Materials and Methods
2. MATERIALS AND METHODS

2.1 Glassware

All the glassware used in the present experiments including test tubes, pipettes, measuring cylinders, culturing flasks, reagent bottles, petri plates, screw cap test tubes, screw cap bottles were of Borosil and Duran brand.

2.2 Cleaning

The glassware used in the experiments were initially soaked in dilute chromic acid (5 % w/v of potassium dichromate in 10 % v/v sulfuric acid) for 24 h and cleaned with tap water and teepol (detergent). After removing all the traces of the detergent, the glassware were rinsed with deionized water and kept in oven for drying at 70 °C.

2.3 Water

Single and double distilled water obtained from Milli-Q plant stored in white carboys was used for rinsing of glassware and for preparation of media, stock solutions and chemical analysis. Milli-Q water was used for HPLC analysis.

2.4 Gases used

Argon, hydrogen and nitrogen gases are used in the present study were of 99.9 % purity while oxygen and acetylene were of commercial grade obtained from Goyal gasses Pvt. Ltd., Hyderabad.

2.5 Chemicals

All the chemicals used in the present study were of analytical grade from Sigma-Aldrich, Lancaster, Himedia, Qualigens, Merck, Genei and GE health care.

2.6 Determination of pH

pH was determined using a digital pH meter (Digisun Electronics, India model DI-707).

2.7 Sterilization

Sterilization of the culture media and glassware was done by autoclaving at 15 lbs for 15 minutes. Heat labile compounds were sterilized by filtration (Millipore) of their aqueous solutions through a 0.45 μm cellulose acetate membrane.

2.8 Collection of sample

Marine sediment water sample was collected from the fishing harbor of Visakhapatnam, India during March 2004, in sterile screw cap polypropylene bottles of 20 ml capacity, brought to the laboratory and were transferred to enrichment broth within 3 to 7 days of sample collection. The water was polluted with fish waste and oil from fishing boats.
2.9 Enrichment, isolation and purification

Water sample collected from sea shore was kept for enrichment in three media compositions with 2 % NaCl, viz photoautotrophic medium with sulfanilate as electron donor, the second with sulfide as electron donor and the third is the photolithoheterotrophic medium with sulfide and pyruvate as electron donor and carbon source respectively. Enrichment culture of the isolate was purified by repeatedly streaking on agar slants with mineral medium (Table 4) under N₂ gas phase in 25 x 150 mm test tubes containing 15 ml medium till all the colonies appearing on two successive slants were identical. Purity of the culture was checked by streaking on nutrient agar (Difco Manual, 1998) plates (g l⁻¹: peptone, 5; yeast extract, 3; NaCl, 5; and agar, 15) and incubating under aerobic and anaerobic illumination (2,400 lx)] conditions at 30±2 C. Contamination from other phototrophic bacteria was checked by monitoring the cultural characters like color of the culture, colony morphology and by microscopic observation.

2.10 Quality check of cultures

Broth culture was checked for purity before and after experimentation by streaking on nutrient agar plates [Difco Manual, 1998] (g l⁻¹: peptone-10, NaCl- 5, yeast extract-3 and agar-20) and incubating (illumination (2,400 lx) at 30±2°C.

2.11 Maintenance of stock cultures

2.11.1 Agar stabs

Stock culture of the purified isolate was maintained as agar stabs or as broth cultures. Stabs were prepared using growth medium (Table 4) with 2 % (w/v) agar as solidifying agent, filled to ¾ volume of 5 ml capacity screw cap test tubes. The culture was stabbed into the agar deeps and incubated under illumination (2,400 lx) at 30±2°C. After 6-8 days of growth, the stab cultures were preserved under refrigeration at 4°C until further use. The stabs were sub-cultured every 90 days and contamination from other bacteria was checked periodically by microscopic examination and by streaking on nutrient agar (Difco Manual, 1998) plates (g l⁻¹: peptone-5, Yeast extract-3 and agar-15) supplemented with 2 % NaCl (w/v) both under aerobic and anaerobic conditions.
2.11.2 Agar slants

Stock culture of the isolate was maintained as agar slants. Slants were prepared by using 2 % (w/v) agar solidified nutrient broth filled to 1/10 volume in test tubes in slanting position. Culture was taken onto the inoculation loop and streaked onto the slant. After 48 h of incubation, slant cultures were preserved under refrigeration at 4 °C until further use. The slants were sub cultured every 90 days and purity was checked periodically by streaking onto nutrient agar plates.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g l⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>0.25</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>20</td>
</tr>
<tr>
<td>NH₄Cl</td>
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<tr>
<td>CaCl₂·2H₂O</td>
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<tr>
<td>Pyruvate</td>
<td>3.0</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.4</td>
</tr>
<tr>
<td>Ferric citrate solution (0.1 %, w/v)</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>*Micronutrient solution (SL7)</td>
<td>1 ml</td>
</tr>
<tr>
<td>**Na₂S·9H₂O/Na₂S₂O₅·5H₂O</td>
<td>2 ml</td>
</tr>
<tr>
<td>***Vitamin B₁₂ (2 mg/100 ml, w/v)</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

*SL7: (mg l⁻¹): HCl (25%, v/v) – 1 ml; ZnCl₂ (70); MnCl₂·4H₂O (100); H₃BO₃ (60); CoCl₂·6H₂O (200) NiCl₂·6H₂O (20); Na₂MoO₄·2H₂O (40); CuCl₂·2H₂O (20)

**Na₂S·9H₂O solution Na₂S·9H₂O (2.4 g) was dissolved in deionized water (10 ml) in 15ml screw cap test tube and flushed with nitrogen gas for 2-3 minutes to replace the air in the tube with nitrogen gas and closed tightly and was autoclaved.

***Vitamin B₁₂ Vitamin B₁₂ (2 mg/100 ml, w/v) was dissolved in distilled water and filter sterilized by using 0.2 µm pore sized Millipore cellulose acetate membrane filters into a sterile screw cap tube.

Final pH of the medium was adjusted to 7.0-7.5 with sterile HCl (1 N)/ NaOH (1 N).

Table 4: Composition of mineral medium (Lakshmi et al., 2009) used for the growth of purple sulfur bacteria.


2.12 Morphological Characterization

2.12.1 Direct microscopic observation

Morphological properties such as cell shape, size, division, aggregate formation and presence of sulfur globules were visualized with a phase contrast microscope (Olympus- B201) on agar-coated slides according to the method of Pfennig and Wagener (1986) and motility of the culture was observed on slides without agar coat.

2.12.2 Gram staining

A loopful of logarithmically growing culture was taken on a clean slide, smear was prepared, dried and was fixed by heat. The slide was then flooded with crystal violet (10 g of crystal violet is dissolved in 100 ml of absolute ethanol, filter sterilized and the volume made to 1000 ml with double distilled water), and stained for a minute, then washed under tap water. In the next step Gram’s Iodine solution (Iodine 1 g, potassium iodide 2 g, dissolved in 300 ml double distilled water), a mordant was added to the slide for a minute. After washing with water decolorization of the dye was done with acetone. In the final step safranin is applied as a counter stain, washed with running water, air dried and the slide was observed under microscope (Olympus- B201) at 100 X magnification.

2.12.3 Negative staining of flagella for Transmission Electron Microscopy

Negative staining of flagella and observation under TEM was done at RUSKA Labs, College of Veterinary Sciences, SVVU, Rajendranagar, Hyderabad. 5 ml of well-grown culture was centrifuged at 4000 rpm (eppendorf AG 22331 Hamburg) for 5 minutes and the pellet was suspended in 0.1 M phosphate buffer, centrifuged at 4000 rpm for 5 minutes. The supernatant was replaced with 1 ml of fresh phosphate buffer. A small drop of sample was placed on a piece of para-film and a carbon coated EM grid was placed on that drop. After 20 minutes the grid was removed and the excess sample was drained with filter paper. The grid was washed with distilled water and stained with 2% (w/v) uranyl acetate. The grid was washed and allowed for air drying. The grid with sample was observed under transmission electron microscope (Model: Hitachi, H-7500).

2.12.4 TEM (sectioning) for Intra cytoplasmic membrane (ICM) structures

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

2.13 Chemotaxonomic Characterization

2.13.1 Pigments analysis

2.13.1.1 Whole cell absorption spectrum

Absorption spectrum of whole cells was measured by the sucrose method of Truper and Pfennig (1981). To 3.5 ml of the liquid culture, 5 g of sucrose was added and mixed thoroughly on a vortex spinner. The absorption spectrum from 300-1100 nm was measured on a Spectronic Genesys 2 spectrophotometer using sucrose in the medium as blank.

2.13.1.2 Identification of carotenoids by HPLC

Ten times concentrated cell suspension was taken and extracted into acetone and methanol (7:2) which was concentrated in vacuum concentrator and analyzed through HPLC analysis using Acetonitrile : Methanol : Ethyl acetate (5:4:1) solvent system, at a flow rate of 1ml/min. was performed at room temperature using Shimadzu SPD 10AVP isocratic system, Luna 5μ C18 100A column (250 x 4.6mm) and the compounds were detected in PDA detector at 450nm.

2.13.2 Quinones analysis

The protocol followed was according to Collins et al., 1977; Hiraishi et al., 1984. Approximately 500 mg of freshly freeze-dried cells were suspended in 30 ml of chloroform:methanol (2:1, v/v) and gently stirred over night. The suspension was then filtered using a Whatmann No.1 filter paper and the filtrate containing menaquinones, rhodoquinones and/or ubiquinones was dried in a rotavapour.
2.13.2.1 Separation of menaquinones, rhodoquinones and ubiquinones by TLC

The above sample was dissolved in acetone and chromatographed on a silica gel TLC plate and developed with a petroleum ether and diethyl ether (85:15, v/v) or benzene. The separated quinones were then visualized under a UV lamp. Menaquinones, ubiquinones and rhodoquinones which appeared as a band were scrapped and eluted with acetone.

2.13.2.2 Separation of ubiquinones, rhodoquinones and menaquinones by HPLC

The ubiquinones, rhodoquinones and menaquinones fraction obtained following TLC purification was dissolved in 500 µl of acetone and 200 µl of the suspension was chromatographed on a Luna 5µm Phenomenex RP C18 100A Reverse Phase Column (250 x 4.6 mm) fixed to a photodiode-array detector (SPD-M20A, 220–800 nm, Prominence, Shimadzu LC 20AT, Japan) connected to the HPLC system. An isocratic gradient of methanol: isopropyl ether (3:1, v/v for ubiquinones and menaquinones; 4:1, v/v for rhodoquinones) was used for elution, the flow rate is 1.0 ml/min and detection is at 250, 280 and 270 nm, respectively.

2.13.3 Cellular fatty acid composition

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

2.13.4 Endometabolome analysis through FT-IR analysis

For metabolome fingerprinting, exponentially growing phototrophically cultures were harvested by centrifugation (23,000g for 15 min) and the resultant pellets were washed twice with distilled water. The cell pellet (1g) was quenched in liquid nitrogen (-196°C) according to the protocol of Chassagnole et al. (2002) and the pellet was freeze-dried. The freeze-dried pellet was mixed with KBr and pelletized, the KBr pellets were used for recording spectra between 4000 and 450 cm-1 (at a resolution of 4 cm-1) by using a Fourier transform infrared (FT-IR) spectrometer (Spectrum 100; Perkin Elmer) equipped with a KBr (Potassium bromide) beam splitter and a DTGS (deuterated
Materials and methods

2.14 Determination of growth

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

2.15 Physiological Characterization

2.15.1 Growth modes

2.15.1.1 Photolithoautotrophy

Photolithoautotrophy of the culture was studied by inoculating the culture in fully filled screw cap tubes (10 × 100 mm) with mineral medium [anaerobically in the light (2,400 lx) with Na₂S.9H₂O (2 mM) / Na₂S₂O₃ .5 H₂O (5 mM), sulfite (2 mM) as the electron donor and NaHCO₃ (0.1 %, w/v) as sole/principal carbon source respectively and incubated at 30±2°C.

2.15.1.2 Photoorganoheterotrophy

Photoorganoheterotrophy of the culture was studied by inoculating the culture in screw cap tubes (10 × 100 mm) fully filled with mineral medium with pyruvate (0.3 %, w/v) as carbon source and incubated anaerobically in the light (2,400 lx) at 30±2°C.

2.15.1.3 Chemolithoautotrophy

Chemolithoautotrophy of the culture was studied by inoculating the culture in to 100 ml of modified mineral medium in 250 ml conical flask [aerobically in the dark with Na₂S₂O₃.5H₂O (5 mM) as the electron donor and NaHCO₃ (0.1 %, w/v) as carbon source and incubated in incubator (micro aerobic conditions) with orbital shaker (aerobic – 100 rpm) at 30±2°C.

2.15.1.4 Chemoorganoheterotrophy

Chemoorganoheterotrophy of the culture was studied with pyruvate as a carbon source and electron donor by inoculating the culture in to 100 ml of mineral medium in a 250 ml conical flask [aerobically in the dark] and incubated in both incubator (micro aerobic conditions) with orbital shaker (aerobic (100 rpm) at 30±2°C.
2.15.1.5 Fermentative mode

Fermentative growth mode of the culture was tested by inoculating the culture in the fully filled screw cap tubes (10 x 100 mm) with mineral medium (Table 4) [with glucose/fructose/pyruvate (0.3 %, w/v) as carbon source] and incubated anaerobically in the dark at 30±2°C.

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

1 % of the inoculum was inoculated into mineral medium (Table 4) with NH₄Cl (0.07 %, w/v) and yeast extract as a source of nitrogen and growth factors, respectively with the test organic or inorganic compound serving as the electron and/or carbon source. Growth was monitored turbidometrically (OD₆₆₀) in fully filled 10 x 100 mm screw cap test tubes after phototrophic (2,400 lx for purple sulfur bacteria) incubation at 30±2°C. Various organic carbon sources viz., sugars, sugar alcohols, fatty acids, alcohols, tricarboxylic acid (TCA) cycle intermediates, aromatic compounds and a few inorganic compounds such as sodium sulfide, H₂ and thiosulfate were tested for their utilization as electron and/or carbon source. Sugars, sugar alcohols and TCA cycle intermediates were used at a concentration of 0.3 % (w/v). Fatty acids, alcohols and thiosulfate (filter sterilized using 0.45 µm cellulose acetate membrane) at 0.1 %, w/v, benzoate at 1 mM, sodium sulfide at 2 mM, while H₂ was used at a concentration of 20 % v/v of gas phase.

2.15.3 Utilization of various nitrogen sources

Mineral medium (Table 4) was used in the presence of various inorganic (sodium nitrite, sodium nitrate and NH₄Cl, 0.07 %, w/v), organic substrates (glutamine, glutamate and urea, 0.07 %, w/v), sulfanilate, anthranilate (1 mM) and N₂ (100 %, v/v of gas phase) serving as sources of nitrogen. Media with out any nitrogen source served as the control. Growth was monitored turbidometrically in fully filled 10 x 100 mm screw cap test tubes after phototrophic incubation at 2,400 lx and 30±2°C.

2.15.4 Utilization of various sulfur sources

Mineral medium (Table 4) was used in the presence of various inorganic compounds [magnesium sulfate (5 mM), sodium sulfite (5 mM), sodium sulfide (1 mM) and sodium thiosulfate (5 mM)], organic compounds [thioglycolate (5 mM), and cysteine...
(5 mM), alkylsulfonates (methanesulfonate, Taurine and butanesulfonate) and arylsulfonates (benzenesulfonate, 4-toluenesulfonate, 4-sulfobenzoate, 5-sulfosalicylate and sulfanilate (1mM)) serving as sources of sulfur. Media with out any sulfur source but with magnesium chloride (0.2 %, w/v) served as the control. Growth was monitored turbidometrically in fully filled 10 x 100 mm screw cap test tubes after phototrophic incubation at 2,400 lx and at 30±2°C.

2.15.5 Vitamin requirement

1 % inoculum (culture) was inoculated in mineral medium (Table 4) with NH₄Cl (0.07 %, w/v) as nitrogen source, devoid of yeast extract and replaced with the test vitamin solutions (Biotin [15 μg l⁻¹], Thiamine [500 μg l⁻¹], Nicotinic acid [500 μg l⁻¹], para-Aminobenzoic acid [300 μg l⁻¹], Pyridoxal Phosphate [15 μg l⁻¹], Calcium pantothenate [10 μg l⁻¹], B₁₂ [15 μg l⁻¹]) filter sterilized on a 0.45 μm cellulose acetate membrane. Growth was monitored in 10 x 100 mm fully filled screw cap test tubes under phototrophic conditions at 2,400 lx and 30±2°C. Repeated subculture without the vitamins was carried out for three subsequent transfers to determine absolute requirement.

2.15.6 Saline requirement and tolerance

To the prepared mineral medium (Table 4) with NH₄Cl (0.07 %, w/v) as nitrogen source, different concentrations (0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 %) of sodium chloride were added before autoclaving. Media with out NaCl served as control. One percent of the culture was inoculated and incubated under phototrophic conditions at 2,400 lx and at 30±2°C.

2.15.7 Sulfide tolerance

Growth of the culture at different concentrations of sulfide (0.5, 1, 3, 5, 7, 9, 10, 15, 17, 20 and 40 mM) was monitored by incubating the inoculated cultures in the growth media (Table 4) under phototrophic conditions at 2,400 lx and 30±2°C. Media without sulfide served as control.
2.15.8 Growth at different temperatures

Growth of the culture at different temperatures (15, 20, 25, 30, 35, 40, 50°C) was monitored by incubating the inoculated cultures in the growth media under phototrophic conditions at 2,400 lx.

2.15.9 Growth at different pH

Growth of the culture at different pH (4, 5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 10) was monitored by incubating the inoculated cultures in the growth media under phototrophic conditions at 2,400 lx, at 30±2°C.

2.16 Genetic Characterization

2.16.1 G+C mol % determination

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

Protocol for DNA extraction and purification

2 to 3 g wet packed cells

↓

Suspended in 50 ml Saline – EDTA (0.15 M NaCl + 0.1 M EDTA pH 8)

↓

Centrifuged (10,000 rpm, 10 minutes)

↓

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

↓

*Lysed the cells

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

OR

10 mg of lysozyme was added to the cells suspended in 25 ml Saline - EDTA. The mixture was incubated at 37°C with occasional shaking till increase in viscosity was observed which indicated the lysis of the cells (30–60 minutes). When lysozyme was used, SDS was also added after the cells had lysed followed by the 60°C heating and cooling].
Materials and methods

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

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

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

Pipetted out upper aqueous layer which contained nucleic acids.

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

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

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

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

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

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

Added 95 % (v/v) ethyl alcohol to the supernatant; dispersed the precipitate in ½ to ¾ of the supernatant volume.

Added ribonuclease [(50 μg ml⁻¹) ribonuclease was dissolved in 0.15 M sodium chloride pH 5 and heated at 80°C for 10 min to inactivate any DNAase present]

Incubated for 30 min at 37°C.
Materials and methods

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

To the supernatant 95 % (v/v) ethyl alcohol was added to precipitate the nucleic acids

Dissolved the precipitate in 9 ml dilute saline citrate

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

While stirring the solution rapidly with glass rod isopropyl alcohol was added drop wise into the vortex

DNA as fibrous material was collected on glass rod

Washed the DNA isolated with ethyl alcohol

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

25µl of the DNA stock in concentrated buffer solution was diluted to standard saline buffer concentration with diluted saline citrate buffer (0.015 M NaCl + 0.0015 M trisodium citrate) and the absorption at 260 nm was noted by spectrophotometry.

(ii) Lysis of the DNA 5 mg of purified DNA was lysed to bases with 0.1 ml of perchloric acid in glass stoppered bottle at 100°C in water bath for 1 h. Standard DNA obtained from Himedia chemicals was also lysed by the same method. After the hydrolysis the black char was homogenized with sterile glass rod, diluted to 0.5 ml with water. The DNA lysate was centrifuged; supernatant was collected and filtered with 0.2 µm cellulose nitrate filter.

(iii) Standard DNA bases preparation and chromatographic conditions Standard bases A, T, G and C (obtained from Hi-media) were dissolved in 0.1 N HCl to a concentration of 1 mM. The flow-rate of the solvent was 1.0 ml/min at a temperature of 37°C. The solvent was prepared by combining 40 ml of 0.5 M triethylamine phosphate (TEAP), pH 5.1, with about 750 ml of Milli-Q water. HPLC-grade methanol (120 ml) was

Catabolism of 4-toluenesulfonate.......
added, and the volume was adjusted to 1 l (Mesbah et al., 1989). The solvent was then filtered through a 0.2 μm Nylon membrane filter. To prepare 0.5 M TEAP solution, triethylamine was diluted with water, the pH was adjusted to 5.1 with 85 % phosphoric acid, and the solution was brought to its final volume.

(iv) HPLC Analysis 25 μl of the lysed DNA sample was injected and the bases were detected based on the retention time of the standard bases (A, T, G and C). The concentrations of the bases were calculated by comparing with that of standards and the mol % G+C was calculated using the concentrations of the bases in the formula:

\[
mol \% \ G+C = \frac{G+C}{A+G+C+T} \times 100
\]

2.16.2 16S rRNA gene sequence analysis

2.16.2.1 16S rRNA gene amplification

(i) DNA extraction DNA was extracted and purified as described earlier (Section 2.16.1.i)

(ii) Agarose gel electrophoresis 10 μl of genomic DNA and 10 μl of standard genomic DNA (as marker) were electrophoresed (Bangalore GENEI) in 0.8 % (w/v) horizontal agarose gel in TAE buffer at 15 V cm⁻¹, stained in 0.5 μg ml⁻¹ ethidium bromide and visualized on UV transilluminator (Bangalore GENEI).

(iii) Amplification of 16S rRNA gene Amplification is performed on 50 μl volumes in 0.2 ml microfuge tubes using a DNA thermal cycler (MJ Mini Personal Thermal Cycler – BIO-RAD). All plastic ware were autoclaved and ultraviolet irradiated. The primers used for the amplification of the 16S rRNA gene are Eub27F (5’GAGTTTAGATCTGCTAG-3’) and Univ1492R (5’-GGTTACCTTGGTACGACT-3’). The concentration and volume of the reaction mixture are as follows.

1. Primers: 2 μl of each primer (10 pmol μl⁻¹); 2. Template: 2 μl of DNA template (25ng μl⁻¹); 3. Water: 21 μl and 4. PCR Master mix: 25 μl (Bangalore GENEI [Cat. No.105908])
Table 5: PCR programme for amplification of 16S rRNA gene

<table>
<thead>
<tr>
<th>No. of cycles</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Elongation</th>
</tr>
</thead>
<tbody>
<tr>
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<td>50</td>
<td>72</td>
</tr>
<tr>
<td>1</td>
<td>42</td>
<td>72</td>
<td>5 min</td>
</tr>
</tbody>
</table>

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

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

(vi) 16S rRNA gene sequencing and assembling of the 4 partial sequences The complete length of the 16S rRNA gene sequence was obtained by sequencing with 4 primers Eub27F (5'-GAGTTTGATCCTGGCTCAG-3’), 5'-372F (5'-TACGGGAGGCAGCGAG-3’), 5'-790F(5'-GATACCCCTGGTAATCTTG-3’) and Univ1492R (5'-GTTACCTTGTAGACT T-3’). The 16S rRNA gene amplicon was sequenced at MWG, Bangalore, India. The four sequences obtained as *scf format were assembled using software SeqMan in the DNA STAR Lasergene 6 package.

(vii) BLAST search The single contig of sequence of length approximately 1350 to 1450 bp was submitted to the NCBI-BLAST search in order to know the nearest phylogenetic relative. EzTaxon server (web based database of 16S rRNA gene sequences – Easiest way to the accurate identification of prokaryotes) is more useful for comparison of 16S rRNA gene sequences with type strain sequences.
2.16.2.2 Phylogenetic analysis

(i) Collection of 16S rRNA gene sequences of the type strains

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

(ii) 16S rRNA gene Sequence Alignment

Sequences (all the closely related type strain sequences along with an out group sequence and the sequence to be analyzed) were aligned using the CLUSTAL X program (Thompson et al., 1997). The alignment file was opened with BioEdit software and the alignment was corrected manually, and the file was saved with “.phy” extension which is an input file for the programs used for phylogenetic analysis.

(iii) Methods for phylogenetic tree construction

Distance based method was used for phylogenetic analysis. **Neighbor joining method in MEGA4 software:** The evolutionary distances were calculated by using the kimura-2 parameter (Kimura, 1980) in a pair wise deletion procedure. The evolutionary tree was constructed using the neighbor-joining method within the MEGA4 software (http://www.megasoftware.net/mega4.pdf) and percentage support values were obtained using a bootstrap procedure based on 100 resamplings.

2.16.3. Multilocus Gene Sequence Analysis (MLSA)

Amplification of four universally present housekeeping and metabolic genes specific for purple sulphur bacteria encoding photosynthetic reaction centre M subunit (*pufM*), molecular chaperonin HSP 60 (*dnaK*), DNA repair and recombination factor recombinase A (*recA*), protein synthesis and translation elongation factor (*fusA*) including the genes for 16S rRNA and 16-23S rRNA internal transcriber spacer (ITS) region was done.

Due to unavailability of specific primers and parameters for amplification of these genes for purple sulfur bacteria, Touchdown PCR amplification (TD-PCR) was carried out for amplification of genes for *fusA*, ITS region, *dnaK*, *recA* and *pufM* as done by Serrano.
et al., (2010) with some modifications. **Table 6** gives the list of primer sequences, approximate size (bp) of the amplified fragments and parameters for amplification of the genes in this study.

**I** Amplification parameters for **fusA**, **ITS region**, **dnaK** and **recA**

A: (TD-PCR): initial denaturation at 94 ºC for 2 min, 10 cycles of denaturation at 94 ºC for 1 min, (initial) annealing temperature ranging 65-55 ºC for 1 min (with progressive lowering at 1 ºC/cycle) and elongation at 72 ºC (1 min), followed by 20 cycles at 94 ºC for 1 min, final annealing temperature 55 - 45 ºC (1 min), elongation at 72 ºC (1 min) plus a final extension for 5 min at 72 ºC.

B: Amplification parameters for **PufM** gene amplification

Initial denaturation at 94-95 ºC for 2-4 min followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing temperature ranging 65- 55 ºC, elongation at 72 ºC for 1-2 min, with a 10-15 min of final elongation time at 72 ºC.

All sequences were deposited in European Molecular Biology Laboratory (EMBL) Nucleotide sequence Database, (www.ebi.ac.uk/EMBL). The accession numbers and length of concatenated sequences of all the genes are listed in **Table 7**.

**II** Phylogenetic analysis of sequences

Individual gene sequences of all the strains were first aligned by ClustalX and the alignments were improved by removing hypervariable regions within each gene sequence by using the online programme Gblocks (Castresana 2000). The similar length gene sequences were concatenated and further Gblocks edited to yield a uniform sequence length of 1869 bp for all strains. The phylogenetic tree of concatenated sequences was constructed using the ClustalW logarithm of MEGA version 4.0 (Tamura et al., 2007), and the distance was calculated with default parameters, the K2P distance model and the neighbor, algorithm, pair-wise deletion procedure (Saitou and Nei 1987).
Table 6: List of target loci and primers used for multilocus sequence analysis (MLSA) of strain JA121

<table>
<thead>
<tr>
<th>Target loci</th>
<th>Function of gene/protein</th>
<th>Amplicon size(bp)</th>
<th>Primer Name</th>
<th>Primer sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S-23S rRNA (ITS)</td>
<td>(16S-23SrRNA)</td>
<td>500</td>
<td>ITSi ITSr</td>
<td>GTGAAGTCGTAACAAGG CGTCACTCGACCATATCC</td>
<td>Garcia et al., 1996</td>
</tr>
<tr>
<td>PufM</td>
<td>Reaction center M</td>
<td>225</td>
<td>pufM.557F pufM.750R</td>
<td>CGCACCTGGACTGGAC CCCATGGTCCAGCCAGAA</td>
<td>Achenbach et al., 2001</td>
</tr>
<tr>
<td>RecA</td>
<td>Recombinase A</td>
<td>450</td>
<td>recABDUP1 recABGDN2</td>
<td>CCCGAGTCCTCGGNAARACN ACNACNACYTCRTCNGGRTT</td>
<td>Santos and Ochman 2004</td>
</tr>
<tr>
<td>DnaK</td>
<td>Chaperonin, HSP 60</td>
<td>850</td>
<td>INT1 INT2</td>
<td>CATCGGCATCATGGCNCAAYTHGA CAGCATCGGCTGCAYNCCYTTRT</td>
<td>Serrano et al., 2010</td>
</tr>
<tr>
<td>FusA</td>
<td>Translation elongation factor G</td>
<td>750</td>
<td>fusAF fusAR</td>
<td>CATCGGCATCATGGCNCAAYTHGA CAGCATCGGCTGCAYNCCYTTRT</td>
<td>Santos and Ochman 2004</td>
</tr>
</tbody>
</table>
### Materials and methods

Catabolism of 4-toluenesulfonate

**Table 7: Genbank accession numbers (gene length) used for MLSA of *Marichromatium* spp.**

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Genbank accession number (gene length)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16S rRNA</td>
</tr>
<tr>
<td><em>Mch. litorei</em> JA349&lt;sup&gt;T&lt;/sup&gt;</td>
<td>AM947937 (1427 bp)</td>
</tr>
<tr>
<td><em>Mch. chrysaorae</em> JA553&lt;sup&gt;T&lt;/sup&gt;</td>
<td>FN813515 (1306 bp)</td>
</tr>
<tr>
<td><em>Mch. gracile</em> DSM 203&lt;sup&gt;T&lt;/sup&gt;</td>
<td>EU850806 (1472 bp)</td>
</tr>
<tr>
<td><em>Mch. indicum</em> JA100&lt;sup&gt;T&lt;/sup&gt;</td>
<td>AJ543328 (1435 bp)</td>
</tr>
<tr>
<td><em>Mch. bheemlicum</em> JA124&lt;sup&gt;T&lt;/sup&gt;</td>
<td>AM180952 (1438 bp)</td>
</tr>
<tr>
<td><em>Mch. purpuratum</em> DSM 1591&lt;sup&gt;T&lt;/sup&gt;</td>
<td>EU850807 (1467 bp)</td>
</tr>
<tr>
<td><em>Mch. fluminis</em> JA418&lt;sup&gt;T&lt;/sup&gt;</td>
<td>FM210274 (1434 bp)</td>
</tr>
<tr>
<td><em>Strain JA121</em></td>
<td>AM179449 (1448 bp)</td>
</tr>
</tbody>
</table>
2.16.4 MLSA barcoding

The concatenated Gblocks edited MLSA (recA, dnaK, pufM, fusA, 16S rRNA and ITS) sequences for each strain were subjected to *in silico* restriction analysis using the NEB cutter V2.0 with NEB restriction enzymes [http://tools.neb.com/NEBcutter2/](http://tools.neb.com/NEBcutter2/) and the restriction bands were used as the MLSA barcode (Shivali *et al.*, 2012).

2.16.5 DNA-DNA hybridization

DNA-DNA hybridization was performed by the membrane filter method (Tourova and Antonov, 1987) involves three steps:

(I) Immobilisation of DNA

About 10 µg of DNA was taken in a microfuge tube to which 20X SSC was added to a final concentration of 6X and the contents were boiled for 10 min. The tube was then chilled immediately on ice and the DNA was immobilized on to a Hybond N+ membrane using a dot blot apparatus. The wells were then washed with 100 µl of 0.5 N NaOH after which the filter was removed, dried and baked in a vacuum oven at 80 °C for 2 h under vacuum.

(II) Radioactive labeling of the DNA to be used as a probe

Nick translation is a procedure by which pre-existing nucleotides in a DNA molecule are replaced by radioactive nucleotides thus, generating $^32$P-labelled DNA with a high specific activity. The procedure takes advantage of the fact that *E.coli* DNA polymerase I is capable of adding nucleotide residues to the 3’ hydroxyl terminus to one strand of a double stranded DNA molecule, which is nicked. Further, the same enzyme by its 5’ to 3’ exonucleolytic activity can remove nucleotides from the 5’ side of the nick. Thus, eliminating nucleotides from the 5’ end and adding nucleotides at the 3’ end and resulting in movement of the nick.

Labelling of the DNA was carried out in a microfuge tube in a 100 µl reaction mix consisting of the ingredients as listed in table 8.
After 2 h of incubation at 15 °C, the reaction was stopped with 8 µl of 0.25 M EDTA and subsequently 56 µl of 5M ammonium acetate, 50 µl of carrier DNA and 500 µl cold alcohol were added. The contents were then incubated at –70 °C for 1 h or over night at –20 °C and spin at 15000 rpm for 20 min at 4 °C. The pellet was washed with 70 % alcohol, briefly dried under vacuum, dissolved in 6X SSC, boiled for 10 min and immediately chilled on ice and used for hybridization.

(III) Hybridization

The baked filter was soaked in the prehybridization buffer (0.5 M Phosphate buffer pH 7.2 and 7 % SDS) for 1 h at a temperature corresponding to 20 °C less than the Tm of the DNA being used for hybridization. Subsequently, the prehybridization buffer was discarded and the probe dissolved in the prehybridization buffer was added and hybridization was done for 16 h at the same temperature as above. The filter was then washed with 0.5X SSC containing 0.1 % SDS for 10 min at room temperature and then with 0.1X SSC containing 0.5 % SDS for 20 min at 50 °C. Subsequently, the filter was dried, exposed to BAS-MS 20/25 cm Imaging Plate (Fuji Photo Film Co., Tokyo, Japan) for 1 h or X-ray film for 24 h and developed using BAS 1800 Bio-Imaging Analyzer, Tokyo, Japan) or an Kodak X-ray film. The autoradiogram was scanned and quantified using a BAS 1800 Bio-Imaging Analyzer (Fuji Photo Film Co., Tokyo, Japan). Simultaneously, the filters were also processed for determination of radioactive counts in the [14C] channel of the Tri-carb liquid scintillation counter (Model No. B1500, Zurich, Switzerland).

### Table 8: List of ingredients and their quantity required for labeling of the DNA in nick translation

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>5 µl (200 – 300 ng)</td>
</tr>
<tr>
<td>10 X Nick translation buffer</td>
<td>5 µl (Supplied by BRIT, India)</td>
</tr>
<tr>
<td>dTTP</td>
<td>7 µl</td>
</tr>
<tr>
<td>dCTP</td>
<td>7 µl</td>
</tr>
<tr>
<td>dGTP</td>
<td>7 µl</td>
</tr>
<tr>
<td>α-32P-dATP</td>
<td>5 µl (50 µCi)</td>
</tr>
<tr>
<td>H2O</td>
<td>64 µl</td>
</tr>
</tbody>
</table>
The percent hybridization was calculated as follows:

\[
\frac{\text{Counts obtained from heterologous hybridization}}{\text{Counts obtained from homologous hybridization}} \times 100
\]

2.16.6 **Plasmid isolation** was done through conventional alkaline lysis method (Sambrook Russell, Molecular cloning, a laboratory manual).

2.17 **Determination of dry weight**

Dry weight of the culture was determined from O.D versus dry weight graph prepared specifically for this culture. An aliquot of the logarithmic culture was centrifuged at 10000 rpm for 10m and the pellet was washed with saline (0.1 % NaCl w/v) and resuspended in distilled water. Known volumes of concentrated cell suspension and its various dilutions were transferred to previously weighed aluminum boats and dried to constant weigh at 60 °C. All weights were determined in a single pan balance. An O.D versus dry wt graph is plotted taking O.D of cell suspension at 660nm. Calculation of the dry wt was done by the empirical formula drawn from the graph.

\[
\text{O.D}_{660} \times 0.1 = 0.3 \text{ mg dry wt.ml}^{-1}
\]

2.18 **Determination of minimum inhibitory concentration (MIC) of arylsulfonates**

MIC of arylsulfonates on growth of *Marichromatium* sp. JA121 was studied in the photoheterotrophic medium along with arylsulfonates at various concentrations in fully filled screw cap test tubes (12x100mm) incubated phototrophically (2,400 lx) at 30 ± 2 °C.

2.19 **Colorimetric Analysis**

2.19.1 **Estimation of sulfite by acidfuchsin method**

Sulfite was quantified colorimetrically as the sulfite-Fuchsin complex. Acidfuchsin reagent was made by mixing the solutions 0.8 M H$_2$SO$_4$, 0.08 %Fuchsin, 1.6 % HCHO in the ratio of 7:2:1. To the 50µl of sample 950 µl of acidfuchsin reagent was added and kept for incubation for 15min at room temperature. The formed sulfite-Fuchsin complex was read at 580 nm (Denger *et al.*, 2001).
2.19.2 Estimation of proteins: Proteins were estimated using Bradford’s method (Bradford, 1976).

2.20 Enzyme Assays

2.20.1 Preparation of cellfree extracts

Forty eight hour old culture was centrifuged (16000xg for 10 min), washed with buffer (0.02 M K-phosphate, pH 7) and the pellet was sonicated (6-8 cycles, probe MS 72 in Bendelin sonoplus sonicator). The sonicated sample was centrifuged (16000xg for 10 min) and the supernatant was used as enzyme source for transformation studies.

2.20.2 4-Toluenesulfonate methyl mono-oxygenase

4-Toluenesulfonate methyl mono-oxygenase activity was assayed as O_2 uptake at 30°C. The reaction mixture contained (in 1.0 ml) 100 µmol of potassium phosphate buffer, pH 6.8, 200 nmol of NADH, 0.5 mg of protein, and the reaction was started by the addition of 15 µmol of 4-toluenesulfonate. Aliquots were drawn at different time intervals, precipitated protein and products were analyzed through HPLC and LCMS analyses (Locher et al., 1991).

2.20.3 4-Sulphobenzoate 3, 4-dioxygenase

The enzyme activity was assayed as O_2 uptake at 30°C with Clarke-type oxygen electrode with a thermostatically controlled (30°C) 1 ml vessel. The reaction mixture contained (in 1.0 ml) 100 µmol of potassium phosphate buffer, pH 6.8, 200 nmol of NADH and 100 µg of protein, and 10 µmol of 4-sulfobenzoate. Aliquots were drawn at different time intervals, precipitated protein and products analyzed through HPLC and LCMS analyses (Thurnheer et al., 1986).

2.20.4 Protocatechuate 4, 5-dioxygenase

Protocatechuate 4, 5-dioxygenase was assayed as O_2 uptake at 30°C. Reaction mixtures contained (in 1.0 ml) 100 µmol of potassium phosphate buffer; pH 6.8, 150 µg of protein and the reaction was started by the addition of 10 µmol of protocatechuate.

2.20.5 Benzylsuccinate synthase

Phototrophically growing (48 h) cells of Marichromatium sp. JA121 on pyruvate (25 mM), succinate (22 mM) and 4-toluenesulfonate (1 mM) was used for assay. Ten ml of the supernatant of sonicated sample was taken in a test tube (25 x 250 mm), sealed with a subba seal and flushed (10 min) with ultra pure argon and incubated for 30 min before
Materials and methods

Catabolism of 4-toluenesulfonate assay. Five ml of the reaction mixture contained 100 µmoles of 50 mM Tris buffer (pH 7.5), 5 µmoles of 4-toluenesulfonate and 10 µmoles of sodium succinate. Reaction mixture was taken in 15 x 150 mm test tubes, sealed with subba seal and bubbled (10 min) with ultra pure argon. Reaction started by the addition of 50 µg of protein (crude cell free extract 100 µl) using a syringe. Samples were withdrawn periodically with a syringe and the reaction was terminated using HCl (1N). The membrane (0.2 um) filtered samples were analyzed using HPLC, LCMS and MS/MS.

2.20.6 Benzylsuccinate synthase assay under aerobic conditions

Similarly with the same reaction mixture contents, benzylsuccinate synthase assay was carried out under aerobic conditions using 2 ml appendors. Samples were withdrawn periodically with a syringe and the reaction was terminated using HCl (1N). The membrane (0.2 um) filtered samples were analyzed using HPLC, LCMS and MS/MS.

2.21 PCR amplification of benzyl succinate synthase (bssA) gene and sequencing

4-Toluenesulfonate induced culture (250 ml) was harvested after 48 h by centrifugation (10000 x g for 10 min). Genomic DNA was extracted from the pellet by the method mentioned in section 2.16.1. PCR amplification was performed using primer sets listed in table 9, designed form alignments of known sequences (EMBL accession numbers: AB285034 [Azoarcus sp. DN11], EF123667 [sulfate reducing bacterium TRM1], EF123666 [Geobacter sp. TMJ1], EF123663 [Desulfobacula toluica], AB167725 [Magnetospirillum sp. TS-6]). PCR amplification for putative bssA gene was performed according to the method of Washer and Edwards (2007) with slight modifications and parameters were mentioned in table10. The products were pooled up and run on a 2 % agarose gel. On agarose gel more than one band was observed the band of the expected length was excised from the gel and purified using a QIAGEN gel purification kit (QIAGEN,.) and given it for sequencing which was outsourced.
Materials and methods

<table>
<thead>
<tr>
<th>Primer sets</th>
<th>Sequence (5’–3’)</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) BSS FP1</td>
<td>ACTGGGTCATAGTGCTGTGATGTCGCCCGG</td>
<td>60</td>
</tr>
<tr>
<td>2) BSS FP2</td>
<td>GTAGACGCAAGACCAAGACCCCGTTCGGAAG</td>
<td>60</td>
</tr>
<tr>
<td>3) BSS RP1</td>
<td>AATCGTGGTGATGGGTTGTGCCAGCCGGTTGGG</td>
<td>60</td>
</tr>
<tr>
<td>4) BSS RP2</td>
<td>GGTTCGCGAGCCCACTTCCATGTAAAGGCCGAC</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 9: Primer sets designed from alignments of known bssA sequences

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
<th>cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>65</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Elongation</td>
<td>72</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Final elongation</td>
<td>72</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Table 10: PCR cycling parameters used to amplify bssA gene in Marichromatium sp. JA121
2.22 Metabolite Analysis through Liquid Chromatography

2.22.1 Extraction of metabolites

Cells of *Marichromatium* sp. JA121 grown in the presence and absence of 4-toluenesulfonate were harvested by centrifugation (16000 x g for 10 min) at 12, 24 and 66 h of phototropic assay was done in fully filled 250 ml reagent bottles under illumination (2400 lux) incubations. The culture supernatant was concentrated to 5 ml in a Rota-evaporator, which was directly used for LC-MS analysis.

2.22.2 Extraction of metabolites from bulk culture supernatant

Metabolites were extracted from the 2L bulk culture supernatant drawn after 48 h of phototropic incubation in the presence of 4-toluenesulfonate by adding 1L of ethyl acetate. The culture supernatant was fractionated into ethyl acetate and aqueous fractions were concentrated in a Rota-evaporator separately, redissolved in methanol. This methanolic extract was analyzed through HPLC and LC-MS analyses.

2.22.3 HPLC Analysis

HPLC analysis of substrates and products was performed at room temperature using Shimadzu SPD 10AVP isocratic system. 50 mM Potassium phosphate buffer and methanol (6:4) mixture was used as solvent at 1 ml.min⁻¹, Luna 5µ C₁₈ 100A column (250 x 4.6mm) and the compounds were detected in UV-VIS detector at 222nm. The retention times (tᵣ in min) of pyruvate and 4-toluenesulfonate was 2.5 and 4.62 respectively.
Materials and methods

Flowchart 1: Isolation of metabolites of 4-toluenesulfonate from the 4-toluenesulfonate induced culture supernatant of *Marichromatium* sp. JA121

AQ layer = Aqueous layer
EA layer = Ethyl acetate layer
2.22.4 LC-MS

Metabolite studies were carried out on Shimadzu LC/MS (LC/MS-2010A). Analysis was performed at 40 °C (LC column oven) and 85 °C (MS ionization chamber). Methanol, water (1:1) was used as a solvent at 0.2 ml.min⁻¹, Luna 5µ C₁₈ (2) 100A column (250X4.6mm) and the compounds were detected (LC) at 254 nm. The column effluent from the LC was mobilized into an Electron Spray Ionization (ESI) region and Atmospheric pressure chemical ionization (APCI) region under N₂ gas for generating molecular masses, which were detected in a negative mode and positive modes.

2.22.5 LC-MS/MS Q-TOF analysis

Liquid chromatography (LC) - mass spectrometry (MS) analysis was performed with Micro mass (Deltanics Brukers), equipped with an auto injector. MS was performed using MS-ESI ion source (Nitrogen flow rate 0.5 /h). Working conditions were in ESI both negative and positive ion mode and the separation was done using Agilent-400 Binary gradient HPLC 1200-series with U.V detector on C18 column (Luna 5 µ 150 x 4.6 mm), analysis was performed at 30 °C (LC column oven) and 85 °C (MS ionization chamber). Mobile phase consisted of acetic acid and water (45:55) the column was equilibrated for 10 min prior to each analysis. Flow rate was 0.8 ml.min⁻¹, injected volume was 10 µl and compounds were detected (LC) at 220 nm. The column effluent from the LC was nebulized into an ion source (ESI) region with collision energy of 10 eV for generating molecular masses.