for 30 sec followed by 34 cycles of 98 ^{0}C for 10 sec, 54^{0}C for 30 sec, 68 ^{0}C for 1min and a final extension at 68. ^{0}C Amplified product 300 bp was documented (BIO-RAD-molecular imager Gel DOC^{TM}U.S.A) and sequenced at Scigenome Lab Pvt., Kochi, Kerala, India.

SOX gene sequence similarity analysis

The sequences obtained were matched with Genbank database using the BLAST search algorithm (Altschul *et al.*, 1990). All the gene sequence of MCCB234 were multiple aligned using the ClustalW algorithms (Thompson *et al.*, 1994). Phylogenetic tree of SOX genes were constructed by (Saitou and Nei, 1987) using the software MEGA 6 (Tamura *et al*., 2007.).

4.2.10 Determination of sulphide removal potential of photosynthetic bacteria MCCB 234 from aquatic sediment system. (Simulated microcosm study)

Preparation of MCCB 234 biomass

MCCB234 biomass was generated in the laboratory condition using BG-11 medium with pH 8. A loopful of MCCB 234 culture was aseptically transferred into the medium and incubated at RT (Room Temperature) for 15 days under light/dark condition. After the incubation period, cell biomass

was divided into three portions on the basis of optical density as 0.1 OD, O.3 OD and 0.5 OD. The experiment comprised three phases.

First phase of experiment

Black sediment was brought from a pond at Arookuty, Cherthala district, Kerala. The sample was allowed to settle in an FRP tank for a couple of weeks. Thereafter the initial hydrogen sulphide in the sediment system was measured by way of methylene blue spectrophotometry.

Weighed out approximately 150g of black sediment into 3.5 L capacity plastic bottles containing 1.5 L of seawater with 25 ppt salinity. The water quality parameters such as ammonia, E*h*, pH, nitrate and nitrite (Grasshoff *et al.*, 1999) of the system were analyzed before inoculation. The cell suspension (10 ml) of MCCB234 (25 ppt) at three optical densities (0.1, 0.3, 0.5) were applied to the bottles provided with 1200 lux light as measured by lux meter (PCE-EM886). The bottles without inoculum were taken as control and triplicates were kept for all the bottles. They were tightly closed using polythene transparent cover for providing anaerobic conditions and incubated for 4 days for the acclimatization of the culture with the environment. After incubation, 5ml of 3.25 g /100 ml concentration of sodium sulphide was added to all the tubes and calculated the initial concentration (0 hr) of H_2S by methylene blue spectrophotometry. Further, the removal rate of H_2S of test samples was checked from 1st to 8^{th} day of the experiment by comparing with the control.

2^{nd} phase of experiment

The water quality parameters were again checked before starting the second phase. An adequate volume of hydrogen sulphide and cell

suspension of MCCB234 could be added to the test bottles depending on the sulfide removal efficiency. The experiment could be continued same as the first phase for a week for checking the removal potency.

3^{ed} phase of experiments

Before starting the third phase, water quality parameters were again measured. Depending on the removal efficiency, inoculum and hydrogen sulphide could be added accordingly. Then the experiment proceeded for one more week as the other two phases.

4.2.11 Water quality parameters

The methodology followed for the estimation of ammonia, nitrate, nitrite, E*h* and pH was the same as described in chapter 2 (section 2.2.18).

4.3 Results

4.3.1 Purification of photosynthetic bacteria MCCB 234

Around 2 years were taken to purify sulphur bacteria from GSB2 consortium. Among the different colonies developed in each tube, one of them was picked from lower dilution by Pasteur pipette and purity was further confirmed microscopically. Purified culture was deposited in MCCB culture collection at National Centre for Aquatic Animal Health, Cochin University Science and Technology and named as MCCB 234.

4.3.2 Determination of Chlorophyll pigments of MCCB 234 by using Sucrose-mediated Absorption Spectra Measurement.

Bacteriochlorophylls have characteristic absorption maxima at specific wavelength, (nm) bcl (Bacteriochlorophyll) “a” (375,590,628,805,830-890), bcl “b” (400-439, 605, 840, 1020-1040) bcl “c”

(745-755) bcl "d" (725-745) and bcl "e" (710-725). In the present study, the peak obtained in the absorption spectra of MCCB 234 was 628nm and 438nm (Fig.4.2) and could be similar with bcl "a" (phycocyanin) and bcl "b" respectively.

4.3.3 Growth of MCCB 234 under different laboratory condition based on the different nutritional parameters

The growth of MCCB 234 under different nutritional conditions was analysed and it could survive with minimum nutrition. High growth was observed in agar plate which was incubated in light and dark compared with that incubated under continuous light. One important observation was that no growth was visually observed under the plates when incubated under dark. Based on the above observations, photosynthetic bacteria MCCB 234 was found to have light essential for its survival (Table 4.6, Table 4.7 and Table 4.8).

Table 4.6

Continues light.	**Results**
Pfennig's medium with distilled water + Trace element solution + Sodium Sulphide	Moderate growth
Pfennig's medium with sea water + Trace element solution + Sodium Sulphide	Moderate growth
Pfennig's medium with distilled water – Trace element solution + Sodium Sulphide	Moderate growth
Pfennig's medium with sea water – Trace element solution + Sodium Sulphide	Moderate growth

("+" indicates include and "-"indicates exclude the compound in the medium)

Table 4.7

Light/dark incubation	Results
Pfennig's medium with distilled water + Trace element solution + Sodium Sulphide	High growth
Pfennig's medium with sea water + Trace element solution + Sodium Sulphide	High growth
Pfennig's medium with distilled water – Trace element solution + Sodium Sulphide	High growth
Pfennig's medium with sea water – Trace element solution + Sodium Sulphide	High Growth

("+" indicates include and "-"indicates exclude the compound in the medium)

Table 4.8

Incubation at dark	Results
Pfennig's medium with distilled water + Trace element solution + Sodium Sulphide	No growth
Pfennig's medium with sea water + Trace element solution + Sodium Sulphide	No growth
Pfennig's medium with distilled water – Trace element solution + Sodium Sulphide	No growth.
Pfennig's medium with sea water – Trace element solution + Sodium Sulphide	No growth

("+" indicates include and "-"indicates exclude the compound in the medium)

4.3.4 Observation of Growth of MCCB 234 without sodium sulphide and different nutritional conditions

In the present study MCCB 234 was found to survive without sodium sulphide and concluded that it was a facultative hydrogen sulphide oxidizer. Based on the growth obtained from different nutritional conditions, it was again confirmed that MCCB 234 required only minimum nutritional conditions (Table.4.9).

Table.4.9 Growth of MCCB 234 without sodium sulphide and different nutritional parameters

Pfennig's medium without Sodium sulphide.	**Results**
Pfennig's medium with distilled water + Trace element solution	Growth observed
Pfennig's medium with distilled water – Trace element solution	Growth observed
Pfennig's medium with sea water + Trace element solution	Growth observed
Pfennig's medium with sea water –Trace element solution	Growth observed
Pfennig's medium with distilled water + Trace element solution – Acetate	Growth observed
Pfennig's medium with distilled water – Trace element solution + Acetate.	Growth observed
Pfennig's medium with sea water + Trace element solution – Acetate.	Growth observed

("+" indicates include and "-"indicates exclude the compound in the medium)

4.3.5 Subculture of MCCB 234 into BG-11 medium for better growth

The BG-11 medium could support better growth as compared to Pfennig's medium (Pfennig *et al*, 1981). It took 25-30 days for attaining growth of MCCB234 in Pfennig's medium (Fig.4.3) however, in BG-11 medium it took only 10 days. (Fig.4.4). Hence BG-11 medium was selected for further analysis (Fig.4.3).

4.3.6 Optimization of physical conditions of MCCB 234 by one factor at a time method.

Biomass was calculated by µg/ml by packed cell volume method as same as MCCB146 and MCCB147 and observed factor value was 5.68. (Fig.4.5) (Refer chapter 2, 2.2.7 Absorbance Vs Biomass by determining packed cell volume).

4.3.6.1 Salinity preference of MCCB 234

The organism MCCB 234 exhibited significant growth in different salinity ranging from 10ppt to 40ppt. Maximum growth was obtained with 20 ppt salinity and minimum growth at 40 ppt (Fig.4.6). Statistically analysed the variations of growth under different salinity range by ANOVA (P <0.05) and found to be highly significant. The growth was considered as biomass µg/ml.

4.3.6.2 pH preference of MCCB 234

The present study clearly demonstrated that the isolate, MCCB234 exhibited growth in different pH ranging from 6 to 13 and the higher growth was observed at pH 7. Acidic pH was not preferred by the organism and alkaline condition was found to be the most preferable for it. An important observation is that it can survive under extreme pH (13) and concluded that it was an alkaliphilic organism and adapted to survive at high pH. The result was shown in (Fig.4.7). The growth was considered as biomass µg/ml. The variations in growth with different pH were statistically analysed and found to be more significant (P < 0.05).

4.3.6.3 Temperature preference of MCCB 234

The favourable temperature for the growth of MCCB234 was found to be 35 ^{0}C and the growth was found within 3 days of incubation (experiment time was fixed as 15 days). MCCB 234 exhibited growth at different temperature ranging from 20 to 35 ^{0}C (Fig.4.8). Growth was considered as biomass µg/ml. The variations in growth at different temperature were statistically analysed and found to be highly significant ($P < 0.05$).

4.3.7 Scanning Electron Microscopy of MCCB 234.

Morphological analysis of MCCB234 was carried out by scanning electron microscopy and demonstrated the organism filamentous embedded in thick matrix or biofilm having no heterotrophic bacteria entangled (Fig. 4.9). Filaments were unbranched, rarely solitary, usually more or less parallel oriented in fine, smooth, trichrome tip slightly tapered and bent, trichrome formed of cylindrical cells and characterized by distinct constrictions at the cross walls (Fig.4.10 and Fig.4.12) composed of cylindrical cells, elongated embedded in the matrix (Fig.4.11).

4.3.8 Determination of hydrogen sulphide utilization ability of MCCB 234 by methylene blue spectrophotometric method (under laboratory condition).

The hydrogen sulphide removal efficiency of MCCB 234 was analyzed in laboratory condition by methylene blue spectrophotometric method. Significant reduction of hydrogen sulphide was observed for the test sample as compared to the control within 24 hrs of the experiment. The removal efficiency of test sample has been calculated as 62%. (Fig.4.13)

The concentration of hydrogen sulphide was found to be decreasing in the control sample also. Pointing to the fact that the gas it getting lost on opening the bottle. This formed the primary level screening for checking the hydrogen sulphide removal efficiency. After statistical analysis by single factor ANOVA, reduction of sulphide after 24 hrs by MCCB234 was found statistically significant ($P < 0.05$).

4.3.9 Molecular characterization of MCCB 234

4.3.9.1 DNA extraction, 16Sr RNA gene amplification and sequencing.

DNA was isolated (Fig. 4.14) and amplified using universal primers and resulted in the PCR product of 1500 bp (Fig. 4.15). The sequences of 16S rDNA region when matched with the GenBank database, it showed 88% similarity with *Geitlerinema* sp. The phylogenetic tree constructed by using MEGA 6 (Tamura *et al*., 2007) is shown in Fig.4.16. The nucleotide sequences determined in this study were deposited in GenBank database of NCBI (www.ncbi.nlm.nih.gov) and assigned the accession number KP019934.

4.3.9.2 Amplification and sequencing of Internally Transcribed Spacer region (ITS).

For the identification of MCCB 234 isolate, ITS region was amplified with a product size of 700 bp (Fig 4.17). The sequence was submitted to Genbank and assigned the accession number KU 761995. The sequence from the ITS region when matched with the GenBank database showed 100% similarity to *Geitlerinema* sp. 7105 16S-23S ribosomal RNA intergenic spacer complete sequence. The phylogenetic tree was constructed

with the aligned sequence of ITS and similar sequences from Genbank database (Fig 4.18).

4.3.9.3 Amplification of cpcBA-Intergenic Spacer Sequence from MCCB 234

For the identification of MCCB234 isolate, cpcBA intergenic spacer sequence was amplified with a product size of 600 bp (Fig 4.19). The sequence was submitted to Genbank and assigned the accession number KF-494336. The sequence results of cpcBA-IGS were multiple aligned using the ClustralW algorithm (Thompson *et al.,* 1994). The phylogenetic tree was constructed by (Saitou and Nei. 1987) using MEGA 6 (Tamura *et al.,* 2007) (Fig.4.20). cpcBA intergenic spacer sequence of MCCB234 was showing 96% similarity with cyanobacteria *Geitlerinema* sp. phycocyanin subunit.

4.3.9.4 Amplification of Rbc-LX-Rubisco largest subunit for the identification of MCCB 234

For the identification of MCCB234 isolate, Rbc-LX-Rubisco largest subunit gene was amplified resulting in a product size of 900 bp (Fig 4.21). The sequence was submitted to Genbank and assigned the accession number KX.784510. The sequence results of Rbc-LX-Rubisco largest subunit was multiple aligned using the ClustralW algorithm (Thompson *et al*., 1994). The phylogenetic tree was constructed by the method (Saitou and Nei. 1987) using MEGA 6 (Tamura *et al.,* 2007) (Fig.4.22). Rbc-LX-Rubisco largest subunit gene of MCCB234 was showing 99% similarity with hypothetical protein (Rbc-LX) *Geitlerinema sp.*

4.3.9.5 Amplification of sulphide oxidizing genes from photosynthetic bacteria MCCB 234.

SOX-like gene was amplified at 300 bp (Fig.4.23) and the nucleotide sequence which could be similar within the SOXD (operon) gene of *Rodovulum sulphidophilum* with 76% similarity but query coverage was comparatively very less (Fig. 4.24). The sequence results of SOX like gene was multiple aligned using the ClustralW algorithm (Thompson *et al.,* 1994). The phylogenetic tree was constructed by the method suggested by Saitou and Nei (1987) using MEGA 6 (Tamura *et al.,* 2007).

4.3.10 Determination of sulphide removal potential of photosynthetic bacteria MCCB 234 from aquatic sediment system. (Simulated microcosm study)

1st phase of experiment

Initially, concentration of H_2S in the sediment systems was found to be negligible. Hence the appropriate volume of H_2S has been provided in both test and control systems. Within 24 hrs of incubation, the concentration of H_2S was found to get elevated in control samples as compared to that of the first day. This might be due to the generation of hydrogen sulphide by the indigenous action of sulphate-reducing bacteria prevailed in the sediment because of the anaerobic conditions. Meanwhile, the concentration of H_2S in the test systems got diminished or sulphide reduction values were more or less equal to zero. As the experiment progressed, the amount of H_2S found to get decreased and finally reached equilibrium on the final day of the first phase (Fig.4.25) indicating H_2S removing potency of the isolate. However, reduction of H_2S was seen in the control also after two days of

incubation suggesting that the sulphide oxidizing organisms already present in the sediment has brought about the change. The assay was continued for further 5 days without changing the conditions (Fig.4.26) and the removal was 100% (Fig.4.27) by which the organism could be designated as an efficient sulphide bioaugmentor for aquatic system. The extend of removal of H_2S in the test samples was significant during the first 8 days (P <0.05) and the last 5 days (P < 0.05).

Second phase of experiment

During the initial stage of the second phase, the concentration of H_2S in the test systems was recorded zero due to sulphide removal potency of the isolate. Hence on the 13th day, an adequate volume of Na_2S was provided to the test systems for further testing. Since sulphide was not removed from the control systems in the first phase, Na_2S was not supplied in control. As the removal rate was getting reduced in the second phase compared with that of the first phase (Fig.4.28), on the 15th day of experiment, a second dose of inoculum of MCCB 234 was supplied with respective optical density into each respective test systems. Next day onwards the concentration of H_2S was found to be gradually declining in the test and the experiment was continued for further 4 days till the concentration of H_2S turned zero (Fig.4.29) (P< 0.05)

Third phase of experiment

In the 3rd phase of the experiment, the concentration of H_2S remained at zero level in the test systems and elevated level in the control sample as in the case of the second phase. Hence on 20th day appropriate volume of sodium sulphide was provided to the test systems to proceed the experiment (Fig. 4.30). Very efficient sulphide removal or sulphide oxidation was

observed for all the test systems and sulphide concentration remained intact in the controls. The percentage of sulfide removal was calculated and expressed graphically (Fig.4.31). Statistical analysis by ANOVA confirmed the significant sulphide removal potency of test samples ($P < 0.05$).

4.3.11 Water quality – Physico -chemical analysis

Even though the ammonia concentration in the test and control system was higher, (Fig. 4.32) it declined from 7th day on wards and reached zero indicating significant ammonia removal efficiency of the system. ($P < 0.05$). The same was the trend with respect to nitrite nitrogen (Fig. 4.33) ($P < 0.05$).

According to Fig. 4.34, concentration of nitrate was higher during the initial days and was found to get gradually declining. ($P < 0.05$)

pH variations during the experiment in both the systems were insigificant (Fig.4.35) ($P > 0.05$).

Redox potential or E*h*, was found to be higher in the tests compared to that in the control (Fig. 4.36). ($P < 0.05$).

4.4 Discussion

Purification of filamentous photosynthetic bacteria MCCB 234 from heterotrophic bacteria and establishment of axenic culture was a tedious, time-consuming process. Purification of MCCB 234 was carried out by deep agar dilution method as discussed earlier and finally obtained filamentous green colonies on Pfennig's soft agar. Millach *et al.* (2015) and Burgos *et al.*, (2013) reported that *Geitlerinema* sp. could be successfully isolated and grown in Pfennig's medium. Ferris & Hirsch (1991) reported that purification and cultivation of cyanobacteria were often arduous and time-consuming to

produce axenic cultures. Various approaches have been taken so far to develop more-efficient methods to purify and cultivate cyanobacteria. However, in our study, BG-11 medium in addition to Pfennig's medium yielded better results. Microscopical examination of the isolate MCCB 234 demonstrated its morphological appearance which seemed too similar to cyanobacteria.

The absorption maxima of MCCB 234 determined by sucrose-mediated absorption method at 628nm corresponded to bcl "a" and bcl "b". The characteristic peak of *Geitlerinema* sp. at 628nm indicated the presence of phycocyanin pigment of Phycobiliproteins (Parmar *et al.*, 2010). Phycocyanin is the most commonly found pigment in *Geitlerinema* sp. and is involved in electron excitation for PS2 photosystem and a similar observation was reported by Anuja & Manjushri (2013). Phycocyanin is a blue colour photosynthetic accessory pigment that absorbs light at about 620 nm and emits fluorescence at about 640 nm and predominantly seen in cyanobacteria species (Gantar *et al.*, 2012; Jodlowska & Latala, 2013). All data demonstrated that the isolate comprised phycocyanin pigment for PS2 photosystem.

In the present study, growth of *Geitlerinema* sp. under different physiological and nutritional conditions indicated that light/dark incubation enhanced the growth compared to continuous light alone and no growth was observed under dark conditions. Accordingly, MCCB234 could be confirmed as photosynthetic and required light for growth. While, Sánchez - Alejandro *et al.* (2014) observed that photoautotrophic members of the genus *Geitlerinema* sp. exhibited growth under any diverse environmental conditions. Besides, Diamond *et al.* (2017) stated that photosynthetic cyanobacteria under 24 hrs cycles of light and darkness (Circadian clock)

were found very essential for its survival. Thus, Cyanobacteria integrate circadian clock signals with natural light/dark cycles to control and regulate the phycobilisome or phycocyanin pigment production.

Anoxygenic cyanobacteria generally use sulfide as the electron donor for sulphide mediated photosynthesis. A recent study (Grim& Dick, 2016) reported that *Geitlerinema* sp. PCC 9228, could switch between oxygenic photosynthesis and sulfide-based anoxygenic photosynthesis. Apart from that Myers *et al.* (2008) stated that the ability of *Geitlerinema* sp. to perform simultaneous oxygenic and anoxygenic photosynthesis permits efficient CO_2 assimilation in environments that exhibit duel transitions between oxic and anoxic conditions. Cyanobacteria that utilize this strategy included *Microcoleus chthonoplastes* and *O. amphigranulata* (currently reclassified as *Geitlerinema).* Facultative anoxygenic CO_2 photoassimilation using sulfide as an electron donor, in addition to the oxygenic "plant-type" photosynthesis, has recently been demonstrated in many different cyanobacteria. Detailed studies of anoxygenic photosynthesis in *O. limnetica* (*Geitlerinema* sp.) showed that sulfide is oxidized to elemental sulfur in the reaction driven solely by photosystem I. The present work was focussed on to study the oxygenic and anoxygenic photosynthetic efficiency of *Geitlerinema* sp., it was found that MCCB 234 was able to carry out photosynthesis under both oxygenic and anoxygenic conditions.

Grim & Dick (2016) reported that *Geitlerinema* sp. as a model organism for studying the physiology of flexible anoxygenic and oxygenic photosynthesis. Accordingly, in this study, metabolic flexibility in response to varying nutritional conditions in-vivo and could be found, and *Geitlerinema* sp. was highly versatile which could survive under minimum

nutrient conditions. Miazek *et al.* (2015) also found that cyanobacteria were able to synthetize numerous compounds in higher amounts, as a response to stress conditions such as high temperature, high salinity, nutrient starvation, and also metal stress.

Geilterinema sp. is a cosmopolitan cyanobacterium that is highly adapted to natural and artificial environments and can withstand extreme conditions of salinity and pH (Rampelotto, 2013). Salinity of water in the aquatic environment is an important physical factor that affects growth and development of living organisms. The acquisition of salt tolerance in *Geitlerinema* sp. is associated with decrease in biomass (Chandra, 2016). The present study also showed that salinity stress (up to 40 ppt) enhanced the biomass.

Geitlerinema sp. is an alkalophilic organism as reported by Sánchez-Alejandro *et al.* (2015). The present study revealed that *Geitlerinema* sp. MCCB 234 exhibited significant growth at pH 7, 8, 9 and 10 and also survived at pH 13.0 and hence classified as extremely alkaliphilic. Negligible growth could be observed at pH 6 also which might be due to the adaptability of *Geitlerinema* sp. under stressed conditions.

Geitlerinema sp. was predominant in the winter season at 30-35 ^{0}C, (Sánchez-Alejandro *et al.,* 2015). Our study also indicated that the optimum temperature of *Geitlerinema* sp. was 35 ^{0}C, and an upper growth temperature limit was 35 ^{0}C as reported by Anuja& Manjushri, 2013.

Traditionally, the classification of cyanobacteria is based on morphological characteristics such as trichome width, cell size, division planes, shape and arrangement, pigmentation and the presence of characteristics such as gas vacuoles and a sheath. Scanning electron

microscopic image of MCCB 234 demonstrated the presence of trichome and its slightly tapered and bent tip structure, characteristic features of *Geitlerinema* sp. as reported by Richert *et al.* (2006). Apart from this, trichomes of *Geitlerinema* sp. MCCB 234 formed cylindrical cells characterized by distinct constrictions at the cross walls as observed in *Geitlerinema* sp. PCC 850 (Moro *et al.*, 2010).

Molecular identification of MCCB 234 using 16S rRNA gene sequences showed 88% similarity to *Geitlerinema* sp. Functional gene sequence analyses using ITS, RbcLX and cpcBA IGS were also employed for the identification of MCCB 234.

cpcBA IGS is the conserved intergenic spacer (IGS) between cpcB and cpcA, and corresponding flanking regions henceforth referred to as cpc BA-IGS (Crosbie *et al.*, 2003). Molecular identification based on cpcBA IGS of MCCB 234 exhibited 96% similarity to *Geitlerinema* sp. RbcLX genecodes for the large subunit of ribulose 1, 5-bisphosphate carboxylase oxygenase of the Calvin cycle and it contains a conserved region, which can be used for molecular identification of cyanobacteria (Tomitani *et al.*, 2006). In the present study, RbcX which is in the intergenic space between the large (rbcL) and small (rbcS) subunit genes of Rubisco 1, 5-bisphosphate carboxylase oxygenase (Onizuka *et al.*, 2004) could be amplified and exhibited 99% similarity to RbcX gene of *Geitlerinema* sp.

ITS-based identification technique has been used for phylogenetic analyses of strains of photosynthetic eubacterial genera. Operons containing the genes coding for the three rRNAs (16S, 23S, 5S) and their associated ITS regions are normally present in multiple copies in the bacterial genome. (Boyer *et al.*, 2001). In this study, amplification of ITS region yielded two

copies at 1000 bp and 700bp. Only 700 bp bands were eluted, sequenced and analysed. ITS internal transcribed region of MCCB 234 exhibited 100% similarity to *Geitlerinema* sp. PCC 7105 16S-23S ribosomal RNA intertrancribed spacer complete sequence.

Generally, cyanobacteria are able to switch between anoxygenic and oxygenic photosynthesis which are found exclusively in systems such as phototrophic microbial mats, where they are regularly exposed to sulphide, eg, *Geitlerinema* sp. and, *Pseudanabaena* (Klatt *et al.,* 2015). SOX protein is a periplasmic protein which catalyses the oxidation of sulphur compound. The importance and function of SOX gene was described and suggested that the gene region of *P. pantotrophus* encoding sulphur oxidizing ability comprises 15 genes, of which seven, SOX X,Y,Z,A,B,C,D, encode the periplasmic proteins SOX Y, Z, SOX B, SOX C,D and SOX X, A which catalyse hydrogen sulfide-, sulfur-, thiosulfate- and also sulphite-dependent cytochrome c reduction *in vitro* (Rother *et al.,* 2008). Another study conducted by Yamamoto *et al.,* 2011 state that SOX gene performed sulphur oxidation pathway at neutral pH in *Proteobacteria.* SOX gene and its metabolism in sulphur oxidizing pathways in various sulphur bacteria now has been reported (Friedrich *et al.,* 2001, Meyer *et al.,* 2007, Friggard *et al.,* 2008a, Sauve *et al.,* 2007, Sukurai *et al.,* 2010, Headd *et al.,* 2013 and Mattes *et al.,* 2013). However, SOX similar gene could be identified in *Geitlerinema* sp. and it could be 76% similar with the SOX gene of *Rodovulum sulphidophilum*. Hence, the present study proved that the isolate could perform oxygenic or anoxygenic photosynthesis depending upon the availability of sulphide, using PS1 or PS2 photosynthetic units simultaneously. Besides, after translation of SOX protein by expasy software, the result could be similar with the "Fe" containing hypothetical

protein of *Geitlerinema* sp. and participating photosynthetic pathways. SOX gene was also a "Fe" containing protein and participating H_2S mediated photosynthesis. However in the present status, still there was no Genebank submission of SOX gene of *Geitlerinema* sp. Based on above mentioned results, we concluded that it might be SOX like gene of *Geitlerinema* sp.which participated in the sulphide mediated photosynthesis. However, further work is needed for its confirmation at molecular level. Apart from that, presence of SOX gene in *Geitlerinema* sp. is not yet reported anywhere else, and this statement is substantiated with the support of NCBI databank and from the literature. Therefore, this study could be considered the first report of a gene in connection with oxidation of sulphide in *Geitlerinema sp*. Moreover, in connection with sulphide removal and SOX gene, very limited literature is available now. Meanwhile, oxidation of sulphide to thiosulfate in *Geitlerinema* sp. and its sulphide removal efficiency have been reported by Klatt *et al.* (2016). Apart from this contribution, Jorgensen 1986 reported anoxygenic photosynthesis in *Geitlerinema* sp., and under this circumstances MCCB234 *Geitlerinema* sp. could be designated to perform oxygenic and anoxygenic photosynthesis simultaneously.

Sulphide oxidation in soil is mediated primarily by microorganisms, sulphide concentration, and composition of sediment (organic and inorganic components) and activity of the microbial community which dictate oxidation rates. Sulphide oxidation is a biological process, it is strongly influenced by factors directly affecting microbial activity including soil temperature, pH, E*h*, nitrate, nitrite and ammonia etc. In many soils, these factors represent the primary constraints to sulphide oxidation. Oxidation of sulphide is also influenced by the effective surface area of the sulphide exposed to microbial activity. Thus oxidation is favoured by the adequate

concentration of sulphide and abundant populations of sulphide oxidizing bacteria were present in sediment for capable of oxidizing Sulphide. Thus the availability of organic substrates from sediment or root exudates may also affect Sulphide oxidation (Germida *et al.,* 1993). Oxidation of sulphide in sediments is depended upon several factors as described earlier, other than these factors some chemical factors also influence sulphide oxidation eg. "Cell permeability". It should be possible to transform chemical energy from sulphide into an electrochemical potential across a membrane called proton motive force (pmf). This pmf is then used to drive the synthesis of ATP via membrane-bound enzymes, the ATPases and this energy is used for cellular metabolisam (Nealson, 1997). Based on this observation, it is postulated that during anaerobic condition the organism become more permeable to sulphide assimilating it for energy. Sulphide oxidation is not a single step, and several organisms are involved making it the most complex pathway in the biogeochemical cycles. Three kinds of micro-organisms play their role in sulphur cycle, autotrophic bacteria, such as those oxidizing sulphur from sulphide to sulphate, the sulphate-reducing bacteria which need sulphur in their metabolism and to get it reduced to sulphide even though it requires considerable energy; and the very important group of heterotrophic bacteria which utilize organic compounds and, as such, serve as the ultimate source of energy for nearly all bacteria. The special groups of bacteria which do not use organic matter for energy are those which have the power of utilizing the energy released when inorganic compounds oxidize. They are called autotrophic bacteria and *Geitlerinema* sp. MCCB 234 comes under this group. These groups of organisms can play in the sulphur cycle (Galliher., 1933). Sulphide is a highly reduced compound and it can act as electron donor for sulphide mediated photosynthetic pathways.

In the present study, 100% removal of H_2S was noticed by *Geitlerinema* sp. in three experimental phases and found to be highly efficient in sulphide removal, unlike the control samples where sulphide concentration increased significantly from the second day onwards because of indigenous organisms. Sulphide oxidation potential of MCCB 234, when compared to other two isolates it could be exhibited higher efficiency from the second day onwards.Thus it could be concluded that the isolate *Geitlerinema* sp. MCCB 234 is potent, versatile and stable for bioremediation of sulphide. This is the first study report of sulphide removal by *Geitlerinema* sp. from aquatic sediments. Apart from sulphide removal, the other water quality parameters were tested in each phase. In the initial day, the anaerobic condition was found developed due to the production of ammonia, nitrite and nitrate in the test as well as in the control samples. But on7^{th} and final day, their concentration was found reduced in both tests as well as in controls. These fluctuations could not interfere with the H_2S removal efficiency of the isolate. During phase one, even before inoculation, elevated pH (8-8.5) was observed which played important role in nitrification, enhanced desorption of exchangeable ammonium ($NH4^{+)}$ through ammonia formation from sediment. Similarly, absorbed ammonium pools and elevated pH levels in the sediment - water, increased effluxes of phosphorous and total ammonium associated with reduction of nitrification, denitrification and oxygen consumption, and increased ammonia concentration in sediment (Gao *et al.,* 2012). In the present study, the system had two phases, upper water and lower sediment, pH of the sediment was 8 to 8.5.Considering the natural environment, sediment is more anaerobic than upper water phase. Similarly, pH might be higher in lower phase sediment than higher water phase. Upper water phase is considered

the microaerobic zone compared to sediment lower part. According to Gao *et al.* (2012) at elevated pH high sediment phase effluxes of phosphorous and total ammonium were associated with a reduction of nitrification, denitrification and oxygen consumption because of which elevated of ammonia is being produced in sediment phase. Notably, constant pH level could be found in test samples which enhanced sulphide oxidation. But pH in the control had the tendency to decline, but was not statistically significantly.

In natural environment, sulphide production by the anaerobic decomposition of organic matter is governed by redox potentials, the availability of electron acceptor and the affinity for common substrate (Piker *et al.*, 1998). In the present study redox potential of treated samples could be low compared with the control which might be due to the sulphide reduction occurred in test samples. This pointed out the successful bioremediation of sulphide in test samples. A similar observation was reported by Hamoutene *et al.* (2013).

The redox potential (E*h*) was fond enhanced on 7^{th} and final day compared to control. In natural environment, sulphide production by the anaerobic decomposition of organic matter is governed by redox potential, the availability of electron acceptor and affinity for common substrate. According to DeLaune *et al.*, (2005) E*h* is determined from the concentration of oxygen, nitrite, nitrate, manganese. Iron, sulphate and CO_2 and the reductant included various organic matter and reduced inorganic compounds like sulphides. The E*h* increment in the treated system positively correlated with the decomposition of organic matter thereby highlighting the bioremediation potential.

Sulphide utilization ability of MCCB 234 in simulated microcosm study proved that MCCB 234 *Geilterinema* sp. has the ability to perform sulphide mediated photosynthesis and the percentage of sulphide is dramatically reduced under anoxygenic conditions. On the primary level of screening in laboratory condition, it could be found out that MCCB 234 was a potent isolate for the bioremediation of H_2S. But under in vitro conditions, there were so many factors interfering with the metabolic pathways which made a significant difference in terms of H_2S utilization potency of MCCB 234.

According to Mass *et al.* (2002), Green sulphur bacteria (GSB) and purple sulfur bacteria (PSB) coexist in different aquatic ecosystems. The interactions between both the groups and the ways in which these are influenced by light conditions. The light conditions in sediments are shaped by scattering and strong attenuation of UV and shorter visible wavelengths and are therefore proportionally enriched in red and near-infrared (NIR) light below the surface. In the present study also, similar conditions might prevail in the system. It was observed that dim light and natural condition (Fig.4.36) enhances the potency of sulphide removal by MCCB 234 similarly as MCCB 146 and MCCB 147. This statement is correlated with that of Mass *et al.* (2002). The organism which exhibited 67% removal of sulphide performed much better under microcosm enhancing the activity to 100%. Similarly, water quality parameters improved gradually in test samples. Based on these observations *Geilterinema* sp. MCCB 234 could be designated as a potent sulphide "bioremediator" capable to improve health and sustainability of aquatic systems.

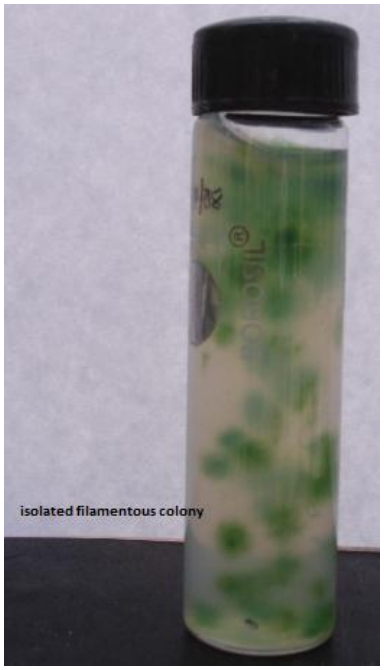


Fig.4.1. Isolated colonies of MCCB234 developed in soft agar tube

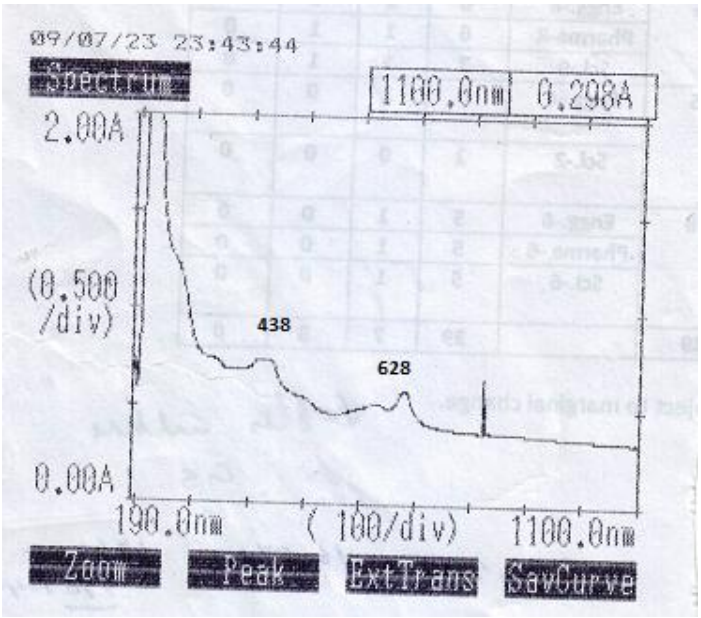


Fig.4.2 Characteristic peak of MCCB234 by sucrose-mediated absorption spectrum

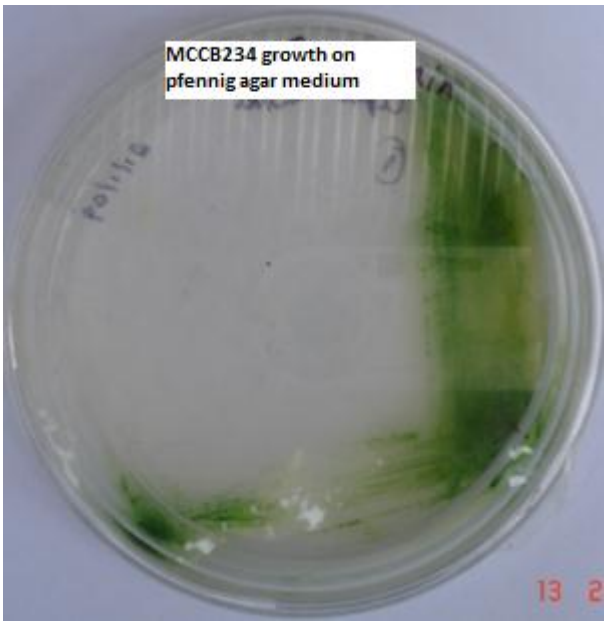


Fig.4.3. Phototrophic growth of MCCB234 on Pfennig's medium

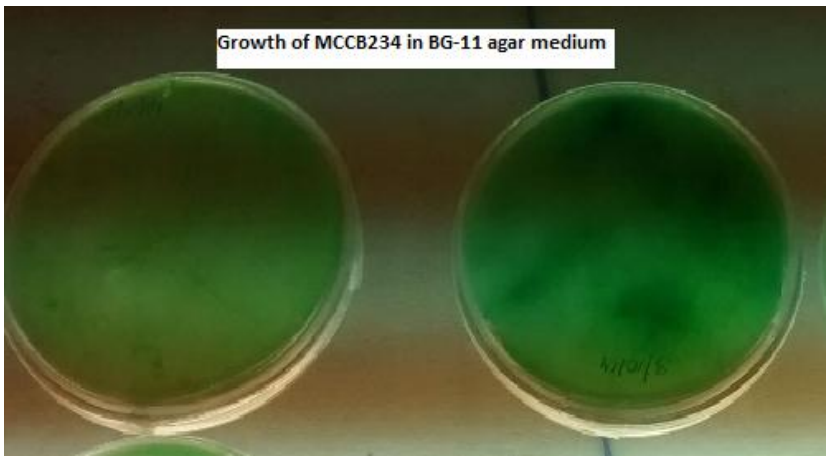


Fig.4.4. Phototrophic growth of MCCB234 on BG-11 medium

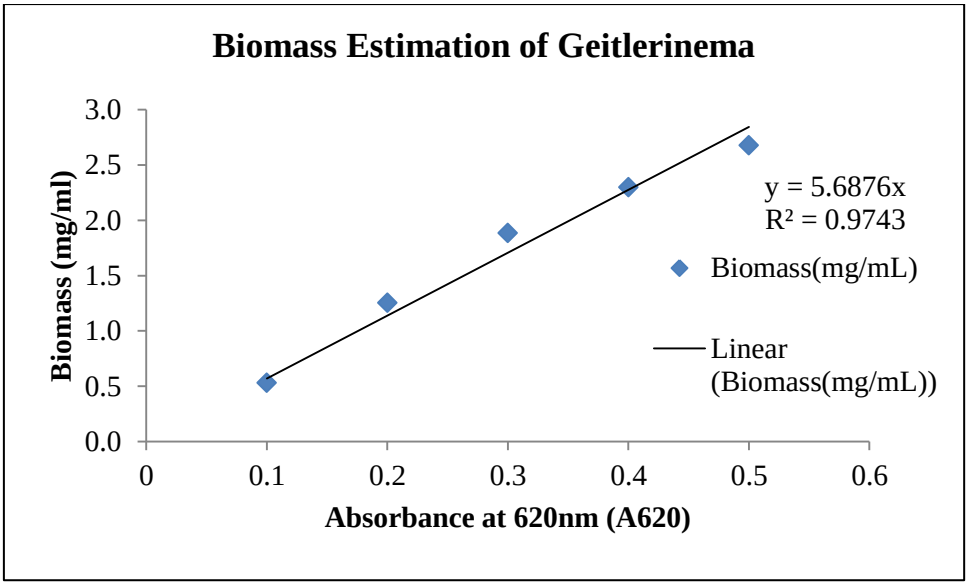


Fig. 4.5 Standards graph for biomass estimation of *Geitlerinema.*

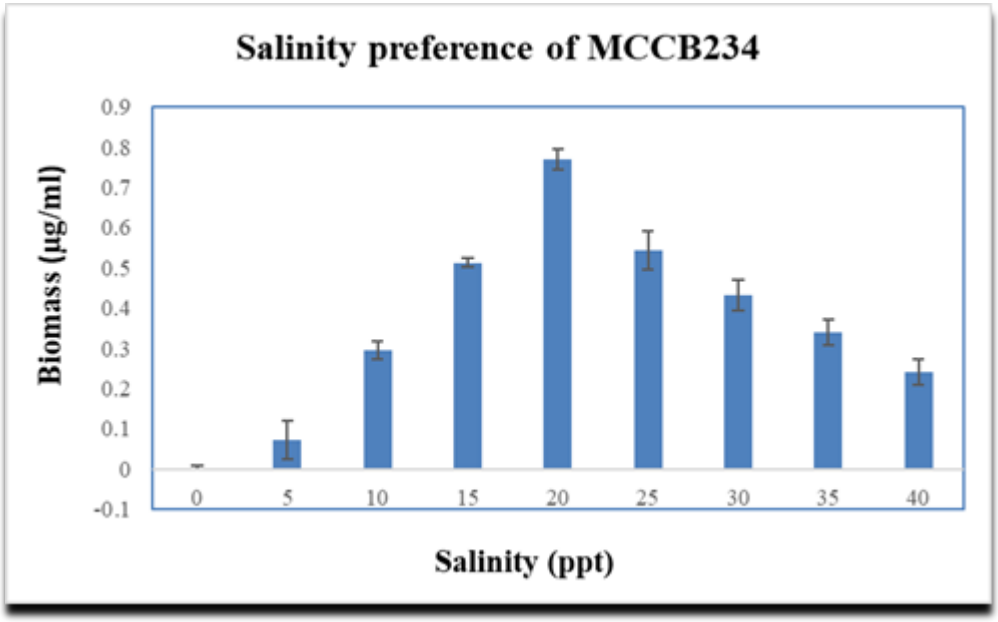


Fig. 4.6. Salinity preference of MCCB 234

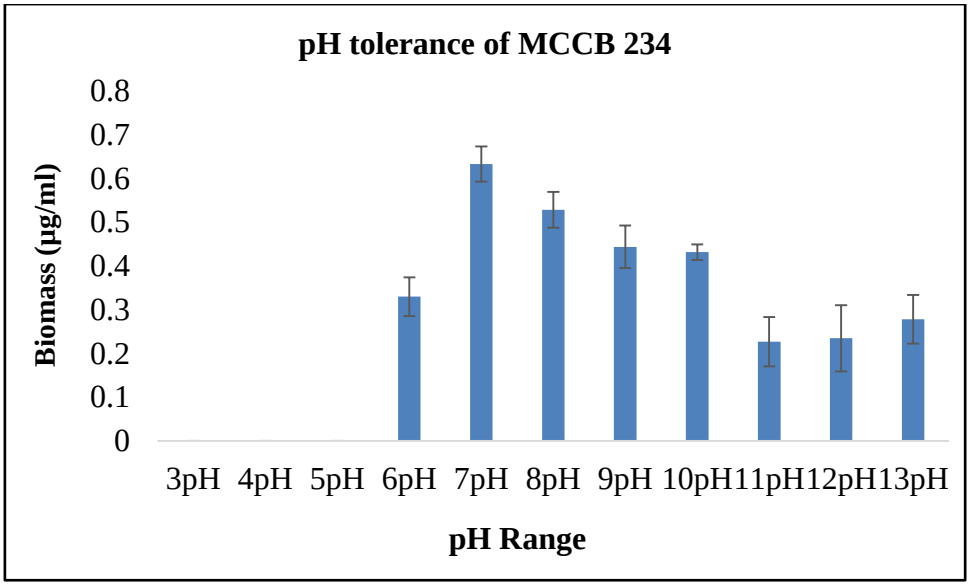


Fig.4.7. pH preference of MCCB 234

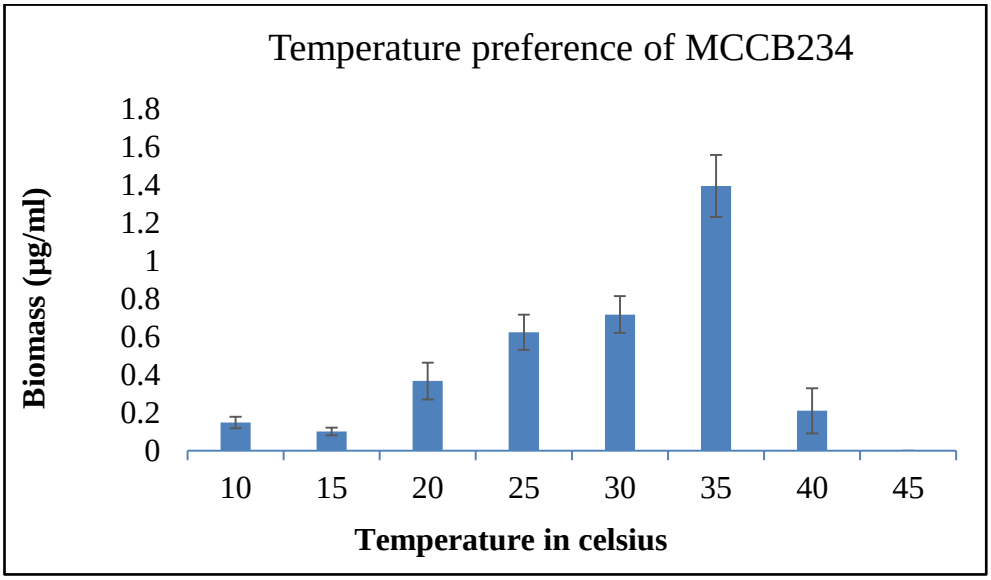


Fig.4.8. Temperature preference of MCCB 234

Fig.4.9

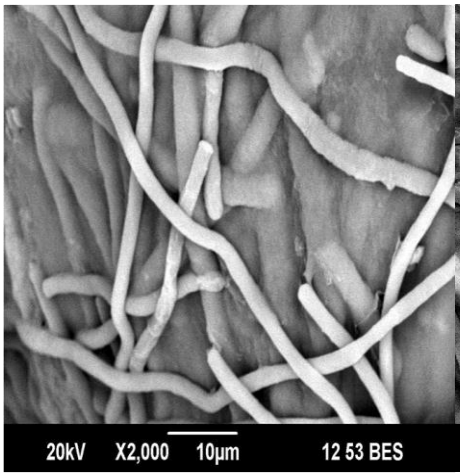


Fig.4.10

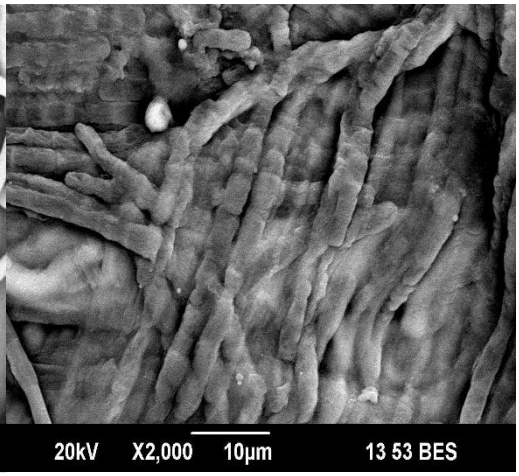


Fig.4.11

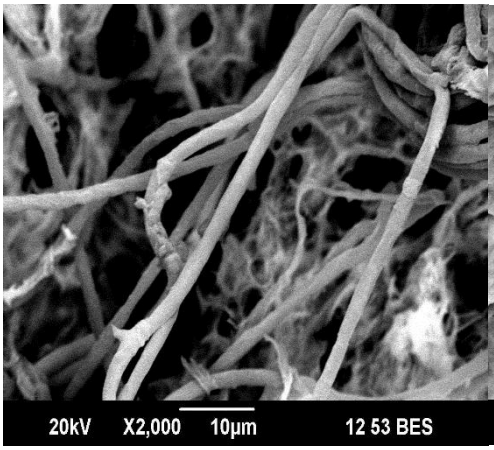


Fig.4.12

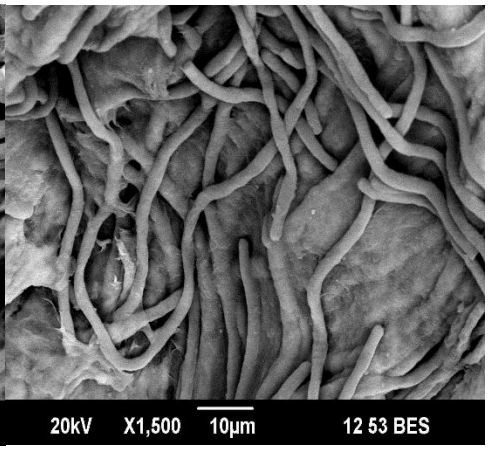


Fig.4.9, Fig.4.10, Fig.4.11 and Fig.4.12. Scanning electron microscopic images of MCCB 234

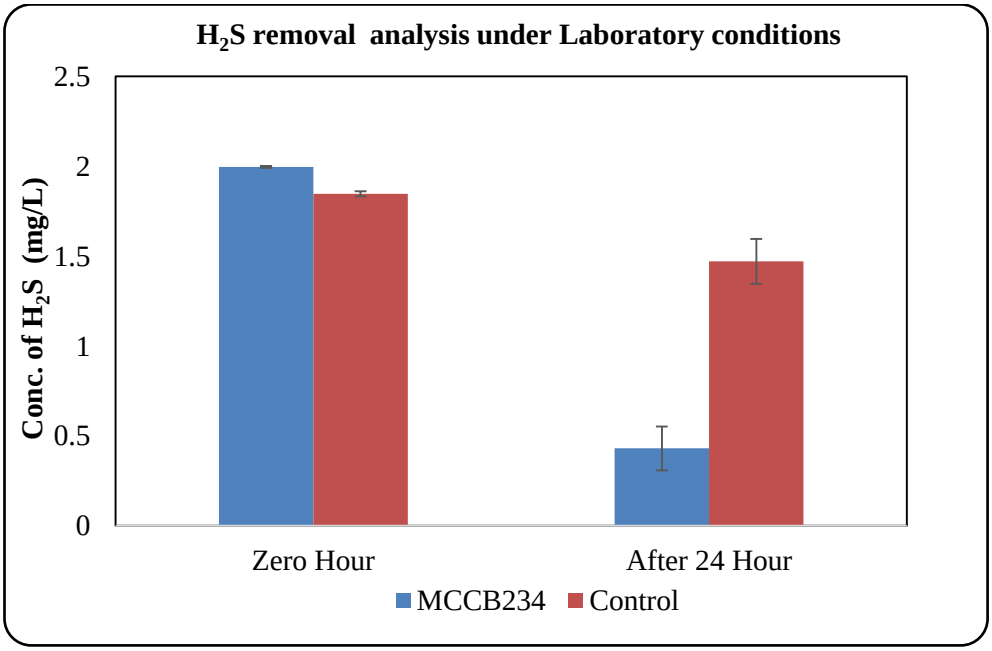


Fig.4.13. Removal of sodium sulphide by MCCB234 in the laboratory condition

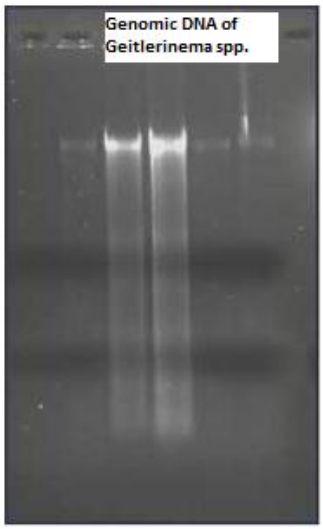


Fig.4.14. Genomic DNA of MCCB 234

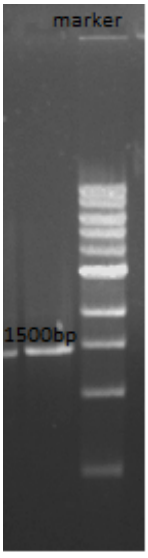


Fig.4.15. The 16S rRNA gene of MCCB 234

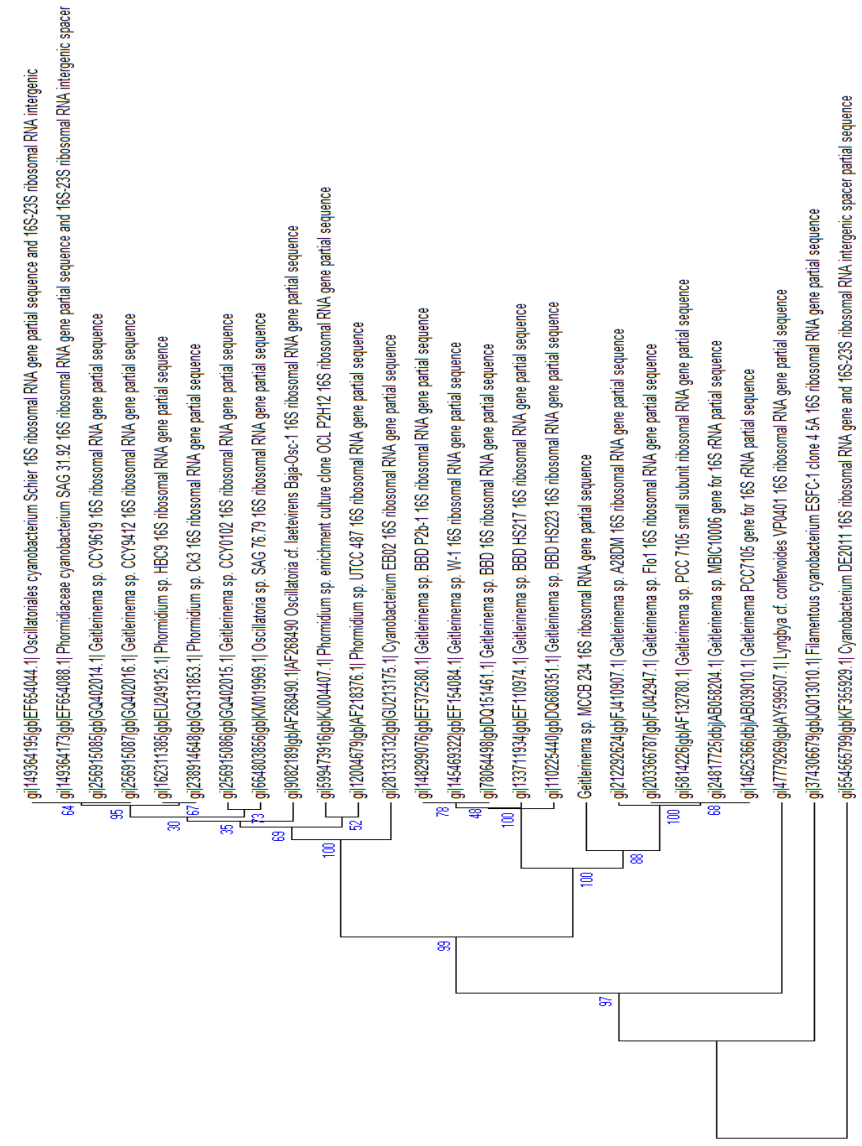


Fig.4.16. Phylogenetic tree of 16SrRNA gene MCCB234

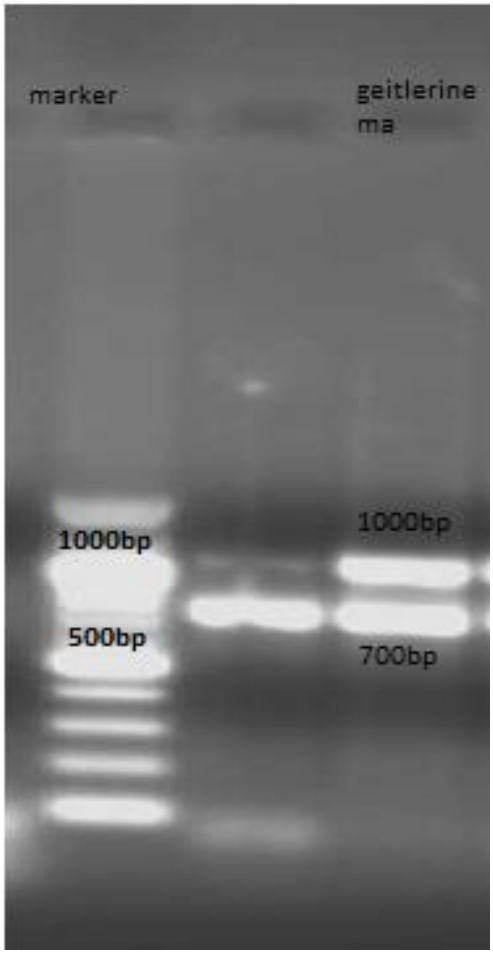


Fig.4.17. Amplification of ITS region of MCCB 234

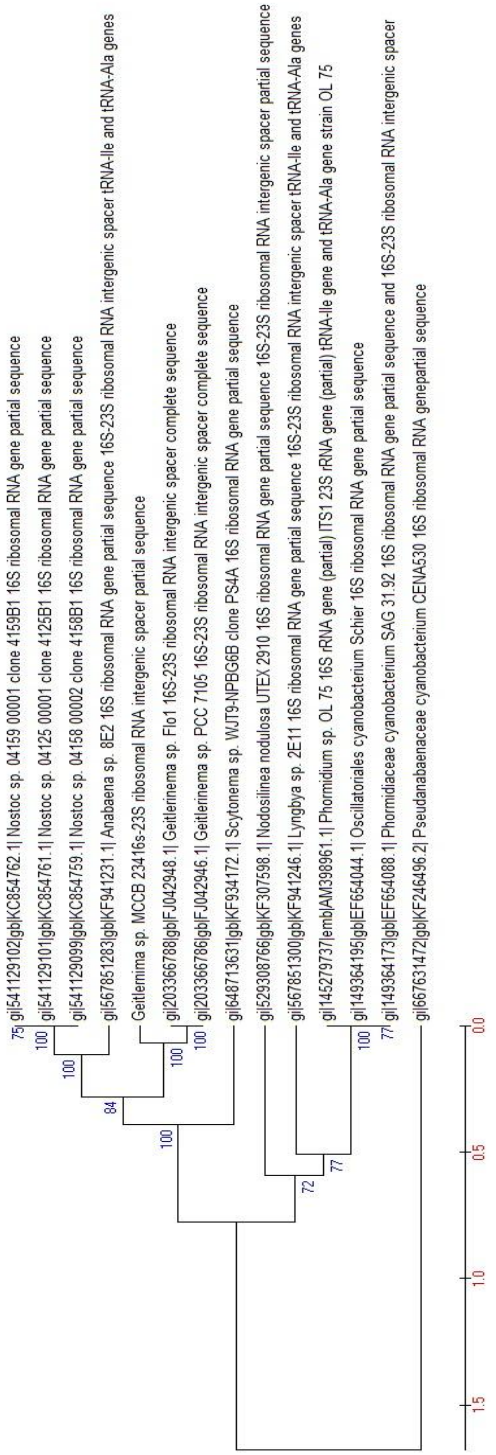


Fig.4.18. Internal transcribed spacer (ITS) phylogenetic tree of MCCB234

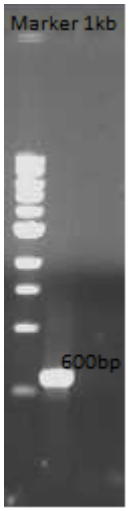


Fig. 4.19. Amplification of *cpcBA*-Intergenic Spacer Sequence from MCCB 234

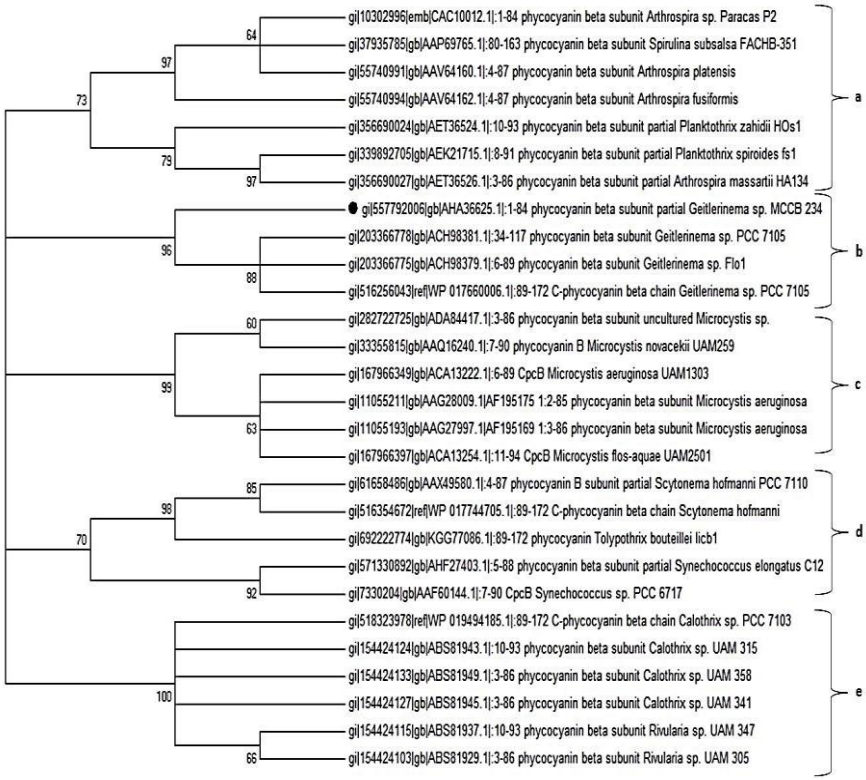


Fig. 4.20. Phylogenetic tree of *cpcBA*-Intergenic Spacer Sequence of MCCB234

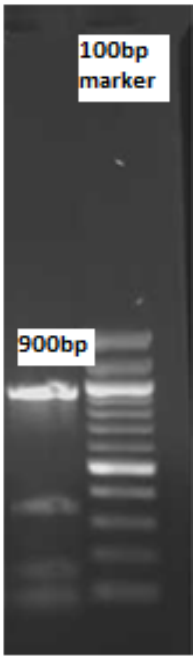


Fig. 4.21. Amplification of Rbc-LX gene from MCCB234

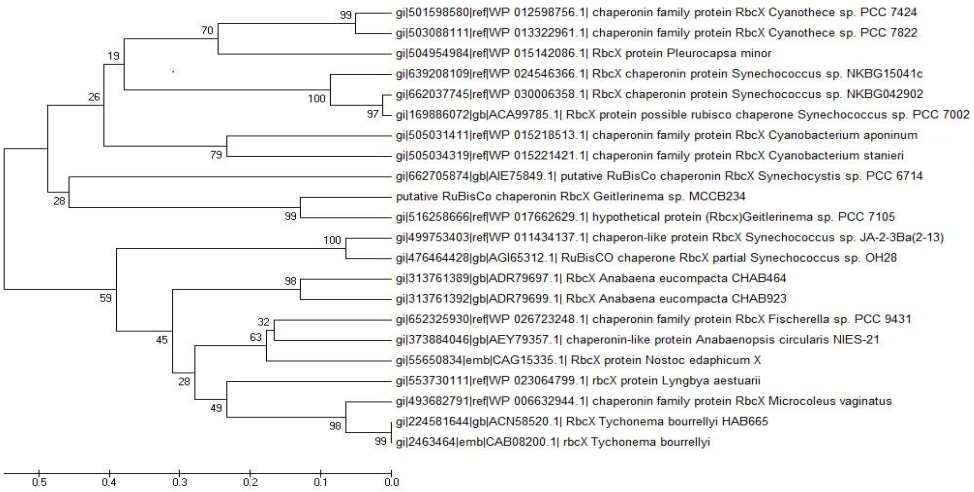


Fig. 4.22. Phylogenetic tree of Rbc-LX Sequence from MCCB234

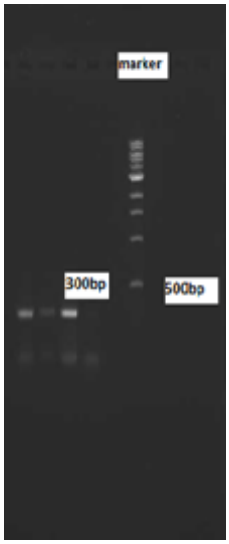


Fig. 4.23. Amplification of SOX gene from MCCB234

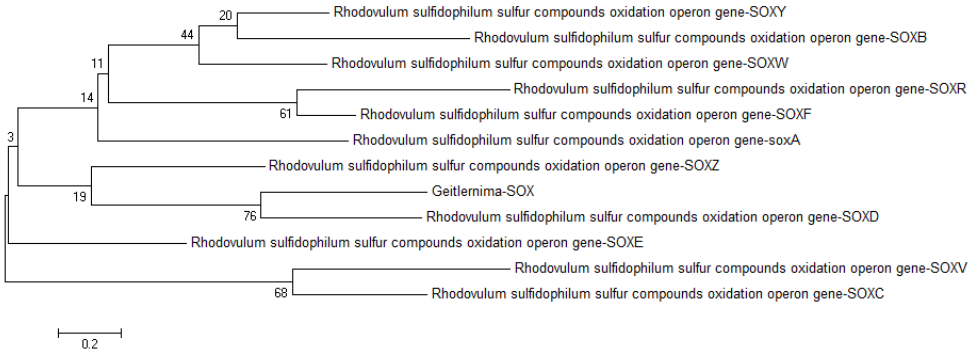


Fig. 4.24. Phylogenetic tree of SOX like Sequence from MCCB234 on the basis of SOX nucleotide sequences from *Rodovulum sulphidophilum*.

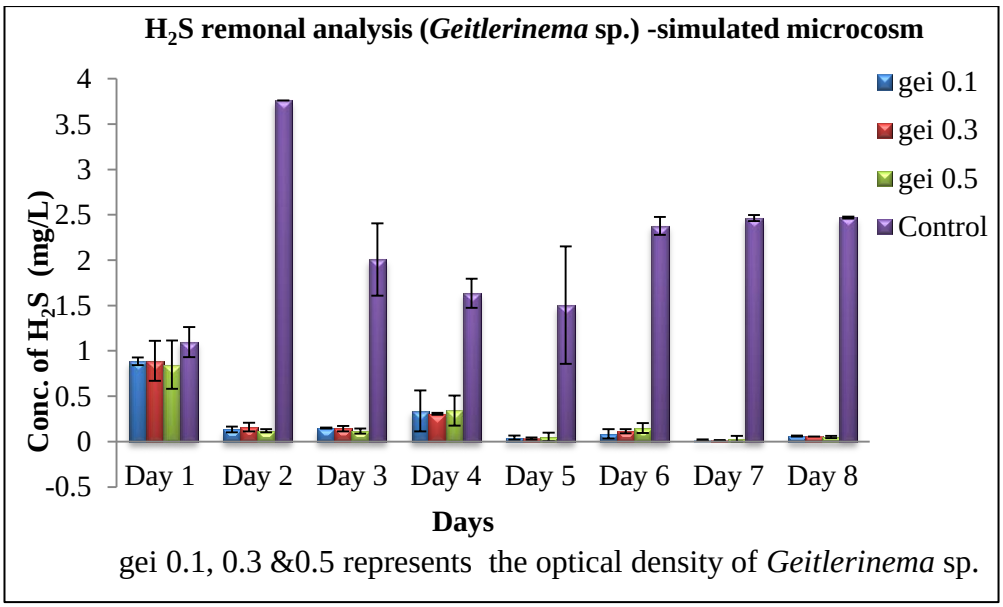


Fig.4.25

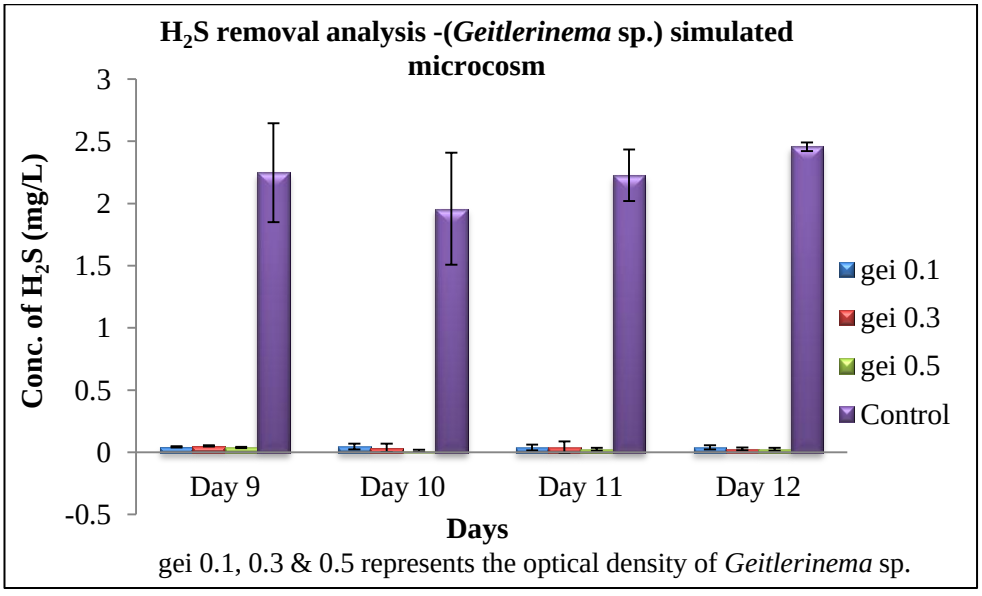


Fig.4.26

Fig.4.25 & Fig.4.26. Sulphide removal efficiency of *Geitlerinema* sp. during in the first phase of the experiment

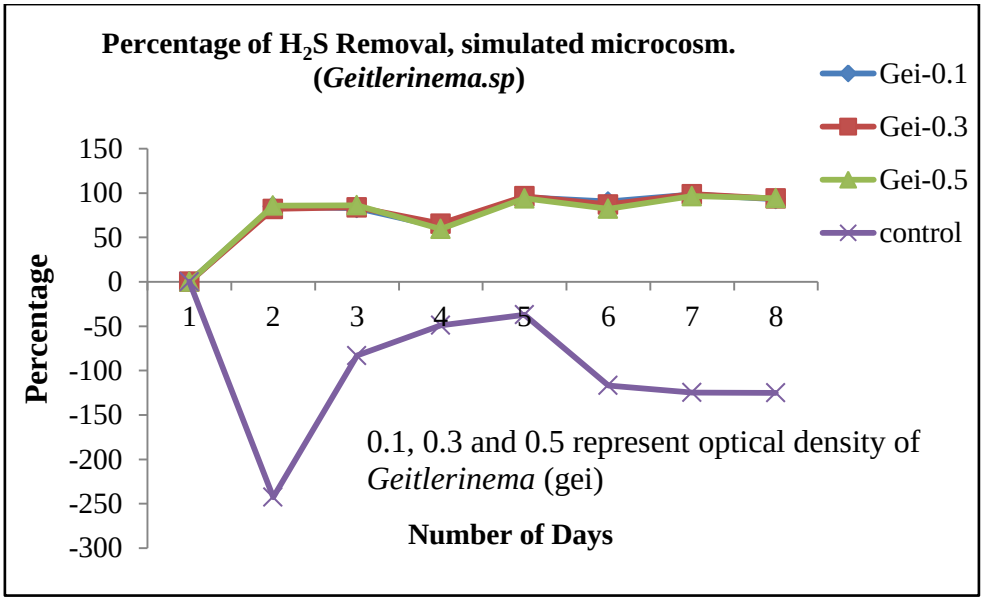


Fig. 4.27. Percentage of sulphide removal by *Geitlerinema* sp. from 1st to 8th day of phase one experiment

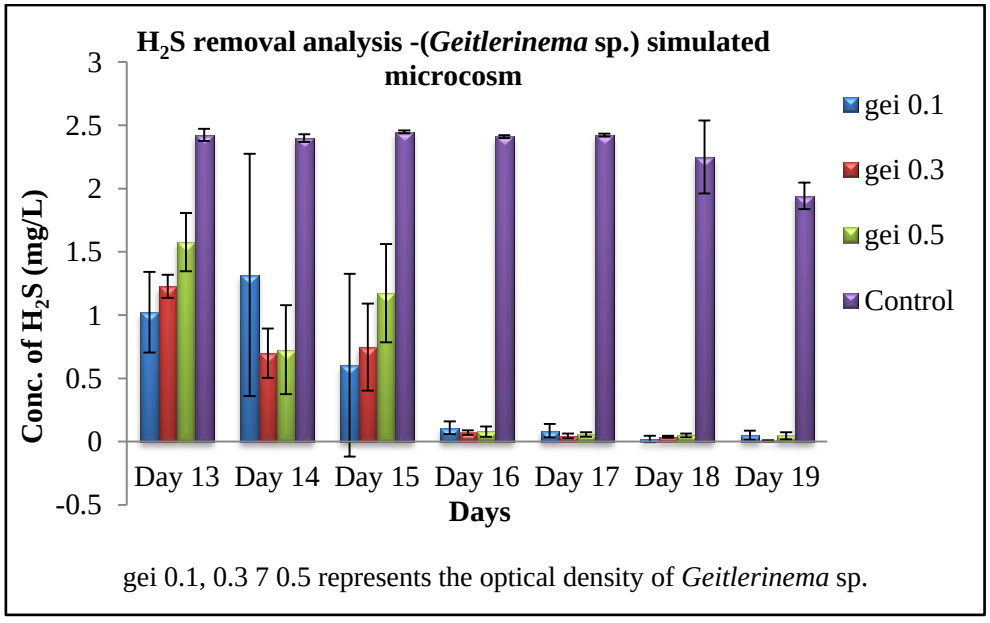


Fig. 4.28. Removal of sulphide by *Geitlerinema* sp.during in the 2nd phase of the experiment

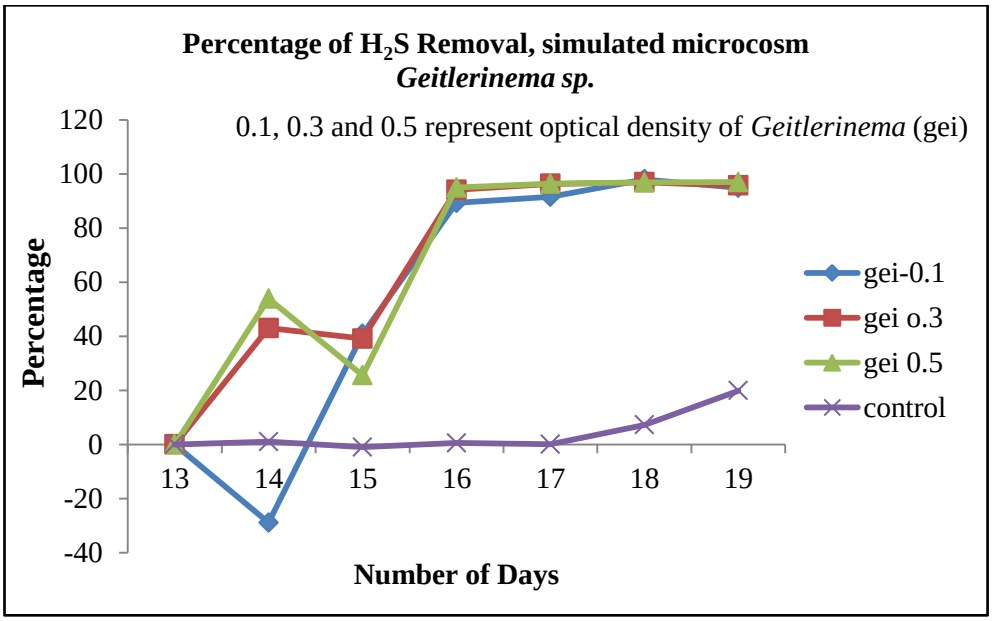


Fig. 4.29. Percentage of sulphide removal by *Geitlerinema* sp. from phase 2 experiment

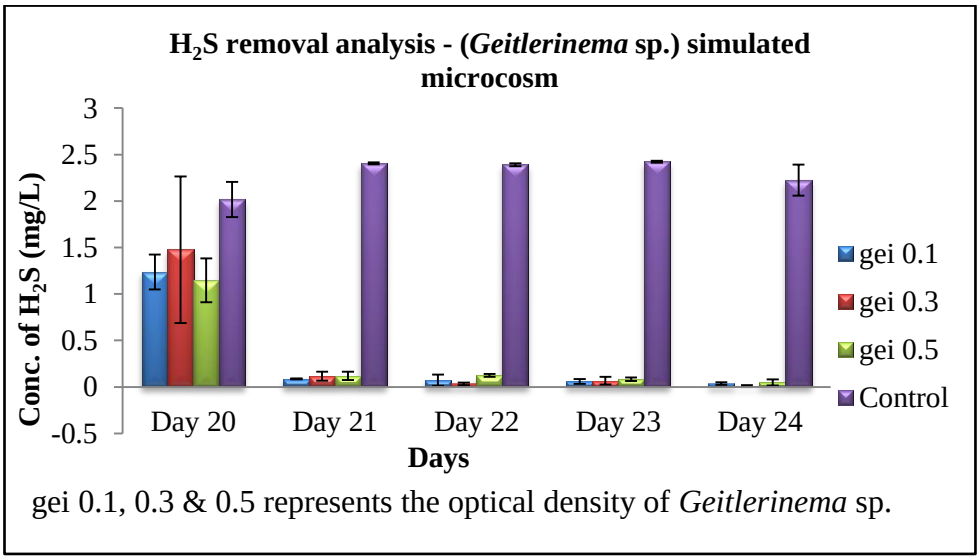


Fig. 4.30. Removal of sulphide by *Geitlerinema* sp. at the 3rd phase of the experiment

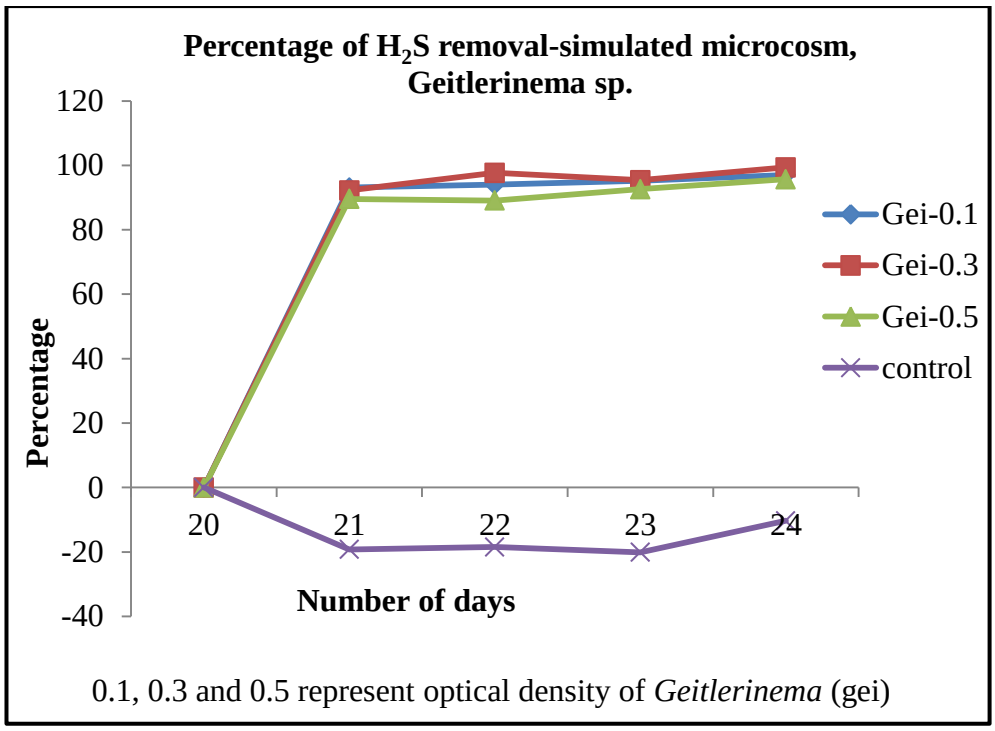


Fig.4.31. Percentage of sulphide removal by *Geitlerinema* sp. during in the phase 3 experiment

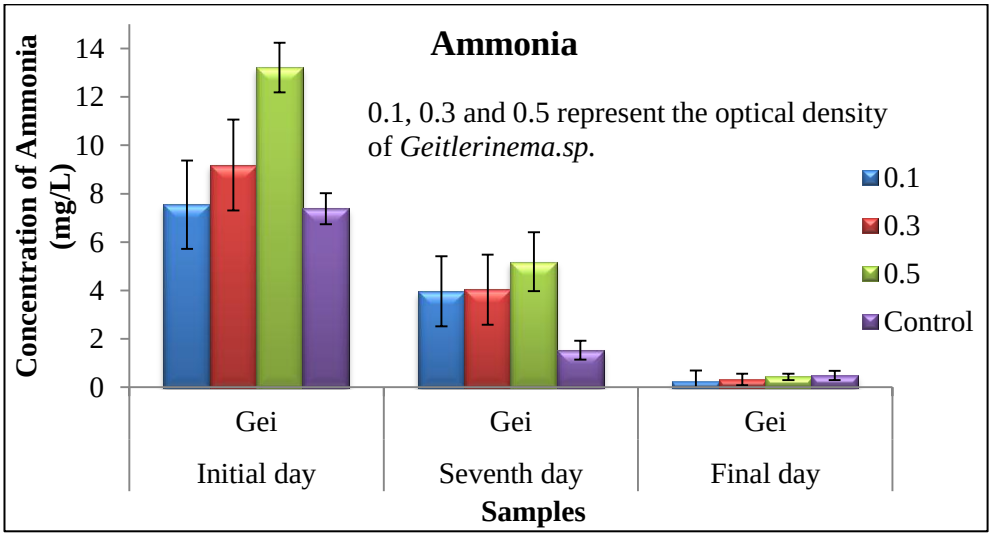


Fig. 4.32. Concentration of Ammonia during in the experiment

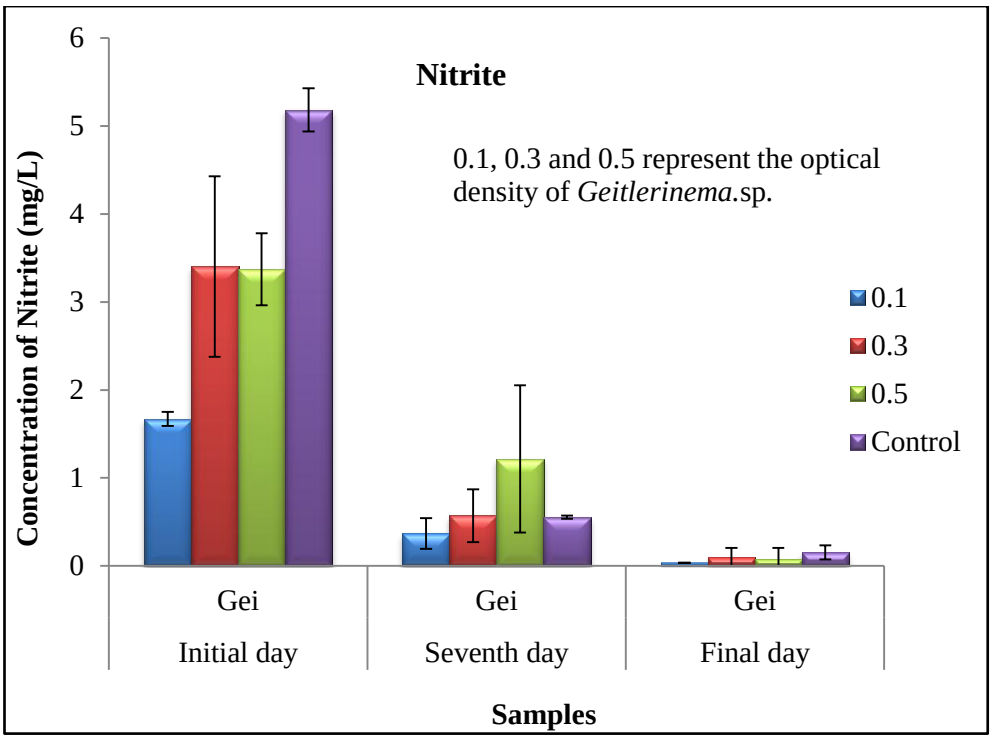


Fig.4.33. Concentration of Nitrite during in the experiment

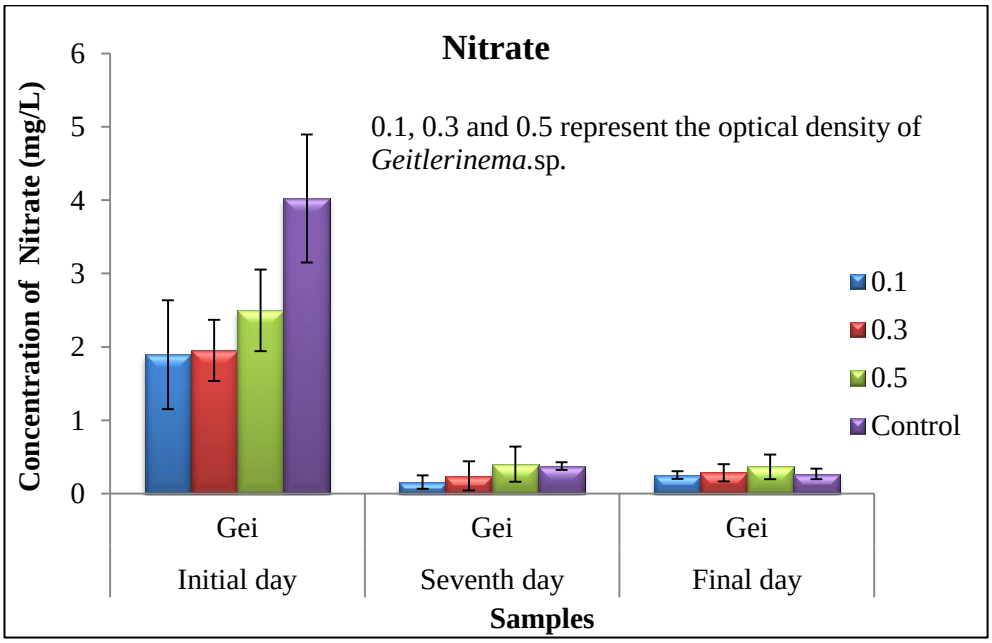


Fig. 4.34. Concentration of Nitrate during in the experiment

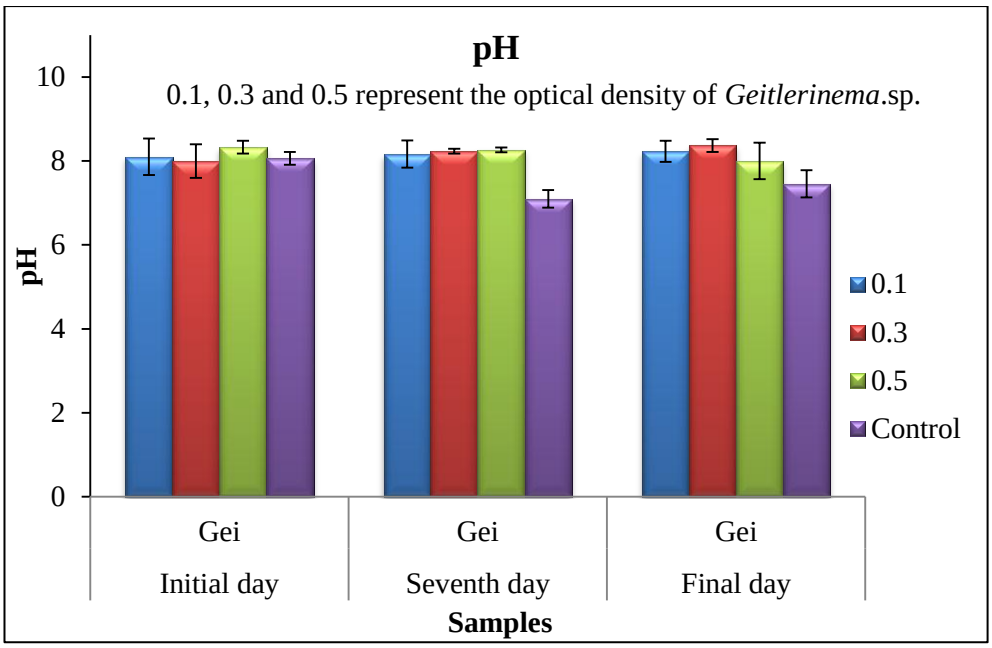


Fig.4.35. pH during in the experiment

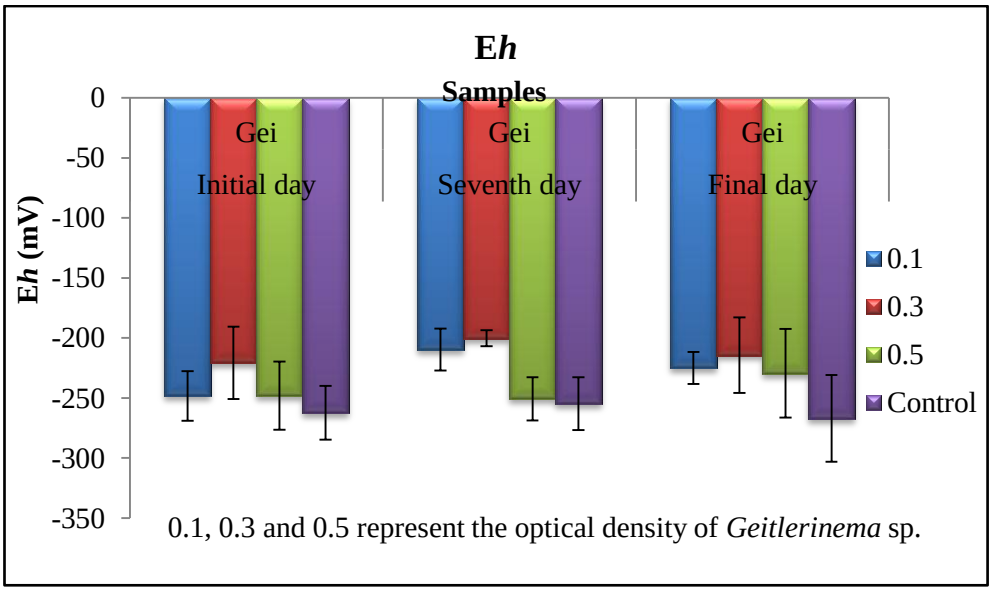


Fig.4. 36. E*h* during in the experiment

Chapter 5

CONCLUSION AND THE WAY FORWARD

Bioremediation of H_2S has generated a great deal of interest in the aquatic system worldwide as sulphur is the most important factor anywhere thatruins life in the anoxic sediments. In aerobic conditions, organic sulphur decomposes to sulphide, which in turn gets oxidized to sulphate. Sulphate is highly soluble in water and so gradually disperses from sediments. Sulphide oxidation is mediated by micro organisms in the sediment, though it can occur by purely chemical processes. Organic loading can stimulate H_2S production and reduction in the diversity of benthic fauna. Hydrogen Sulphide is soluble in water and has been suggested as the cause of gill damage and other ailments in fishes. Unionised H_2S is extremely toxic to fish that may occur in natural waters as well as in aquaculture farms. Bioassays of several species of fish suggest that any detectable concentration of H_2S should be considered detrimental to fish production.

The photosynthetic benthic bacteria that break H_2S at pond bottom have been recently used in the aquatic system to maintain a favourable environment. These bacteria contain bacteria-chlorophyll that absorb light and perform photosynthesis under anaerobic conditions. They are purple, green sulphur bacteria and cyanobacteria that grow at the aerobic as well as anaerobic portion of the sediment-water interface as previously described in Chapter-1. Photosynthetic purple non-sulphur bacteria can also

utilizesulphide as an electron donor for H_2S mediated photosynthesis and it can decompose organic matter, NO_2 and harmful wastes of ponds. The green, purple sulphur bacteria and purple non-sulphur bacteria can split H_2S to utilize the wavelength of 900-1200 Infra-Red light from the solar spectrum. The purple, non-sulphur bacteria and green sulphur bacteria obtain reducing electrons from H_2S at a lower energy cost than H_2O splitting photoautotroph and thus require lower light intensities for carrying out photosynthesis.

Chromatiaceae, *Rhodospirillaceae* and *Chlorobiaceae* are the families of photosynthetic sulphur bacteria that favour aerobic as well as anaerobic conditions for growth while utilizing solar energy and sulphide as described earlier. Apart from this some cyanobacteria *Geitlerinema* sp. can use sulphide as an electron donor for sulphide mediated photosynthesis. The common examples of photosynthetic bacteria of importance in the aquatic system for using for the bioremediation of hydrogen sulphide are *Rhodospirillum, Rhodopseudomonas, Chromatium, Thiocystis, Thiospirillum, Thiocapsa, Lamprocystis, Thiodictyon, Thiopedia, Amoebobacter, Chlorobium, Prosthecochloris, Pelodictyon* and *Clathrochloris.*

Several microorganisms have been studied for application in hydrogen sulphide removal processes as described above. In this context, two consortiawere developed by National Centre for Aquatic Animal Health (Manju, 2005) and its potency was calculated by chemical analysis. Based on the data obtained from chemical analysis it could be found that these two consortia had the potency to utilised sulphide as electron donor through sulphide mediated photosynthesis.

In this research, various studies were undertaken for the purification of bacteria from the consortium, its characterization, and the confirmation of sulphide mediated photosynthesis. Accordingly, the work had the following objectives:

1. Isolation, Purification and Characterization of Photosynthetic Bacterium, MCCB147 as Bioaugmentor for Bioremediating Hydrogen Sulphide in Aquatic Systems.
2. Isolation, Purification and Characterization of Photosynthetic Bacterium MCCB146 as Bioaugmentor for Removal of Hydrogen Sulphide in Aquatic System
3. Isolation, Purification and Characterization of Green Filamentous Photosynthetic Bacterium MCCB234 as Bioaugmentor for Removal of Hydrogen Sulphide in Aquatic System

Major findings are summarized as follows:

1. Isolation, Purification and Characterization of Photosynthetic Bacterium, MCCB147 as Bioaugmentor for Bioremediating Hydrogen Sulphide in Aquatic Systems.
   - ✓ Purification of MCCB147 was done by deep agar dilution method from the consortium PF2.
   - ✓ Purified culture of MCCB147 exhibited slow growth in Pfennig's mineral medium and better growth could be obtained in modified Pfennig's medium incorporating yeast extract.

- ✓ Nutritional assay of MCCB147 revealed that it exhibited photolithohetrotrophic and photolithoautotrophic mode of nutrition. Meanwhile, light was the essential factor for its growth.
- ✓ The organism was identified as facultative hydrogen sulphide oxidizer.
- ✓ Substrate utilizing efficiency of MCCB147 could be evaluated, and among 24 substrates valerate, methionine and nicotinamide could exhibit negative results. These observations demonstrated that, a wide range of ecological adaptability was characteristic of MCCB14. Out of 24 substrates, sodium lactate, glucose and fructose, mannose and glutamic acid supported higher growth compared to that with rest of nutrients.
- ✓ Ammonium chloride and urea in the medium could enhance growth of MCCB147 in terms of biomass production.
- ✓ Presence of reduced sulphur compounds in the medium never enhanced growth of MCCB147.
- ✓ Presence of nitrate and nitrite never exhibited enhancement of growth of MCCB147 at the same time glutamate lead to positive result.
- ✓ Bacteriochlorophyll measurements based on absorbance peak indicated that MCCB147 had bchl "a" and "b".
- ✓ *R. julia* (MCCB147) has LHC - I (Light Harvesting System)judged based on the presence of peak obtained at 888 nm in absorption spectrophotometric analysis. The major

carotenoid pigment present in *R. julia* wasbelonging to spirilloxanthin series due to the presence of the peak at 553 nm.

- ✓ The organism showed high level of cellular aggregation as it preferred to grow under anaerobic conditions, and when the oxygen concentration would be high it had been taking a defensive mechanism against oxygen toxicity by way of aggregate formation. This made a protective shield around them and helped to reduce intercellular space against oxygen toxicity.
- ✓ Cellular hydrophobicity was found to be the reason behind cellular aggregation having 55% hydrophobicity index.
- ✓ Optimized the primary culture conditions of *R.julia* such as pH, temperature and salinity. During the time of the experiment, preferred salinity for biomass production could be found between 20 to 35 ppt. It preferred to grow in alkaline conditions, pH 7.5 or 8. Similarly, optimum temperature range was at 25^0C to 30^0C.
- ✓ TEM analysis revealed MCCB147 to have the several intracytoplasmic membranes, circular vesicles, cytomembranes, lamellar and tubular cytoplasmic membranes with inclusion bodies, poly hydroxyl butyrate structures, and reproduction was found to take place through budding as well, all characteristic photosynthetic bacteria. .
- ✓ H_2S ability of *R. julia*could demonstrate 62% H_2S utilization ability under the laboratory conditions.
- ✓ The isolate MCCB 147 has similarity to both *R. julia* and *A. pfennigii* under same genus *Rhodospeudomonas* at 16S rRNA

gene sequence level, however, the morphological features are more towards *R. julia.*

- ✓ On analysis of PUFM (Photosynthetic Unit Forming gene) gene encoding a protein for the "M" subunit of the photosynthetic reaction center was found to belong to photosynthetic bacteria *Marichromatium* sp. and *Thiocapsa imhoffii.*
- ✓ Its absorption peak was at 880 nm having LHC 1 with Bchl "a".
- ✓ Molecular characterization of Guanylate cyclase (c-di-GMP), a regulatory protein that plays an important role in the regulation of SOX and DSR sulphide oxidizing gene of *R. julia* could be accomplished, regarded as the first report
- ✓ Under simulated microcosm the organism was found a promising facultative H_2S remover with 100% removal rate. Apart from this natural environmental conditions especially light (faint light) could enhance the sulphide removal efficiency when compared with laboratory conditions. Meanwhile water quality parameters such as nitrate, nitrite, ammonia, E*h* and pH remained stable throughout the study period.

2. Purification and molecular charecterization of Photosynthetic bacterium MCCB146 as bioaugmentor for removal of hydrogen sulphide in aquatic system

- ✓ Purification of MCCB146 was done by deep agar dilution method and characterizedand identified as *A. pfennigii* formerly known as *Rhodobium pfennigii* belonging to phylum Proteobacteia, class *Alphaproteobacteria*, order *Rhizobiales*

and family *Rhodobiaceace,* genus *Afifella.* For attaining better growth, the pfennig's medium was modified by incorporatingyeast extract.

- ✓ The absorption maxima indicated it to have chl "a" and chl "b".
- ✓ MCCB146 was found to have LH-1 photo harvesting system with bacteriochlorophyll "a" and the major carotenoid pigment was belonging to spirilloxanthin series as same as MCCB147.
- ✓ The organism was found to form aggregates indicating better resilience to stresses factors such as presence of oxygen, pH, extreme temperature etc.
- ✓ Hydrophobic effect, the factor responsible for aggregation was determined and found to have 63% per cent hydrophobicity index.
- ✓ On assaying the nutritional requirements sodium lactate, pyruvate, trehalose, glycerol, glucose and glutamic acid enhanced growth significantly, and not methionine and valerate.
- ✓ *A.pfennigii*was found to utilize glucose, trehalose, fructose and alcohols for its biomass generation.
- ✓ Reduced sulphur compound cysteine stimulated growth than other compounds such as thioglycolate and thiosulfate.
- ✓ Biomass generation of MCCB146 in the presence nitrate was found to have enhanced.
- ✓ MCCB146is a facultative hydrogen sulphide oxidiser and does not need H_2S as a growth factor and in the absence of H_2S, it can survive under the photolithohetrotrophic mode of nutrition.

- ✓ MCCB146 can adapt to photolithohetrotrophic, photolithoautotrophic and photoorganohetrotrophic conditions as it belongs to alphaproteobacteria having high degree of adaptation to specific environmental conditions and light is an the essential factor for its survival.
- ✓ MCCB146 can tolerate salinity to 35 - 40 ppt beyond which it shows significant biomass reduction. However, highest biomass was obtained at 10 ppt.
- ✓ On the basis of biomass production, optimum pH range of MCCB146 observed was 7-8, and it prefered to grow in slightly alkaline conditions but never beyond pH at 10.
- ✓ Optimization of temperature in MCCB146indicated that it was a temperature sensitive organism and it should be grown in temperatures between 25^0C to 30^0C.
- ✓ Capability of MCCB146 to utilize H_2S was analysed by methylene blue spectrophotometric method and according to which 75% removal efficiency under laboratory conditions was noticed.
- ✓ The organism possessed several intra-cytoplasmic membranes, circular vesicle cytomembranes, lamellar and tubular cytoplasmic membrane with inclusion bodies same as MCCB147 and it was considered as the characteristic features of photosynthetic sulphur bacteria.
- ✓ Based on 16S rRNA gene sequence analysis and phylogenetic tree constructed photosynthetic bacteria MCCB146 was identified asnon-sulphur bacteria, *A.pfennigii*.

- ✓ Translated sequence of PUF M gene of MCCB146 exhibited 43% similarity with PUF M gene of *A. pfennigii.*
- ✓ The isolate MCCB 146 is a facultative sulphide oxidizer useful as bioremediator for sulphide removal from aquatic systems.

3. Purification and molecular charecterization of Green filamentous Photosynthetic bacterium MCCB234 as bioaugmentor for removal of hydrogen sulphide in aquatic system

- ✓ Purification of MCCB234 was done by deep agar dilution method as described as earlier and characterized and identified as *Geitlerinema* sp.
- ✓ BG-11 was found to be a better medium in terms of biomass production of *Geitlerinema* sp. when compared with Pfennig's medium.
- ✓ Absorption maxima of MCCB234 determined through sucrose mediated absorption showed that it was more similar to bcl "a" and bcl "b". The characteristic peak of *Geitlerinema* sp. at 628 nm indicated the presence of phycocyanin, the Phycobiliproteins.
- ✓ Growth of *Geitlerinema* sp. under different physiological and nutritional conditions indicated that light/dark incubation enhanced growth compared with that under continuous light. Similarly,under darkness, no growth was observed, hence the present study proved that it was a photosynthetic organism requiring minimum nutrients for survival.
- ✓ Genus *Geitlerinema* sp. has the ability to perform oxygenic photosynthesis in the absence of sulphide. Meanwhile, during

anoxygenic condition, photosynthesis is carried out by using sulphide as electron donor suggesting that the photosynthetic organism MCCB234 is a facultative hydrogen sulphide oxidizer.

- ✓ Biomass generation in terms of salinity was found to be higher in 20ppt and concluded that MCCB234 was a salinity tolerant organism having upper growth limit up to 40ppt.
- ✓ Basically *Geitlerinema* sp. is an alkalophilic organism due to which it could survive at pH 13, however the optimum pH range was between 7 -10.
- ✓ The optimum temperature of *Geitlerinema*sp.is 35^0C.
- ✓ Scanning electron microscopic image of MCCB234 demonstrated the presence of trichome and its tip's structure was slightly tapered and bent, the characteristic feature of *Geitlerinema* sp.
- ✓ Based on the molecular characterization of MCCB234, 16SrRNA, ITS, RbclX and cpcBA IGS the organism could be placed as photosynthetic cyanobacteria *Geitlerinema* sp.
- ✓ Sulphide oxidizing SOX like gene could be for the first time isolated from *Geitlerinema* sp.
- ✓ Sulphide utilization ability of MCCB234 demonstrated in a simulated microcosm proved that MCCB234 *Geilterinema* sp. had the ability to perform sulphide mediated photosynthesis. As high as 100% removal efficiency was found at the end of the experiment, and one of the important observations was that dim light and natural conditions enhanced sulphide removal rate

compared to that under laboratory conditions same as seen in MCCB146 and MCCB147. By combining all the results, it could be demonstrated that the selected isolates of MCCB146, MCCB147 and MCCB 234 were potent enough for bioremediation of sulphide from aquatic system. Amplification and sequencing of "Guanylate cyclase" (c-di-GMP) and "SOX**"** like gene from *R.julia* and *Geitlerinema* sp. indicated that the organism could actively involve in sulphide bioremediation.

The Way forward

- ✓ Mass production of three selected isolates and development of a cocktail bio-augmenter for hydrogen sulphide bioremediation in aquaculture systems. .

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APPENDIX

SEQUENCES OF AMPLIFIED GENES

1. Photosynthetic unit forming gene of *R. Julia* (PUFM), partial cds

Accession Number- KX-784507

TCGCACCTGGACTGGACCGCGGCGTTCTCGATCCGCTTAGGCAAC
CTCTACTACAATCCCTTCCATATGCTTTCGATTGTCTTCCTGTATG
GCTCGACGCTTCTGTTTGCCATGCATGCGGCGACGATCCTGGCGG
TGAGCCGCTTCGGCGGCGAGCGGGAAGCGGAGCAGGTTGTCGAC
CGCGGAACGGCCGCCGAGCGTGCCGCGCTCTTCTGGCGCTGGAC
CATGGGA

2. Organism-*R. julia* (MCCB 147) Diguanylate-cyclase (DGC) domain, partial cds

Accession Number-KX784509

AACGACCGCCGCACCATCGCCGTCACGGTGGAGGCCGCCAAGGA
TGGCGGCATGGTGCTGGTGGCCGAGGACGTGACGGAGCGCAAGC
GCTCCGAAGCGCGCATCGCCTATATGGCGCATCATGACGACGTG
ACCGGCCTGCCCAACCGCGTCAAGCTGAAGGAACAGCTGGAAGA
GGCGGTGAAGCGGGCGATCGAGGCCGATAAGAGCTTCGCCGTCT
TCTGCATCGATCTCGACCATTTCAAGAGCGTCAACGACACGCTCG
GCCATCCGGTCGGCGACAGCCTCTTGCGCGCCACGGCGCGGCGG
CTTTCGGCGATGACGCGCAAGAGCGACGTGCTGGCACGCTTCGG
GGGCGATGAGTTCGTGCTCATGGCGACCGGCATTGAGGACGCGG
AGGAGGCTGGCCTTTTCGCCACGCGGATCATCGCGGCGCTGACC
CAGCCCTACGCCCTCAACGGCCATTCCATCGTCGCCGGGGCGAG
CGTGGGCATCGCGCTCGCCCCCGCCGACGGACGCAACGCCGACG

CTCTCATCAAGAACGCCGACATGGCGCTCTACCGGGCCAAGGCC
AACGGGCGGGGCACCTTCTGCCTGTTCGAGCGGGAGATGGACGT
GAAGGCGCAGGAGCGGCGCTCGCTGGAAATGGAGCTCAGAAGC
GCCTTCGCCAACGAGGAGCTGCAACTGCATTATCAGCCGCTGCTC
AATCTCAGACGACGCAACATCTCCACCTGCGAGGCGCTCTTGCGC
TGGCCGGGCGGCCGGCGCGGCCCGGTGCCGCCCTCAGAATTCAT
CCCGCTCGCCGAGGAGATGGGCCTCATCGTGGAAATCGGCGACT
GGACGTTGCTGCAAGCCTGCCGCGAGGCGAATAAAT

3. Photosynthetic bacteria *R. julia* MCCB147 16S ribosomal RNA gene, partial Sequence

Accession Number- KU 7661996

TCGATTGAGTTTGATCCTGGCTCAGANCCAACGCTGGCGGCAGG
CTTAACACATGCAAGTCGAACGCGCTCTTCGGAGCGAGTGGCAG
ACGGGTGAGTAACGCGTGGGAATCTACCCAATGGTATGGAATAA
CTCGAGGAAACTCGAGCTAATACCGTATACGCCCTTCGGGGGAA
AGATTTATCGCCATTGGATGAGCCCGCGTCGGATTAGCTTGTTGG
TGGGGTAATGGCCTACCAAGGCGACGATCCGTAGCTGGTCTGAG
AGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATCTTGGACAATGGGCGAAAGCCT
GATCCAGCCATGCCGCGTGAGTGAAGAAGGCCCTAGGGTTGTAA
AGCTCTTTCAGCGGGGAAGATAATGACGGTACCCGCAGAANAAG
CCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGG
CTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGG
ATTGTTAAGTCAGGGGTGAAATCCCGAGGCTCAACCTCGGAACT
GCCTCTGATACTGGCAATCTCGAGTCCGGAAGAGGTTGGTGGAA
TTCCGAGTGTAGAGGTGAAATTCGTAGATATTCCGGAGGAACAC
CAGAGGCGAAGGCGGCCAACTGGTCCGAGACTGACGCTGAGGCG
CGAAAGCGTGGGGAGCCAACAGG

4. Organism-*A.pfennigii* (MCCB 146) photosynthetic unit forming gene (PUFM), partial cds

Accession Number- KX 784508

TCGCACCTGGACTGGACCGCGGCGTTCTCGATCCGCTACGGCAAC
CTCTACTACAATCCCTTCCATATGCTTTCGATTGTCTTCCTGTATG
GCTCGACGCTTCTGTTTGCCATGCATGCGGCGACGATCCTGGCGG
TGAGCCGCTTCGGCGGCGAGCGGGAAGCGGAGCAGGTTGTCGAC
CGCGGAACGGCCGCCGAGCGTGCCGCGCTCTTCTGGCGCTGGAC
CATGGGAAGCTCGCT

5. Photosynthetic bacteria *A.pfennigii* MCCB146 16S ribosomal RNA gene, partial Sequence

Accession Number- KM 921803

GAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGGCTTAAC
ACATGCAAGTCGAACGCGCTCTTCGGAGCGAGTGGCAGACGGGT
GAGTAACGCGTGGGAATCTACCCAATGGTATGGAATAACTCGAG
GAAACTCGAGCTAATACCGTATGCGCCCTTCGGGGGAAAGATTT
ATCGCCATTGGATGAGCCCGCGTCGGATTAGCTTGTTGGTGGGGT
AATGGCCTACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATG
ATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATCTTGGACAATGGGCGAAAGCCTGATCCA
GCCATGCCGCGTGAGTGAAGAAGGCCCTAGGGTTGTAAAGCTCT
TTCAGCGGGGAAGATAATGACGGTACCCACAGAAGAAGCCCCGG
CTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCG
TTGTTCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGATTGTTA
AGTCAGGGGTGAAATCCCGAGGCTCAACCTCGGAACTGCCTCTG
ATACTGGCAATCTCGAGTCCGGAAGAGGTTGGTGGAATTCCGAG
TGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGAGGCG
AAGGCGGCCAACTGGTCCGAGACTGACGCTGAGGCGCGAAAGCG
TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGGATGCTAGCCGTTGGTGGGTATACTCATCAGTGGCGCA
GCTAACGCATTAAGCATCCCGCCTGGGGAGTACGGTCGCAAGAT
TAAACTCAAGGAATTGACGGGGCCCGCACAAGCGGTGGAGCATG

TGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACA
TCCCGGTCGCGGTTTCCAGAGATGGATTCCTTCAGTTAGGCTGGA
CCGGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTG
CCAGCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGC
CGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACG
GGCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGAAAA
TCCCCAAAAACCGTCTCAGTTCGGATTGTCCTCTGCAACTCGAGG
GCATGAAGGTGGAATCGCTAGTAATCGTGGATCAGCATGCCACG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATG
GGAGTTGGTTCTACCCGAAGACGGTGCGCTAACCCGCAAGGGAG
GCAGCCGGCCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAAC
AAGGTAGCCGT

6. *Geitlernima* sp. MCCB234 - Sulphide Oxidizing gene SOX

TTAAAATTTAGTCCAATACCGTGAAAGTCACCATCGGCGGCTCGT
TTTTCGCGACGACGGCGAACAGCGTAGTGTTTGAGAACGGGTTG
GAGTTTAACGCAGCCAATCCCGAAAACAGTACAGCACTGTTGAG
TATCAACGTGCCGTTGGGATTGCAGTTTGGAGAAAATCCGGGAG
CGATTCAGGGGATGGGGTCTGTGTTAAGCGTGCCAGCGGGTGAA
GCCTTGGCTCTCATGGGCGGATCGATCGATCTCGAGGGGGTCGC
GATCGAAGCACCGGGCGGACGAGTCGACCTGGGAGAAGGGCCG
CGAGATCTGGATCCTTTATAA

7. *Geitlernima* sp. MCCB 234, 16s-23S ribosomal RNA partial sequence

Accession Number-KP 019934

GCCTGCCGAGTATTACTCTAACCGTTCGCGGAGGAGGGTGCCGA
AGGCAGGGCTGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
ACCGGAAGGTGTGGCTGGATCACCTCCTTTTAGGGAGACCGTTCC
CCAATCGAGTGAAGGATGTAAGTAAGCCCGCTCGATTGAGGTCA
ACCGAGGTCGGTCGAAGAGAATGCGTCGTGTTTTCAAACGAACG
AGGAACGGACTCCGGGGAGAAATTCCCAACCTCAGAGTCGAGAA
CGTGGGCTATTAGCTCAGGTGGTTAGAGCGCACTCCTGATAATGG
GTGAGGTCCCTGGTTCAAGTCCAGGATGGCCCACCTAACTCAGTC

AGCAGTGAACGGCGAACAGCGAACAGTGAAAACTGATGACTGTG
GGCTGGTGACGGTTGACCGAACTTGTGGGGGTATAGCTCAGTTG
GTAGAGCGCCTGCTTTGCAAGCAGGATGTCAGCGGTTCGAGTCC
GCTTACCTCCACCTCGTCTCGAAACTCAAATCCCAAGAGCGAGA
GTCTCCAGCACCTGAGAAAGCTCAGACTGCTGGACTTCGAGTCC
AGTGAGAACCTTGAAAACTGCATAGTCATCAAAACAATCCTAAG
AAAAGCCGAGACCTCGAGTGATGAGTCAAGAACCAGTTC

8. RuBisCo chaperonin RbcX gene of *Geitlerinema* sp. MCCB 234, partial cds.

Accession Number-KX 784510

CTCTTGAGATTCTTCCGTCGAATCGATTTCAGGATGGGTTTCCGT
CGAATCGATTCAGGATGGGTTTCCGTCGAAGACGTAGGGGCAAC
CCCTGGGGCTGTTTCTTTCACGTTCCAGAATTTGGCGGCGACAGT
CAGTATTCGATTTCCCAATGTTCGATCGAACCATTTCGGGAAGAA
AATCCAACACGGCTTCAGCAAGGTGTTCGCGAACCGTCAAAATT
CGCAAAACCCATTCTTTGTTCTCCTGCATCAACGATTGCAGAAAG
GCTTCGCCATCTTGGACGCGGTTTTCCGCCGATTGCTGCCTCAGC
CAAATCGCTTGGGACGGGTTCGTCTCCGCCAGTTGGTCGATAATC
GTCCGCACGGCTTGATAGGTCAAGTAACTTT
GCAAAACCTTCGCCGTGTCG TTCGCCACTT TTTTCGGATACAT

9. *Geitlerinema* sp. MCCB 234 phycocyanin beta subunit (cpcB) and phycocyanin alpha subunit (cpcA) genes, partial cds

Accession Number-KF 494336

CATCCTGCGCTACGTCACCTACGCGATCTTTGCTGGCGATGCCAG
CGTTCTCGACGATCGCTGCTTGAACGGTCTGCGTGAAACCTACGT
GGCTCTCGGAACCCCCGGTGCATCTGTAGCTGTGGGCGTTCAAAA
AATGAAAGAAGCGGCTCTGCAAATCGTCGGCGATCCCGCTGGTA
TCACCCCCGGAGATTGCAGCAGCCTGATGTCTGAAATCGGCACCT
ACTTCGATCGCGCGGCGGCTGCGGTTGCGTAATTCGCACCCGAA
AGCGTGCTGTCAAGTCCGCCAAGCGGACGATTCAATTTGAAAAT
CGTTTTGACACCCAAAAAGGAGATTACTCAATGAAGACCCCGTT
AACCGAAGCAGTCGCTGCCGCAGATTCTCAAGGTCGCTTCCTCAG
CAGCACCGAACTCCAAACCGCGTTCGGTCGCTTCCGTCAAGCCA
ATGCTGGCTTGCAAGCCGCTCGTCAACTGACTGACAAATCTCAGT

CTCTCATCGACGGCGCTGCACAAGCCGTGTATAACAAATTCCCCT
ACACCACGTCA

10. *Geitlernima* sp. MCCB 234, ITS gene (16S-23S ribosomal RNA intergenic space partial sequence)

Accession Number- KU761995

GCCTGCCGAGTATTACTCTAACCGTTCGCGGAGGAGGGTGCCGA
AGGCAGGGCTGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
ACCGGAAGGTGTGGCTGGATCACCTCCTTTTAGGGAGACCGTTCC
CCAATCGAGTGAAGGATGTAAGTAAGCCCGCTCGATTGAGGTCA
ACCGAGGTCGGTCGAAGAGAATGCGTCGTGTTTTCAAACGAACG
AGGAACGGACTCCGGGGAGAAATTCCCAACCTCAGAGTCGAGAA
CGTGGGCTATTAGCTCAGGTGGTTAGAGCGCACTCCTGATAATGG
GTGAGGTCCCTGGTTCAAGTCCAGGATGGCCCACCTAACTCAGTC
AGCAGTGAACGGCGAACAGCGAACAGTGAAAACTGATGACTGTG
GGCTGGTGACGGTTGACCGAACTTGTGGGGGTATAGCTCAGTTG
GTAGAGCGCCTGCTTTGCAAGCAGGATGTCAGCGGTTCGAGTCC
GCTTACCTCCACCT

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