Catabolism of 4-toluenesulfonate by a newly isolated phototrophic gammaproteobacterium, *Marichromatium* sp. JA121

Thesis submitted for the award of

Doctor of Philosophy

By

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DECLARATION

I E. Shobha, hereby declare that the work embodied in this thesis entitled **"Catabolism of 4-toluenesulfonate by a newly isolated phototrophic gammaproteobacterium, *Marichromatium* sp. JA121"** has been carried out by me under the supervision of Prof. Ch. Venkata Ramana and this has not been submitted to any other Institute or University for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled **"Catabolism of 4-toluenesulfonate by a newly isolated phototrophic gammaproteobacterium, *Marichromatium* sp. JA121"** is a record of bonafide work done by **E. Shobha, a research scholar** for Ph. D programme in Department of Plant Science, School of Life Sciences, University of Hyderabad under my guidance and supervision. We recommend her thesis for submission for the degree of Doctor of Philosophy of the University.

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Dedicated to my
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List of abbreviations

ABRC	Anaerobic Bacterial Resource Center
ABS	Amino benzene sulfonate
ACN	Acetonitrile
AOP	Advanced oxidation process
APB	Anoxygenic phototrophic bacteria
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BChl	Bacteriochlorophyll
BLAST	Basic Local Alignment Search Tool
BSS	Benzylsuccinate synthase
Da	Dalton
DAD	Diode array detector
DDBJ	DNA Data Bank of Japan
DDH	DNA-DNA hybridization
DMSO	Dimethylsulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
ESI	Electron spray ionization
FAME	Fatty acid methyl esters
FMO	Fenna-Matthews-Olson
FT-IR	Fourier transform infra red spectroscopy
GPS	Global Positioning System
GSB	Green sulfur bacteria
HPLC	High-performance liquid chromatography
ICM	Intracytoplansmic membrane
JCM	Japan Collection of Microorganisms
KCTC	Korean Collection for Type Cultures
KEGG	Kyoto encyclopedia of genes and genomes
LAS	Linear alkyl benzenesulfonate

LC-MS	Liquid chromatography mass spectroscopy
m mol	Milli moles
m/z	Mass-to-charge ratio
MEGA	Molecular Evolutionary Genetic Analysis
MeOH	Methanol
mg	Milligram
MIC	Minimum inhibitory concentration
MK	Menaquinone
MLSA	Multilocus sequence analysis
mM	Milli molar
MPa	Megapascal
MS/MS	Tandem mass spectrometry
NADH	(Reduced) nicotinamide adenine dinucleotide phosphate
NBRC	NITE Biological Resource Center
NCBI	National Center for Biotechnology Information
NITE	National Institute of Technology and Evaluation
PCR	Polymerase chain reaction
PDA	Photodiode array
PNSB	Purple non sulfur bacteria
PPM	Parts per million
PSB	Purple sulfur bacteria
RQ	Rhodoquinone
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TCA	Trichloroacetic acid
TDPCR	Touchdown Polymerase chain reaction
TEAP	Triethylamine phosphate
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
TMAO	Trimethylamine-N-oxide
TSA	Toluenesulfonate

Abstract

Arylsulfonates are a class of hydrocarbons with a sulfone functional group covalently attached to the benzene ring by a carbon–sulfur bond, most of these are anthropogenic and cause ubiquitous pollution problem. Biodegradation of arylsulfonates is less understood and much of the work was concentrated on its aerobic degradation while their degradation by anaerobic bacteria is ignored. Anoxygenic phototrophic bacteria (APB) are a diverse group of anaerobic phototrophic prokaryotes primarily contributing to the aquatic habitats and play an important role in the anaerobic nutritional cycles. Among the anoxygenic phototrophic bacteria, purple non-sulfur bacteria (PNSB) metabolizing a wide range of organic compounds including aromatic hydrocarbons and *Rhodopseudomonas palustris* is a model organism for catabolic studies of aromatic hydrocarbons. Utilization of alkylsulfonates (mercaptomalate and taurine) was also possible by this group of bacteria, while metabolism of arylsulfonates was not demonstrated till date. For the present thesis work, a purple sulfur bacterium was isolated from a marine sediment through sulfanilate enrichments and was designated as strain JA121. It has the capability to utilize 4-toluenesulfonate as sole source of sulfur and derives the sulfur through a process of desulfonation. The strain was characterized through polyphasic taxonomic approach and the metabolic pathways of 4-tolueneuslfonate is discussed.

Phylogenetic tree based on the 16S rRNA gene sequence analysis indicated that strain JA121 is a member of the genus *Marichromatium* belonging to the class *Gammaproteobacteria*. 16S rRNA gene sequence similarity of strain JA121 with type strains of *Marichromatium* lies between 94-98 %. However, strain JA121 is 65 % related (based on DDH) to the nearest type strains of *Marichromatium*. Since the value of 56-65 % relatedness is close to the recommended standards (<70 % DDH) to delineate a bacterial species, multilocus sequence analysis (MLSA) was carried out for four protein coding genes viz *recA*, *fusA*, *dnaK*, *pufM*, together with ITS and 16S rRNA genes. Based on the phylogenetic tree constructed for *recA*, *fusA*, *dnaK*, *pufM* and ITS, strain JA121 has 84-99 %, 66-98 %, 49-99 %, 85-100 % and 51-100 % similarity, respectively, with the type strains of the genus *Marichromatium*. The concatenated sequence similarity of the MLSA was 88-99 % along with the MLSA barcoding indicate strain JA121 to be a close

relative of *Marichromatium chrysaorae* JA553^T/ *Marichromatium gracile* DSM203^T but differs with respect to the utilization of arylsulfonates.

Utilization of alkyl/arylsulfonates as sole source of carbon/electron donors by strain JA121 could not be demonstrated. However, arylsulfonates *viz.*, benzenesulfonate, 4-toluenesulfonate, 4-sulfobenzoate, 4-aminobenzenesulfonate and 5-sulfosalicylate were utilized for growth by strain JA121 when used as sole source of sulfur. 4-Sulfobenzoate and protocatechuate were the major metabolites identified from the culture supernatant of strain JA121 when 4-toluenesulfonate was used as sole sulfur source. 4-Toluenesulfonate-methyl monooxygenase and 4-sulfobenzoate 3,4 dioxygenase activities in strain JA121 could be demonstrated, which indicates an oxidative metabolism of 4-toluenesulfonate. In addition, conjugative metabolism of 4-toluenesulfonate was demonstrated in strain JA121 when succinate or fumarate was used as carbon source with the formation of benzylsuccinate and 4-hydroxybenzylsuccinate. Benzylsuccinate synthase activity could be demonstrated in strain JA121 and ~365 bp gene of *bssA* was amplified using designed primer sets. The gene sequence was deposited with NCBI (GQ923885) and the gene has sequence similarity of 40-50% with all reported *bssA* genes.

Based on the experimental evidences, two major pathways of 4-toluenesulfonate metabolism is proposed in strain JA121 which includes an oxidative pathway involving 4-sulfobenzoate and protocatechuate as intermediates and a conjugative pathway involving benzylsuccinate and 4-hydroxybenzylsuccinate as intermediates. These metabolic intermediates were also confirmed by stable isotope probing. In addition, the work concludes in discussing the ecological significance of purple sulfur bacteria in the degradation/transformation of arylsulfonates.

Introduction

1. General introduction

There are over 70,000 synthetic organic chemicals, of which easily biodegradable organic chemicals are generally considered to be non-toxic, while non-biodegradable organic chemicals are considered as recalcitrant, persist in the environment, spread throughout the ecosystems and cause pollution problems. Among the first synthetic organic chemicals to create environmental problems were synthetic detergents (organosulfonates), developed in Germany during World War II because of lack of fats, the normal material from which soap is made.

Organosulfonates are the salts of strong acids, having widespread natural and xenobiotic origin. They have a common trait of a charged sulfonate group, which is regarded as Nature's way to prevent a compound from crossing biological membrane. Naturally occurring sulfonates constitutes O-sulfonates, N-sulfonates and aliphatic C-sulfonates. These are few in number and have important biological functions (Table 1) where as anthropogenic sulfonates include aromatic C-sulfonates (Table 2).

1.2 Naturally occurring organosulfonates

1.2.1 O-Sulfonates: Compounds containing sulfonate moiety attached to the O atom (O-S bond) are termed as O-sulfonates, include tyrosine sulfate in eukaryotic proteins, cerebroside sulfate in human nervous tissue and estrone sulfate in humans (Dodgson *et al.*, 1982)

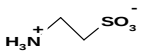
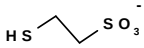
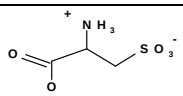
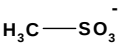
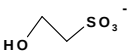
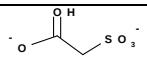
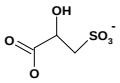
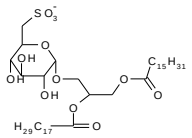
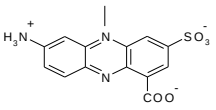
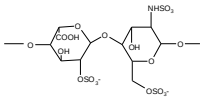
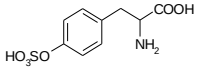
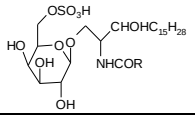
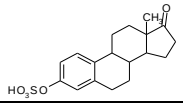
1.2.2 N-Sulfonates: Compounds containing sulfonate moiety attached to the N atom (N-S bond) are termed as N-sulfonates, for example heparin (glycosylaminoglycan of anticoagulant) (Gatti *et al.*, 1979).

1.2.3 Aliphatic C-sulfonates: Compounds containing sulfonate moiety attached to the aliphatic carbon atom (C-S bond) are termed as C-sulfonates, include taurocholate in mammalian gut, capnine a bacterial sulfolipid and few were listed in table 1.

1.2.4 Naturally occurring aromatic sulfonates

Aeruginosin B, a phenazine derivative synthesized by *Pseudomonas aeruginosa* (Herbert and Holliman, 1964), dihydropyoverdin-7-sulfonicacid is a bacterial metabolite (Budzikiewiez *et al.*, 1998), bromoindole sulfonicacid of *Echinodictyum* an Australian marine sponge (Ovendenn and Capon, 1999) and petrobactin sulfonate present in the siderophore of *Marinobacter hydrocarbonoclasticus* (Hickford, 2004) are the naturally occuring aromatic sulfonates reported.

Table 1: Naturally occurring organosulfonates

Compound	Structure	Occurrence	Reference
Taurine		Insects, algae, arthropods and mammals	Huxtable 1992
Coenzyme M		Methanogenic archaea	White 1986
Cysteate		Weathering product in wool, spiders web	Cook *et al.*, 1999
Methanesulfonate		Atmospheric photo oxidation product of dimethylsulfide, marine algae	Baker 1991
Isethionate		Major anion in the squid nerve, red algae	Koechlin 1954
Sulfoacetate		Major spore component	Martelli and Benson 1964
Sulfolactate		Bacterial spores	Benson *et al.*, 1969
Sulfoquinovose		Plant sulfolipid	Benning 1998
Aeuginosin B		Bacteria (*Pseudomonas aeruginosa*)	Herbert & Hollaman 1964
Heparin		A representative of glycosylaminoglycan	Kertesz 2000
Tyrosine sulfate		Eukaryotic proteins	Dodgson *et al.*, 1982
Cerebroside sulfate		Human nerve tissue	Kertesz 2000
Estrone sulfate		Humans	Kertesz 2000

1.3 Anthropogenic sulfonates include arylsulfonates or sulfonated aromatic compounds, in which a sulfonate functional group is covalently attached to the benzene ring by a covalent C-S bond. Aromatic sulfonates are highly acidic and strongly hydrophilic in nature. These are widely used as precursors for synthesis of dyes (e.g. orange II), optical brighteners, pharmaceuticals (e.g. sulfanilamide) (Hooper *et al.*, 1991), softening agents (e.g. phenolsulfonates), preservatives (e.g. sulfanilicacid) and super plasticizers i.e. water reducing admixtures in concrete (e.g. lignosulfonates), synthetic detergents (e.g. linear alkylbenzenesulfonates, LAS) (Schwitzguebel *et al.*, 2002). Environmental relevance of the anthropogenic sulfonates was listed in table 2. Anthropogenic sulfonates can be divided into two main groups; the first group comprises the linear alkylbenzenesulfonates. Their fate in the environment was studied while the second group comprises the aromatic sulfonates.

1.3.1 Recalcitrance of anthropogenic (xenobiotic) sulfonates

The sulfonate group attached to the benzene ring plays an important role in increasing the solubility and dispersion properties of the xenobiotic molecule there by increases its recalcitrance, because of the thermodynamically stable carbon-sulfur bond. Poor biodegradability of arylsulfonates is primarily due to the quaternary carbon that attaches the aromatic structure to the alkyl part of the ABS molecule. Secondarily due to the sulfonate group and the high degree of branching in the alkyl side chain.

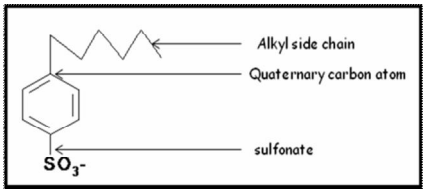


Fig 1: Structure of alkyl benzene sulfonate

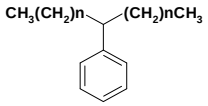
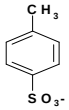
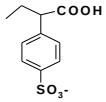
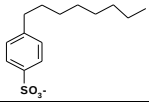
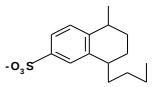
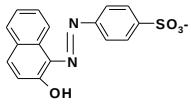
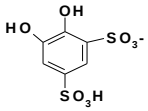
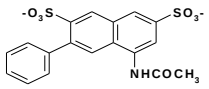
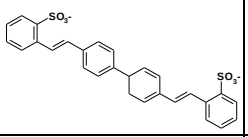
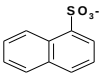
The chemical cleavage of C-S bonds requires high temperatures of 300-320 ^{o}C (Dudley and Frost, 1994). Owing to their excellent water solubility and hydrophilic properties, it is difficult to remove them completely from water in the wastewater treatment works. The problem cannot be solved by physical processes like adsorption because no chemical degradation occurs during these processes (Stoffler and Luft, 1999). As a result, they are discharged and accumulated into the aquatic (Altenbach and Giger, 1995) and terrestrial environments even after treatment (Ruff *et al.*, 1999).

1.3.2 Environmental accumulation of anthropogenic (xenobiotic) sulfonates and its effects

Organosulfonates are produced in large amounts as surfactants (2.5 x 10^6 metric tons of linear alkylbenzenesulfonates per annum [Schulze 1996]), dyestuffs (3 x 10^5 metric tons per annum [Anliker, 1977]), dyestuff precursors and additives in oils and inks (Elvers *et al.*, 1994). The major compound is 4-toluenesulfonate (about 2.7 x 10^4 metric tons per annum in Europe (Behret, 1991) used in household detergent formulations, preparation of foundry molds and syntheses of pharmaceuticals. These compounds were detected in forest soils and marine sediments (Lie *et al.*, 1996), rivers (Zerbinati *et al.*, 1997), waste dump leachates (Riediker *et al.*, 2000) and sewage sludge amended soils. Dudley et al (1994) reported that synthetic detergents pose approximately 10 % of the pollution load in the Rhine River and Italian river Bromida was polluted by sulfonated azo dyes (Zerbinati *et al.*, 1997). Vairavamurthy et al (1994) have reported the accumulation of organosulfonates in marine sediments to a level of 20–40 % of the total organic sulfur, while sulfonate sulfur exceeded 40 % of total S in the O1 horizon of forest soils (Autry and Fitzgerald 1990). It was found that 4-toluenesulfonate was accumulated in Swiss land fill leachates; benzenesulfonate and naphthalenesulfonates were also found in groundwater samples contaminated by percolating leachates (Reidiker *et al.*, 2000).

Anthropogenic sulfonates are nontoxic to higher organisms but toxic to bacteria, algae invertebrates and fish even at a concentration of 1mg/l (Lewis 1993), hence they bioaccumulate and concomitantly cause adverse environmental problems which include destruction of the external mucus layer of fish protecting from bacteria and pathogens, severe damage to the gills, lowering of the surface tension of the water and decrease in the

breeding ability of aquatic organisms. These are of particular environmental concern in purification of drinking water and the development of biological methods to remove them completely during water treatment would be desirable because microbial metabolism provides a safer, more efficient, and less expensive alternative to physico-chemical methods for pollution abatement. Microbial degradation of the arylsulfonates is superior to physical and chemical treatment owing to their more expensive way of physical and chemical treatment; hence there is a need for a better understanding of mechanisms of arylsulfonates biodegradation. Cationic pharmaceuticals were formulated with both aromatic and aliphatic sulfonates (Neil 2001), used as a major aid in assisting patients to abstain from alcohol is dosed at 2 g day^{-1} (Cook *et al.*, 2006). Candidate drugs (sulfonates) for stroke and Alzheimer's disease are also dosed at high levels (Cook et al., 2006).

Compound	Structure	Environmental relevance	Reference
LAS (linear alkyl benzenesulfonate)		Surfactant	Schwitzguebel *et al.*, 2002
Benzenesulfonate		Optical brightener	Cook *et al.*, 1999
4-Toluenesulfonate		Hydrotropic agent	Cook *et al.*, 1999
Sulfophenylbutyrate		Intermediate in LAS biodegradation	Kertesz *et al.*, 1994
Sulfophenyloctane		LAS detergent	Kertesz *et al.*, 1994
DATS		By product in LAS synthesis	Kertesz *et al.*, 1994
Orange II		Dyestuff	Hooper *et al.*, 1991
Tiron		Chelating agent	Kertesz *et al.*, 1994
Acid red I		Dyestuff	Kertesz *et al.*, 1994
4,4'-Bis(2-sulfo-styryl) biphenyl		Optical brightener	Kertesz *et al.*, 1994
Naphthalene sulfonate		Concrete admixtures Contd. in page 7	Cook *et al.*, 1999

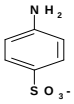
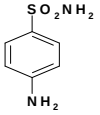
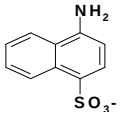
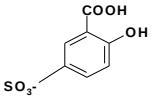
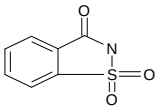
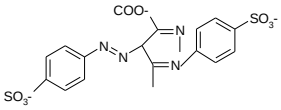
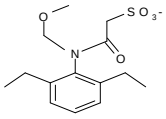
Sulfanilate		Pesticides and azodyes	Schwitzguebel *et al.*, 2002
Sulfanilamide		Pharmaceuticals	Hooper *et al.*, 1991
Aminonaphthalene sulfonate		Dye precursor	Alonso and Barcelo 2000
5-Sulfosalicylate		Integral color anodiser	Cook *et al.*, 1999
Saccharine		Food stuffs, sweetener	Cook *et al.*, 1999
Tartrazine		Dyestuffs	Cook *et al.*, 1999
Alachlor		Herbicides	Cook *et al.*, 1999

Table 2: Environmental relevance of anthropogenic or synthetic (xenobiotic) sulfonates.

1.4. Biodegradation of anthropogenic sulfonates

Early studies of arylsulfonates degradation indicated that these compounds were recalcitrant to normal degradation and were reported as non biodegradable (Bretcher 1981), or poorly biodegradable (Schwitzguebel *et al.,* 2002). LAS compounds were not mineralized in anaerobic sediment (Federle and Schwab, 1992), likewise there was no anaerobic mineralization of various amino aromatic sulfonates (Tan *et al*., 2005). There is little evidence for degradation of aromatic sulfonates under anaerobic conditions where desulfonation of arylsulfonates was reported (Shcherbakova *et al.,* 2003).

Bacterial metabolism of anthropogenic (xenobiotic) sulfonates

1.4.1 Benzenesulfonate (Phenylsulfonate)

Phenylsulfonate used as optical brightener and in the preparation of pharmaceutical drugs known as besylates or besilates. Degradation of benzenesulfonate was studied in *Pseudomonas aeruginosa* A, where degradation was channeled through catechol 2, 3-dioxygenase meta pathway with 2-hydroxymuconic semialdehyde, acetaldehyde and pyruvate as products (Fig 2) (Cain and Farr 1968). Ripin et al (1971) proposed a degradative pathway (ortho pathway) of benzenesulfonate in *Comamonas testosteroni* H-8 to catechol, 2-hydroxy muconate semialdehyde, formate, 4-hydroxy -2- oxovalerate, acetaldehyde and pyruvate. In *Alcaligens* sp. CAT498 and *Comamonas testosteroni* H-8 sulfate accumulation was reported in benzenesulfonate degradation (Johnston 1975). Utilization of benzenesulfonate as a carbon source was reported in a bacterium isolated from activated sludge from Sosei river sewage purification plant at Sapporo (Endo *et al*., 1977).

Benzenesulfonate, 4-hydroxybenzenesulfonate, 3-nitrobenzenesulfonate were desulfonated to corresponding phenols by *Pseudomonas* sp. S-313 (Zurrer *et al*., 1987) (Fig 2) and *Klebsiella oxytoca* KS3D (Dudley and Frost 1994) (Fig 2). *Alcaligenes* sp. O-1 was reported to degrade benzenesulfonate to catechol by benzenesulfonate dioxygenase system or benzenesulfonate: NADH: oxygen oxidoreductase (Thurnheer *et al*., 1990) and also by 2-aminobenzenesulfonate dioxygenase system / orthanilate dioxygenase system (Cook *et al.,* 1998) (Fig 2).

Mixed bacterial culture from the River Elbe, Hamburg, degraded benzene 1, 3-disulfonate to catechol 4-sulfonate. Catechol 4-sulfonate was further metabolized to 3-sulfomuconate and 4-carboxymethyl-4-sulfobut-2-en-4-ol (Contzen *et al*., 1996).

Rhodococcus opacus ISO-5 utilized benzenesulfonate as a sulfur source (David *et al.*, 2003). *Clostridium pasteurianum* DSM 12136 utilized benzenesulfonate, benzene 1, 3-disulfonate as sole source of sulfur (Chih-Ching Chien 2005).

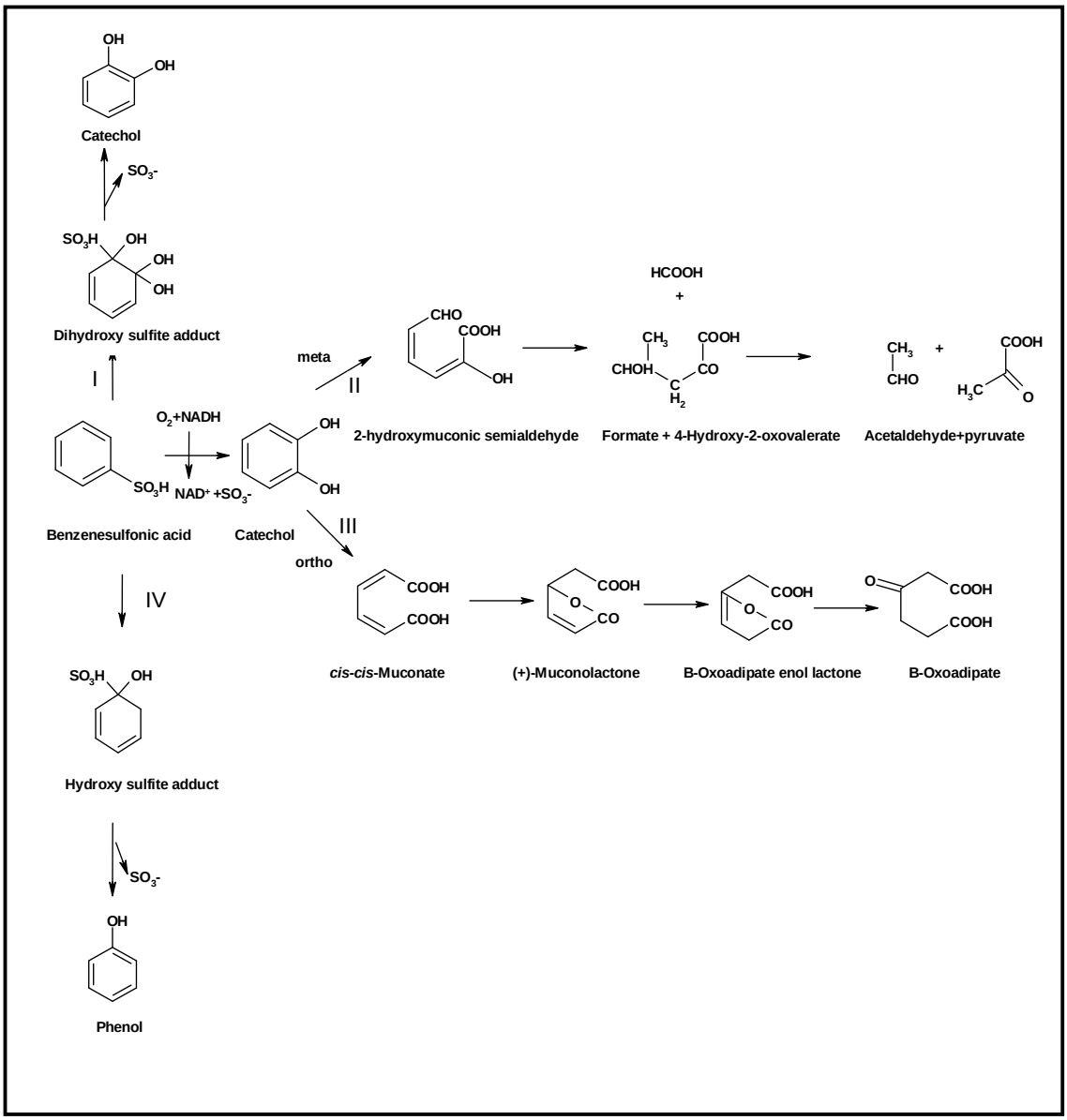


Fig 2: Degradative pathways of benzenesulfonate

(I) *Alcaligenes* sp. O-1 (Thurnheer *et al*., 1990)

(II) *Pseudomonas aeruginosa* A (meta pathway) (Cain and Farr, 1968),

(III) *Comamonas testosteroni* H-8 (ortho pathway) (Ripin *et al*., 1971)

(IV) *Pseudomonas* sp. S-313 (Zurrer *et el*., 1987).

1.4.2 4-Toluenesulfonic acid: (Tosic acid, 4-methylbenzenesulfonicacid, p-toluenesulfonicacid, Tolylsulfonate)

4-Toluenesulfonate has a methyl group para to the sulfonate group. It is a white crystalline, hygroscopic solid used in manufacture of dyes, hydrotropes, used as a catalyst in textile industries, stabilizer for pharmaceuticals intermediates, cleaning agents and plating additive. These compounds enter the environment through the domestic wastes and wastes generated from textile, dye, pharmaceutical industries etc. In humans, ingestion may cause oesophageal burns, dermal contact produce severe burns, inhalation may result in dyspnea, pleuritic chest pain, pulmonary edema, hypoxemia, bronchospasm, pneumonitis, tracheobonchitis and persistent pulmonary dysfunctions, eye contact may cause corneal erosions (http://www.chemadvisor.com)

1.4.2.1 Abiotic degradation

Direct photolysis of 4-toluenesulfonate is not known, since the compound does not absorb light beyond 290 nm. Advanced oxidation processes (AOP) are rapid and non-selective methods for the quantitative chemical breakdown of compounds at high temperature and pressure. The degradation mechanism of 4-toluenesulfonate through AOP is a complex process, involve the generation of highly reactive hydroxyl radicals generated by thermal activation of hydrogen peroxide, where desulfonation occurs at a high temperature and pressure of 180 ^{0}C and 2 M Pa respectively (Stoffler and Luft, 1999).

1.4.2.2 Biotic degradation

1.4.2.2.1 Bacterial metabolism of 4-toluenesulfonate

(i) Aerobic biodegradation of 4-toluenesulfonate

Aerobic degradation of 4-toluenesulfonate was studied in various bacteria, especially those belonging to the genus *Pseudomonas.* Different pathways have been proposed for different strains of *Pseudomonas*, *Pseudomonas aeruginosa* A, *Comamonas testosteroni* H-8, *Pseudomonas putida* S-313 (Fig 3). Overall five pathways were proposed for aerobic degradation of 4-toluenesulfonate in various chemotrophic bacteria. These pathways

involve an initial reaction comprising the oxidation of 4-toluenesulfonate catalyzed by oxygenases.

In *Pseudomonas aeruginosa* A 4-toluenesulfonate was degraded by catechol 2, 3-dioxygenase pathway with intermediates 4-methylcatechol and 2-hydroxy, 5-methylmuconic semialdehyde followed by ring cleavage resulted in formate, propionaldehyde and pyruvate (Cain *et al.,* 1968) (Fig. 3). *Pseudomonas* sp. was reported to degrade 4-toluenesulfonate involving 2, 3 dihydro 4-sulfonate as an intermediate to 3-methyl catechol (Focht and Williams 1970). In *Comamonas testosteroni* H-8 reported that the overall oxidation system for 4-toluenesulfonate is inducible (Ripin *et al.,* 1971).

Comamonas testosteroni T-2 completely degraded 4- toluenesulfonate to cell material, CO_2 and sulfate with a series of intermediates 4-sulfobenzylalcohol, 4-sulfobenzaldehyde, 4-sulfobenzoate, protocatechuate followed by meta ring cleavage products pyruvate and succinate (Locher *et al.,* 1989) (Fig 3). In *Comamonas testosteroni* T-2 a novel outer membrane anion channel (porin) for 4-toluenesulfonate was identified (Mample *et al.,* 2004). The enzymes involved in 4-toluenesulfonate degradation such as 4-toluenensulfonate methyl monoxygenase (EC 1.12.4) and 4-sulfobenzoate 3,4 dioxygenase (EC 1.14.12.8) were purified and characterized (Locher *et al.,* 1991a and Locher *et al.,* 1991b) in *Comamonas testosteroni* T-2. These two enzymes encoded catabolic IncP1b plasmid pTSA was also mapped by subtractive analysis (Tralau *et al.,* 2001). *Achromobacter xylosoxidans* TA12-A and *Ensifer adhaerens* TA12-B were identified as the 4-toluenesulfonate degraders showed the same pathway and contain *tsa* genes from the Tntsa cluster as described previously in *Comamonas testosteroni* T-2 (Tralau *et al.,* 2011).

Alcaligenes sp. O-1 was reported to desulfonate 4-toluenesulfonate by toluenesulfonate dioxygenase or 4-toluenesulfonate: NADH: oxygen oxidoreductase to 4-methylcatechol (Thurnheer *et al.,* 1990; Junker *et al.,* 1994) (Fig 3). However *Pseudomonas putida* S-313 (DSM 6884), *Klebsiella oxytoca* KS3D were also reported to desulfonate 4-toluenesulfonate to *p*-cresol when used as a sulfur source (Kertesz *et al.,* 1994; Dudly and Frost, 1994). *Rhodococcus opacus* ISO-5 utilized 4-toluenesulfonate as a sulfur source for growth (David *et al.,* 2003), however products were not identified.

(ii) Aerobic biodegradation of toluene (methyl benzene)

Similarly the structural and substrate analogue of 4-tolueneusulfonate such as toluene degradation under aerobic conditions was studied in various bacteria, especially those belonging to the genus *Pseudomonas.* Five different pathways were proposed for different strains of *Pseudomonas*, *P. putida* mt-2 (Worsy *et al.*, 1975), *P. mendocina* KR (Whited *et al.*, 1991), *P. cepacia* G4 (Mars *et al.*, 1996), *P. picketti* PK01 (Ronald *et al.*, 1994) and *P. putida* F1 (Zylstra *et al.*, 1988). These pathways involve an initial reaction comprising the oxidation of toluene catalyzed by oxygenases. The aerobic toluene degradation pathway in *P. putida* mt-2 involves oxidation of the methyl group of toluene to produce benzoic acid. Subsequently, cis-benzoate dihydrodiol dehydrogenated to catechol. Aerobic toluene degradation in *P. mendocina* KR, *P. cepacia* G4 and *P. picketti* PK01, involves oxidation of toluene to produce cresol isomers (*p*-cresol, *o*-cresol and *m*-cresol, respectively) as intermediate products. *p*-Cresol is further converted into protocatechuate, while *o*-cresol and *m*-cresol are transformed into 3-methylcatechol. *P. putida* F1 can metabolize toluene by producing cis-toluene dihydrodiol, which was converted to 3-methylcatechol. Catechol, protocatechuate and 3-methylcatechol were undergo aromatic ring cleavage by either ortho-cleavage or meta-cleavage pathways, to produce non-aromatic intermediates (like acetate, pyruvate and succinate) which are metabolized by the Tricarboxylic Acid Cycle (TCA cycle).

(iii) Anaerobic biodegradation of 4-toluenesulfonate

Anaerobic degradation of 4-toluenesulfonate was reported in few of the chemotrophic bacteria belongs to the genus *Clostridium, Methanosarcina, Desulfovibrio* and *Cupriavidus.*

Anaerobic desulfonation of 4-toluenesulfonate was reported in *Clostridium* sp. EV4, where metabolic intermediates were not identified (Denger *et al.*, 1996). *Methanosarcina mazei* MM demethylate 4-toluenesulfonate to benzenesulfonate, forming methane under anaerobic conditions (Shcherbakova *et al.*, 2003). Pure cultures of three strains of *Clostridium* sp. 14 (VKM B-2201), 42 (VKM B-2202), 21 (VKM B-2279), two methanogens, *Methanobacterium formicicum* MH (VKM B-2198) and *Methanosarcina mazei* MM (VKM B-2199) and one sulfate-reducing bacterium, *Desulfovibrio* sp. SR1

(VKM B-2200) were isolated from an anaerobic microbial community reported to degrade 4-toluenesulfonate. 4-Toluenesulfonate was desulfonated to toluene by *Clostridium* sp. VKM B-2202 (Fig 3). The sulfate-reducing strain *Desulfovibrio* sp. SR1 utilized 4-toluenesulfonate as an electron acceptor (Shcherbakova *et al.,* 2003). 4-Toluenesulfonate was used as sole source of sulfur by *Clostridium pasteurianum* DSM 12136 (Chih-Ching Chien 2005), *Cupriavidus metallidurans* and *Variovoraxparadoxus* T (Schmalenberger 2007).

(iv) Conjugative metabolism of toluene (methylbenzene)

Anaerobic toluene degradation has been reported in a wide range of bacteria including methanogenic bacteria, iron reducing bacteria, sulfate reducing bacteria and phototrophic bacteria (Table 3). Benzylsuccinate synthase is an oxygen sensitive, glycyl-radical enzyme that catalyzes the first step of anaerobic toluene degradation by adding fumarate or succinate to the methyl group of toluene (Beller and Spormann. 1997; Biegert *et al.,* 1996; Krieger *et al.,* 2001; Leuthner and Heider 2000; Leuthner *et al.,* 1998). The enzyme has been purified from *Thauera aromatica* K172 and *Azoarcus* sp. strain T (Krieger *et al.,* 2001; Leuthner *et al.,* 1998). Electronic paramagnetic spectroscopy (EPR) has indicated the presence of an oxygen-sensitive, stable organic radical in benzylsuccinate synthase of *Azoarcus* sp. strain T (Krieger *et al.,* 2001). Benzylsuccinate synthase is comprised of three subunits ($\alpha_2\beta_2\gamma_2$) which are responsible for its heterohexameric structure (Krieger *et al.,* 2001; Leuthner *et al.,* 1998).

Benzylsuccinate synthase shows a significant homology with other well characterized enzymes viz. pyruvate formate lyase (PFL) and anaerobic ribonucleotide reductase (ARR) from *E.coli* (Krieger *et al.,* 2001; Leuthner *et al.,* 1998). PFL is a key enzyme that is involved in anaerobic glucose metabolism (Knappe *et al.,* 1984). ARR is required for bacterial DNA replication under anaerobic conditions (Sun *et al.,* 1993).

Biochemical analysis of the purified enzyme benzylsuccinate synthase comprises three subunits; alpha, beta, and gamma, in *Thauera aromatica* T1 are encoded by the *tutD*, *tutG*, and *tutF* genes, respectively. The large alpha-subunit contains the essential glycine and cysteine residues that are conserved in all glycyl radical enzymes. However, the

function of the small beta- and gamma-subunits has remained unclear (Li *et al.*, 2009). FeS clusters in the glycyl-radical enzyme benzylsuccinate synthase were identified through EPR and Mossbauer spectroscopy (Hilberg *et al.*, 2012).

Further, benzylsuccinate synthase is also involved in the degradation of *m*-xylene and cresol isomers in *Azoarcus* sp. T and cresol isomers but not xylene in *T. aromatica* K172 (Verfurth *et al.*, 2004). Analysis of a *bssA* defective mutant in *Azoarcus* sp. T indicated that *bssA* is essential for metabolism of both toluene and *m*-xylene (Achong *et al.*, 2001).

Nutritional mode	Organism	References
Methanogenic	Members of *Methanosaeta* and *Methanospirillum* genera	Beller and Edwards 2000 Edwards and Grbic 1994
Denitrifying	*Thaura aromatica* strain T1 *Thaura aromatica* strain K172 *Azoarcus sp*. strain T *Azoarcus sp*. strain EbN1	Evans *et al.*, 1991a,1991b Schocher *et al.*, 1991 Achong *et al.*, 2001 Rabus *et al.*, 1995
Sulfate-reducing	Strain PRTOL1 *Desulfobacula toluolica* *Desulfobacterium cetonicum* *Desulfosarcina cetonica*, Desulfotomaculum sp.	Beller *et al.*, 1992 Rabus *et al.*, 1993 Muller *et al.*, 1999 Winderl *et al.*, 2007 Winderl *et al.*, 2007
Iron reducing	*Geobacter metallireducens* *Geobacter grbiciae*,	Lovely and Lonergan. 1990 Lovely *et al.*, 1993 Winderl *et al.*, 2007
Phototrophic	*Blastochloris sulfoviridis* strain ToP1	Zengler *et al.*, 1999

Table 3: Bacteria involved in anaerobic degradation of toluene

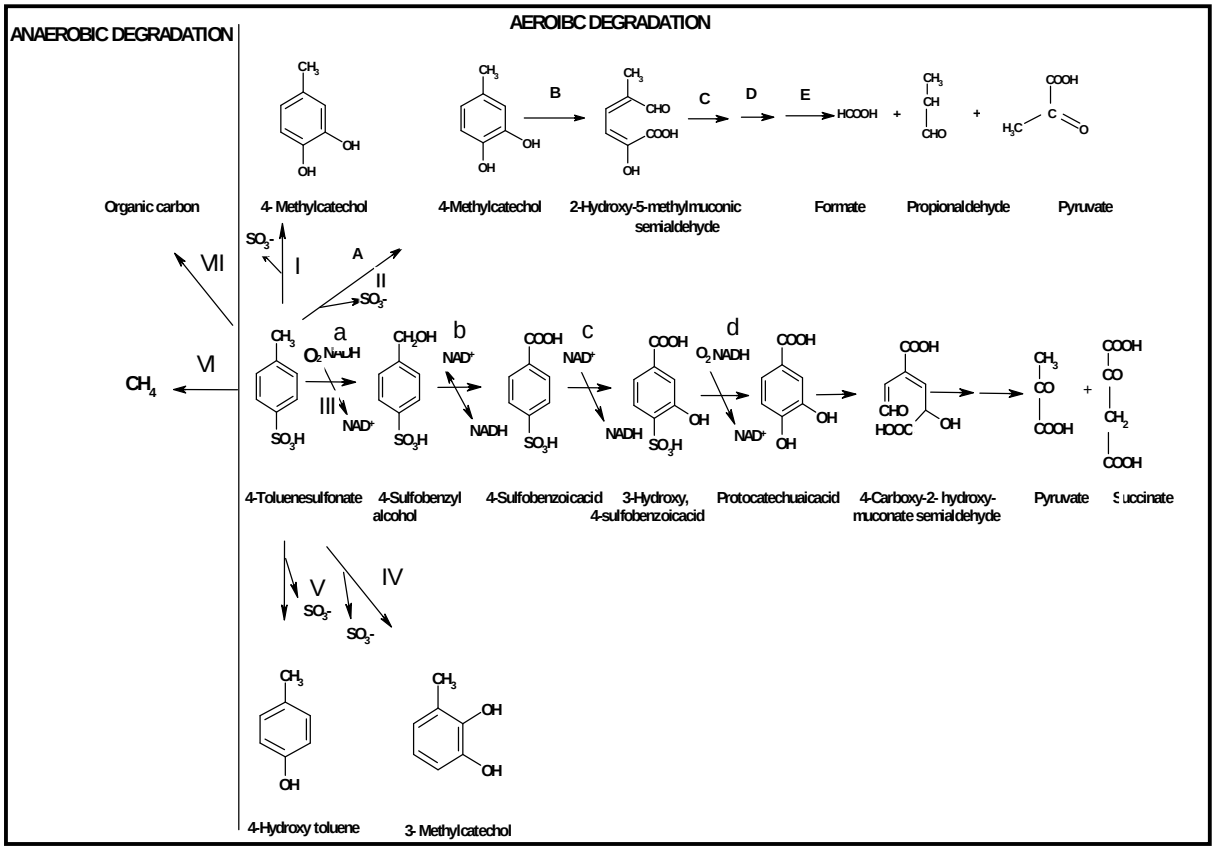


Fig 3: Degradative pathways of 4-toluenesulfonate in

I. ***Alcaligens* sp. O-1 (Junker *et al.*, 1994),**

II. ***Pseudomonas aeruginosa* A (Cain and Farr 1968),**

(A) 2-Amionbenzenesulfonate dioxygenase (2ASDOS) or Benzenesulfonate dioxygenase (BSDOS)

(B) Catechol 2, 3 dioxygenase

(C) 2-Hydroxymuconate semialdehyde hydrolase

(D) 2-oxopent-4-enolate hydratase

(E) 4-hydroxy-2-oxovalerate aldolase

III. ***Comamonas testosteroni* T-2 (Locher *et al.*, 1989),**

(a) 4-toluenesulphonate methyl-monooxygenase system (TSMOS)

(b) 4-Sulphobenzylalcoho1 dehydrogenase, (SOLDH)

(c) 4-sulphobenzaldehyde dehydrogenase (SYDDH)

(d) 4-sulphobenzoate 3,4-dioxygenase system (PSBDOS)

IV. ***Klebsiella oxytoca* KS3D (Dudly and Frost 1994),**

V. ***Pseudomonas* sp. (Focht and Williams, 1970),**

VI. ***Methanosarcina mazei* MM (Shcherbakova *et al.*, 2003) and**

VII. ***Clostridium sp.* EV4 (Denger *et al.*, 1996)**

1.4.3 Aminobenzenesulfonates (ABS) are widely used as precursors in the manufacture of dyes and optical brighteners

1.4.3.1 2-Aminobenzenesulfonate: orthanilicacid (2ABS)

2-Aminobenzenesulfonate was reported in *Pseudomonas, Alcaligens* and bacterial consortium having *Acinetobacter* and *Flavobacterium. Pseudomonas* sp. O-1 completely mineralized 2-aminobenzenesulfonate (as carbon source) to cell material and CO_2 (Thurnheer *et al.,* 1986). *Alcaligens* sp. O-1 degrading 2-aminobenzenesulfonate to sulfite, ammonia and 3-sulfocatechol (Thurnheer *et al.,* 1990) (Fig 4I). 2-Aminobenzenesulfonate dioxygenase system/ orthanilate dioxygenase system catalyzed the first reaction and 3-sulphocatechol 2,3-dioxygenase (EC 1:13:11:2) catalyzed the second reaction. 3-Sulphocatechol 2,3-dioxygenase (EC 1:13:11:2) was purified (Junker *et al.,* 1994) and Mampel et al (1999) purified the oxygenase component of the 2-aminobenzenesulfonate dioxygenase system from *Alcaligenes* sp. O-1. *Rhodococcus opacus* ISO-5 utilized 2-aminobenzenesulfonate as a sulfur source for growth (David *et al.,* 2003).

A bacterial consortium degrading 2-aminobenzenesulfonate was reported by Singh *et al* (2008). *Acinetobacter* and *Flavobacterium* in a bacterial consortium utilized 2-aminobenzenesulfonate as the sole carbon and energy sources under aerobic conditions, (Awasti *et al.,* 2009).

1.4.3.2 3-Aminobenzensulfonate (3-ABS): Metanilic acid is commonly used as a mild oxidant

Pure cultures of putative pseudomonads and two unidentified rods strains M-1 were completely degraded 3-aminobenzenesulfonate and 3-nitrobenzenesulfonate to biomass, SO_4^{2-}, NH_4^+ and CO_2 (Locher *et al.,* 1989, Thurnheer *et al.,* 1988). *Pseudomonas* sp. S-313 utilized 3-aminobenzensulfonate as the source of sulfur yielding the product 3-aminophenol (Zurrer *et al.,* 1989) (Fig 4II). Similarly *Rhodococcus opacus* ISO-5 utilized 3-aminobenzenesulfonate as a sulfur source for growth (David *et al.,* 2003), however products were not identified.

Pseudomonas aeruginosa CLRI, BL22 were able to degrade 3-aminobenzensulfonate to aniline and β-keto adipic acid with dioxygenase (Valli Nachiyar *et al.,* 2007). 3-Nitrobenzenesulfonate (used in reduction as resistant agent for printing thickeners in textile dyeing factories and as a precursor of synthetic dyes in dye producing factories) mineralization and desulfonation was reported in *Alcaligenes* sp. GA-l (Takeo *et al.,* 1997). *Rhodococcus opacus* ISO-5 utilized 2-, 3- and 4-nitrobenzenesulfonate as a sulfur source for growth (David *et al.,* 2003).

1.4.3.3 4-Aminobenzenesulfonate (4-ABS) or Sulfanilic acid

4-ABS is an intermediate in the production of pharmaceuticals, pesticides and is a component of many azo dyes. A co culture of *Hydrogenophaga palleronii* S1 and *Agrobacterium radiobacter* S2 degraded 4-aminobenzenesulfonate where strain S1 deaminated sulfanilate to catechol-4-sulfonate, which served as growth substrate for strain S2 (Burkhard *et al.,* 1988; Burkhard *et al.,* 1993 and Dange man *et al.,* 1996) (Fig 4III). 4-Aminobenzenesulfonate degradation was reported in *Hydrogenophaga intermedia* $S1^T$ (= DSM 5680) (Contzen *et al.,* 2000), *Pseudomonas paucimobilis* [(sulfanilate as sole source of carbon and nitrogen to biomass) (Pieri *et al.,* 2001)], *Rhodococcus opacus* ISO-5 [(as a sulfur source for growth) (David *et al.,* 2003)] and *Clostridium pasteurianum* DSM 12136 [(as sole source of sulfur) (Chih-Ching Chien 2005)]. *Agrobacterium* sp. PNS-1 was reported to completely mineralize sulfanilate with stoichiometric release of sulfite and ammonia (Singh *et al.,* 2004; 2006 a & b). *Pannonibacter* sp. W1 degraded 4-aminobenzenesulfonate as sole carbon as well as energy, nitrogen and sulfur source to 4-sulfocatechol and followed by ring cleavage (QingWanga *et al.,* 2009).

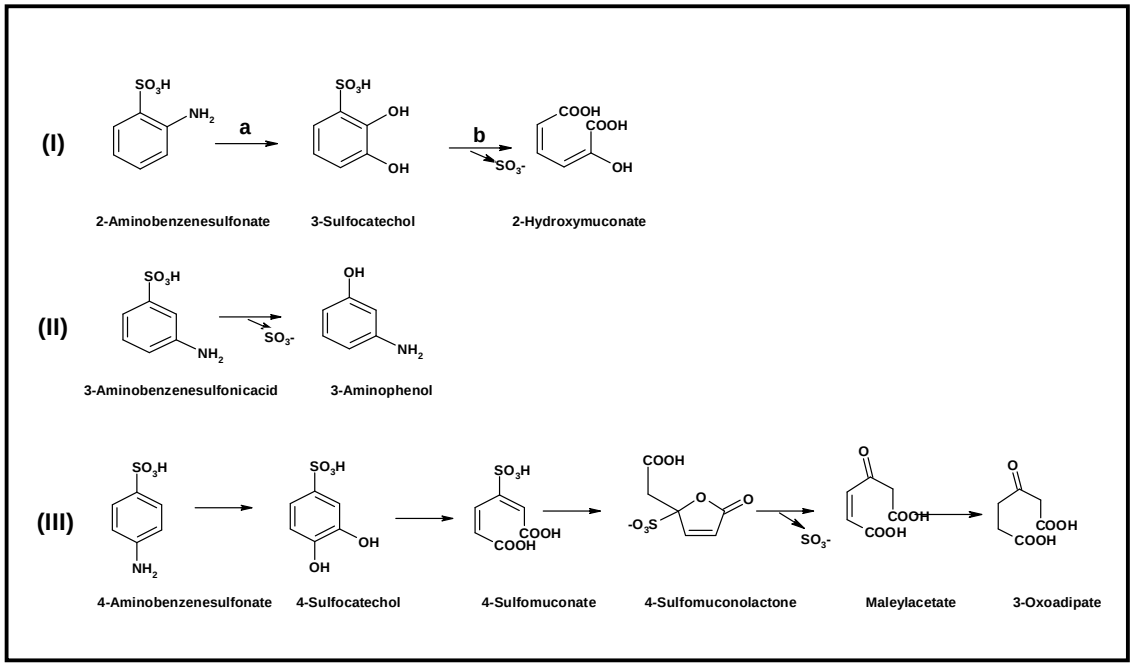


Fig 4: Degradative pathways of isomers of aminobenzenesulfonate

(I) 2-Aminobenznesulfonate by *Alcaligens* sp. O-1 (Thrunheer *et al.*, 1990)

a, 2-aminobenzenesulfonate dioxygenase system / orthanilate dioxygenase system

b, 3-sulfocatechol 2,3-dioxygenase

(II) 3-Aminobenznesulfonate by *Pseudomonas* sp. 313 (Zurrer *et al.*, 1989).

(III) 4- Aminobenzenesulfonate by a mixed culture of *Hydrogenophaga palleronii* S1 and *Agrobacterium radiobacter* S2 (Feigel and Knackmuss 1993).

1.4.4 Substituted naphthalene sulfonates

Isomers of sulfonated naphthalene are used as an intermediate in industrial production of a wide range of chemicals, including dispersants, detergents, azo dyes, and wetting agents. Naphthalene-1-sulfonate and naphthalene-2-sulfonate were desulfonated by dioxygenation to gentisate by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22 (Brilon *et al.*, 1981). *Sphingomonas xenophaga* BN6 was mineralized naphthalene-2-sulfonate (2NS) to salicylate (Fig 4), 5-hydroxynaphthalene- 2-sulfonate to 6-hydroxysalicylate, 6-hydroxynaphthalene- 2-sulfonate to 5-hydroxysalicylate (gentisate) and 7-amino- and 7-hydroxynaphthalene-2-sulfonate to the corresponding 3-substituted salicylate (Nortemann *et al.*, 1986). 1,2-Dihydroxynaphthalene dioxygenase (DHNDO) was purified, the amino terminal amino acid sequence was determined and characterized in *Sphingomonas xenophaga* BN6 (Soltz 1999). The genes encoding the 1, 2-dihydroxynaphthalene dioxygenase, 2-hydroxychromene-2-carboxylate isomerase and 2- hydroxybenzalpyruvate aldolase of the naphthalenesulfonate pathway were identified on the chromosomal DNA of *Sphingomonas xenophaga* BN6 (Keck 2006). *Pseudomonas* sp. S-313 degraded 1-naphthalenesulfonic acid, 2-naphthalenesulfonic acid, 5-amino-l-naphthalenesulfonic acid to l-naphthol, 2-naphthol and 5-amino-l-naphthol respectively (Zurrer *et al.*, 1987).

Disulfonated naphthalenes are more resistant to biodegradation. However, *Pigmentiphaga daeguensis* ASL4 (formerly *Moraxella*) completely metabolized naphthalene-1,6-disulfoante and naphthalene-2,6-disulfonate (Wittich *et al.*, 1988). *Pigmentiphaga* sp. NDS-2 metabolized naphthalene-2,6-disulfonate to gentisate via 5-sulfosalicylic acid, gentisate, maleylpyruvate, fumarate and pyruvate as the products (Fig 5) (Uchihashi *et al.*, 2003). *Rhodococcus opacus* ISO-5 utilized naphthalene-2-sulfonate as a sulfur source for growth (David *et al.*, 2003). *Pseudomonas* sp., *Arthrobacter* sp. and an unidentified bacterium were desulfonated sixteen different sulfonated aromatic compounds, none of which served as a carbon source (Song 2005).

(I) **(II)**

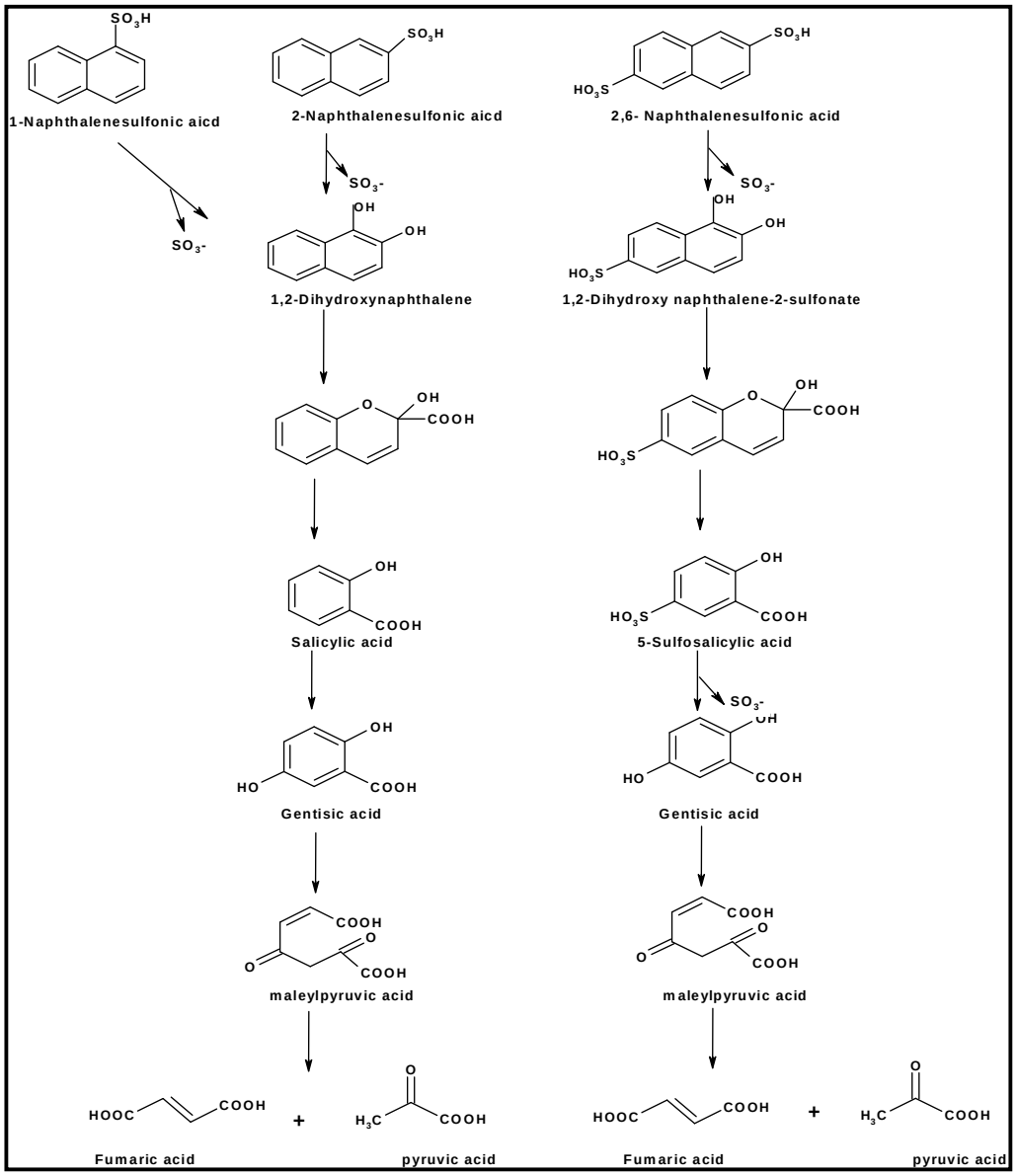


Fig 5: Degradative pathway of 2 -naphthalenesulfonate and 2, 6-naphthalenedisulfonate by *Pseudomonas* sp. BN6 (I) (Nortermann *et al.*, 1986) and *Pigmentophaga* sp. NDS-2 (II) (Uchihashi *et al.*, 2003) respectively.

1.5 Algal metabolism of anthropogenic sulfonates

Chlorella fusca utilized linear sulfonic acids and arylsulfonates as sources of sulfur such as l-naphthalene sulfonate and 2-naphthalene sulfonate (Biedlingsmeier and Schmidt 1983) and l-naphthol was identified as a metabolite of 1- naphthalene sulfonate (Soeder *et al.,* 1986). *Scenedesmus obliquus* was reported to utilize 1,2-naphthoquinone-4-sulfonic acid, l-naphthalenesulfonic acid, 2-naphthalenesulfonic acid, 1,5-naphthalenedisulfonic acid, 1,6-naphthalenedisulfonic acid, 2,6-naphthalenedisulfonic acid, 2,7-naphthalenedisulfonic acid, l-hydroxynaphthalene-5-sulfonic acid, l-hydroxynaphthalene-6-sulfonic acid, 2-hydroxynaphthalene-5-sulfonic acid, 2-hydroxynaphthalene-6-sulfonic acid, 2-hydroxynaphthalene-7-sulfonic acid and naphthalenetrisulfonic acid as sulfur sources (Luther *et al.,* 1991). Under sulfate limitation, the green alga *Scenedesmus obliquus* metabolized 1- naphthalenesulfonic acid and partially used the sulfonate as a source of sulfur. The main metabolite, 1-hydroxy-2- naphthalenesulfonic acid, which was not metabolized further in the algal culture, was formed by hydroxylation of the substrate in position 1 and by migration of the sulfonic acid group to position 2 of the naphthalene ring (NIH shift). A smaller amount of 1-naphthalenesulfonic acid was desulfonated. The resulting 1-naphthol was mostly transformed into 1-naphthyl b-D-glucopyranoside (Kneifel *et al.,* 1997).

1.6 Fungal metabolism of anthropogenic sulfonates

The degradation of sulfonated dyes like Tropaeoline {4-[(2,4-dihydroxyphenyl)azo]benzenesulfonic acid}, Orange II {4-[(2-hydroxy-1-naphthyl) azo] benzenesulfonicacid}, and Congo Red {3,3'-[[1,1'biphenyl]- 4,4'diylbis-(azo)]bis[4-amino-1-naphthalenesulfonic acid]} was reported in *Phanerochaete chrysosporium* (Cripps *et al.,* 1990). Sulfanilic acid, 4-(3-methoxy-4-hydroxyphenylazo)-benzenesulfonic acid, Acid yellow 9 {4-(3-sulfo-4-aminophenylazo)-[benzenesulfonic acid}, 4-(2-sulfo-3'-methoxy-4-hydroxyazobenzene-4-azo)-benzenesulfonic acid, Orange II {4-(2-hydroxynaphthylazo)-benzenesulfonic acid} and Orange I {4-(4-hydroxynaphthylazo)-benzenesulfonic acid} were mineralized to CO_2 by *Phanerochaete chrysosporium* (Paszczynski *et al.,* 1992). Desulfonation of 3, 5-dimethyl-4- hydroxyl and 3, 5-dimethyl-4-aminobenzenesulfonic acid was reported in *Phanerochaete chrysosporium*

(Muralikrishna and Renganathan 1993). The enzymatic mechanism for the oxidation of sulfonated azo dyes by lignin peroxidases from *Phanerochaete chrysosporium* was studied by Goszczynski *et al.* (1994) (Chivukula *et al.,* 1995). These degradations have been attributed to extra cellular peroxidases (Muralikrishna and Renganathan, 1993; Goszczynski *et al.,* 1994).

Phanerochaete chrysosporium oxidize and shortens the side chain of LAS resulting in the formation of sulfophenyl carboxylates (Jagjit *et al.,* 2001). Some of the fungi like *Phanerochaete chrysosporium, Geotrichum candidum, Trametes versicolor, Bjerkandera adusta, Penicillium* sp., *Pleurotus ostreatus, Pycnoporus cinnabarinus,* and *Pyricularia oryza* were decolorized sulfonated dyes such as Direct Blue 1 (Chicago Sky Blue 6B), reactive dye Reactive Black 5. Comparisons of different fungi suggested that *Trametes* or *Bjerkandera* species were superior compared to *Phanerochaete chrysosporium* for the decoloration of different dyes (Stolz 2001).

1.7 Anthropogenic sulfonates biodegradation by plants

The cultured cells of *Rheum rabarbarum* (rhubarb) were reported to accumulate and biotransform 2-chloro-5-nitrobenzenesulfonate, 2-hydroxy-4-sulfonaphthalene-diazonium, 2-hydroxy-4-sulfo-6-nitro-naphthalene-diazonium and 1, 3-naphthalene disulfonates, containing either an amino or a nitro group in position 7, without releasing the metabolites (Romain Duc *et al.,* 1999). Schwitzguébel et al (2008) reported that *Rheum rabarbarum* (rhubarb) accumulate and transform sulfonated anthraquinones. The biochemical mechanisms involved in the metabolism and detoxification of sulfonated anthraquinones in rhubarb (*Rheum rhaponticum*), maize (*Zea mays*) and celery (*Apium graveolens*) were reported (Valerie *et al.,* 2009). The role of antioxidant and detoxification enzymes of *Phragmites australis,* in the degradation of an azo dye, acid orange 7 (AO7), was studied. Increase in activity of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), and glutathione S-transferase (GST) was observed in crude extracts of leaves of *Phragmites australis* during degradation of AO7. *Blumea malcolmii* was found to decolorize Malachite green, Red HE8B, Methyl orange, Reactive Red 2 and Direct Red 5B .The phytotransformation of Direct Red 5B products identified were 4-(4-amino-phenylazo)- benzene sulfonic acid, 3-amino-7-carboxyamino-4-hydroxy-

naphthalene-2-sulfonic acid and 7-carboxyamino-naphthalene-2-sulfonic acid (Anuradha *et al.*, 2009). *Portulaca grandiflora* Hook was reported to decolorize and degrade a sulfonated diazo dye Navy Blue HE2R (NBHE2R) NBHE2R into metabolites viz. N-benzylacetamide and 6-diazenyl-4-hydroxynaphthalene-2-sulfonic acid (Rahul *et al.*, 2011).

1.8 Anoxygenic phototrophic bacteria

Anoxygenic phototrophic bacteria (APB) are physiologically and phylogenetically heterogeneous group of bacteria perform photosynthesis under anoxic conditions in the presence of light with the help of bacteriochlorophyll without the liberation of oxygen. Unlike oxygenic phototrophs (cyanobacteria, algae and green plants), they use only one photosystem and are unable to use water as an electron donor (Imhoff, 1995). They require electron donors of lower redox potential than water like mainly sulfide and other reduced sulfur compounds (thiosulfate, tetrathionate, sulfite etc), and also molecular hydrogen and many small organic compounds (Imhoff, 1995) or sometimes-ferrous iron (Heising *et al.*, 1999; Heising and Schink, 1998) and instead of oxygen, the corresponding oxidized products are sulfate, protons (which are not observed because they are reused for the reduction of CO_2 to cell material), and organic compounds and CO_2, respectively.

Classification of APB was conventionally based on a number of morphological and physiological properties (Trüper and Pfennig, 1981; Imhoff and Trüper, 1989) with some important characteristics like dissimilatory sulfur metabolism, deposition of sulfur granules inside or outside the cells, oxidation of sulfur used for the differentiation major groups of phototrophic purple and green bacteria (Imhoff 1984; Pfennig 1989). Based on phenotypic characteristics, anoxygenic phototrophic bacteria were classified into the green sulfur bacteria, the green nonsulfur bacteria, the purple sulfur bacteria, the purple non sulfur bacteria and the heliobacteria. Based on 16S rRNA gene sequence analysis, they were distributed in five distantly related phyla viz., phylum *Proteobacteria* (which includes the purple bacteria), *Chloroflexi* (which includes the filamentous green nonsulfur bacteria), *Chlorobi* (which includes the green sulfur bacteria), *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).

The Phototrophic purple bacteria are separated into purple sulfur bacteria with two families, *Chromatiaceae*, *Ectothiorhodospiraceae*, BChl *b*-containing anoxygenic phototrophic bacteria, aerobic anoxygenic phototrophic bacteria and purple non sulfur bacteria. The photosynthetic pigments and the photosynthetic apparatus in all purple bacteria are located in intracytoplasmic membranes, which are considered to be originating from, and being continuous with the cytoplasmic membrane. These intracytoplasmic membranes are in the form of small fingerlike intrusions, vesicles, tubules or lamellae. *Chromatiaceae* family members are able to grow with sulfide and elemental sulfur as e- donor and deposit globules of elemental sulfur inside the cells (Imhoff 1984). *Ectothiorhodospiraceae* members deposit elemental sulfur outside the cells (Imhoff *et al.,* 1991). Based on the *pufLM* gene sequence analyses of the metagenome from samples of salt lakes in Chile, South America Thiel et al (2010) indicated the existence of purple sulfur bacteria outside the well-recognized families of *Chromatiaceae* and *Ectothiorhodospiraceae.* However, this is only based on the metagenome (uncultured) analysis, which needs to be confirmed by pure cultures.

1.8.1 Photometabolism of aromatic compounds by anoxygenic phototrophic bacteria

Anoxygenic phototrophic bacteria (APB) are diverse and important members of microbial communities (Imhoff, 2001a; Morris *et al.,* 2004; Yutin *et al.,* 2007), play a major role in anaerobic nutritional cycles. APB are metabolically highly versatile organisms capable of growth on substances ranging from simple aliphatic organic acids to complex polysaccharides (Hiraishi *et al.*, 1989).

Rhodopseudomonas palustris (Harwood and Gibson 1988), *Rhodospirillum fulvum* (Pfennig *et al.,* 1965, renamed *Phaeospirillum fulvum* by Imhoff *et al.,* 1998), *Rhodocyclus purpureus* (Pfennig *et al.,* 1965), *Rhododmicrobium vannielli* (Wright and Madigan 1991), *Rubrivivax gelatinosus*, *Rhodobacter capsulatus* (Blasco and Castillo 1992) *Rhodopseudomonas acidophilus* (Yamanaka *et al.,* 1983 renamed *Rhodoblastus acidophilus* in Imhoff 2001b), *Rhodobacter blasticus* and *Rhodospirillum rubrum* are

known to metabolize monocyclic aromatic compounds. Many of the reported *Rhodopseudomonas* spp. were grown on methylbenzenes, amino benzenes and phenolics, while few of the *Rhodopseudomonas* spp. were reported for degradation of halo carboxylic acids,, polychlorinated biphenyls, dinitrophenols, pyrazines and chlorobenzoates (Blasco and Castillo 1992; Harwood and Gibson 1988; Kamal and Wyndham 1990; Khanna *et al.,* 1992; McGrath and Harfoot 1997; Montgomery and Vogel. 1992; Sasikala *et al.,* 1994, Kusalatha *et al.,* 2010). Zengler et al (1999) reported that *Blastochloris sulfoviridis* ToP1 is the first phototrophic bacterium shown to utilize an aromatic hydrocarbon, toluene.

Rhodobacter sphaeroides OU5 was reported to metabolize 2-aminobenzoate (Nanda *et al.,* 2000; Sunayana *et al.,* 2005), *trans*-cinnamate (Usha *et al.,* 2007), L-phenylalanine, tyrosine (Ranjith *et al.,* 2007a), and photo transform aniline to indole esters (Shankaer *et al.,* 2006). The novel compounds with biotechnological applications were identified in strain OU5 include Sphestrin (a novel indole ester) (Sunayana *et al.,* 2005a), Rhodestrin (a novel indole terpenoid phytohormone) (Sunayana *et al.,* 2005b) Rhodethrin (novel indole terpenoid ether has cytotoxic and phytohormonal activities) (Ranjith *et al.,* 2007b) were identified. Phenols and gallate esters were identified in *Rhodobacter sphaeroides* OU5 in presence of l-phenylalanine used as sole source of nitrogen (Ranjith *et al.,* 2010). *Rhodobacter spheroides* Z08 was effective in ameliorating hazardous pollutants in pharmaceutical wastewater with over 80 % COD reduction (Madukasi *et al.,* 2010).

Ramana et al (2006) reported that *Rubrivivax benzoatilyticus* JA2 grown on benzoate, 2-aminobenzoate (anthranilate), 4-aminobenzoate, 4-hydroxybenzoate, phthalate, phenylalanine, *trans*-cinnamate, benzamide, salicylate, cyclohexanone, cyclohexanol and cyclohexane-2-carboxylate as carbon sources and/or electron donors. Aromatic aminoacids viz. l- tryptophan, l-phenylalanine catabolism and production of tryptophan and indole derivatives in presence of aniline (Mujahid *et al.,* 2010) was reported in *Rubrivivax benzoatilyticus* JA2, where L-tryptophan was metabolized through 2-oxoglutarate, indole-3-pyruvic acid, indole-3-acetaldehyde, indole-3-acetic acid, isatin, benzaldehyde, gallic acid and pyrogallol as intermediates (Ranjith *et al.,* 2011) and l-phenylalanine with l-phenylpyruvic acid and l-phenyllactate as metabolites (Prasuna *et al.,* 2012). Mujahid et al (2011a) reported that *Rubrivivax benzoatilyticus* JA2 produces

indole-3-acetic acid and related indole derivatives from L-tryptophan. Rubrivivaxin, a cytotoxic and cyclooxygenase-I inhibitory phenol terpenoid ester was reported (Ranjith *et al.,* 2011). Due to these properties, PNSB have been utilized to treat different types of wastewaters such as concentrated latex wastewater (Choorit *et al.,* 2002), odorous swine wastewater (Myung *et al.,* 2004), tuna condensate (Prasertsan *et al.,* 1997), oil-containing sewage wastewater, and latex rubber sheet wastewater (Kantachote *et al.,* 2005). Kasomu and Obst (2009) studied the influence of photosynthesis on calcite precipitation.

1.8.2 List of anoxygenic phototrophic bacterial genomes sequenced

1) *Allochromatium vinosum* DSM 180
2) *Chlorobaculum tepidum* TLS
3) *Chlorobium phaeovibrioides* DSM 265
4) *Chlorobium limicola* DSM 245
5) *Chloroflexus aggregans* DSM 9485
6) *Chlorobaculum parvum* NCIB 8327
7) *Chloroflexus aurantiacus* J-10-fl
8) "Candidates chloracidobacterium thermophilum B"
9) *Chloroherpeton thalassium* ATCC 35110
10) *Chlorobium phaeobacteroides* DSM 266
11) *Chlorobium phaeobacteroides* BS1
12) *Chlorobium chlorochromatii* CaD3
13) *Chlorobium luteolum* DSM 273
14) *Heliobacterium modesticaldum* Ice1
15) *Pelodictyon phaeoclathratiforme* BU-1
16) *Rhodopseudomonas palustris* CGA009
17) *Rhodopseudomonas palustris* TIE-1
18) *Rhodopseudomonas palustris* BisA53
19) *Rhodopseudomonas palustris* BisB18
20) *Rhodopseudomonas palustris* BisB5
21) *Rhodomicrobium vannielii* ATCC 17100
22) *Rhodospirillum rubrum* ATCC 11170
23) *Rhodospirillum centinum* SW
24) *Rhodobacter sphaeroides* ATCC 17029
25) *Rhodobacter* sp.SW2

26) *Rhodobacter sphaeroides* KD131
27) *Rhodobacter sphaeroides* 2.4.1
28) *Rhodobacter capsulatus* SB 1003
29) *Rubrivivax gelatinosus* IL144
30) *Rubrivivax benzoatilyticus* JA2
31) *Roseiflexus castenholzii* DSM 13941
32) *Thiocystis violascens* DSM 198
33) *Marichromatium purpuratum* 984

Among the phototrophic bacterial genomes sequenced listed above, only few of the bacterial genomes have the genes for aromatic hydrocarbon metabolism. *Allochromatium vinosum* DSM 180^T complete genome sequence verified that aromatic hydrocarbon degrading genes were not identified (Thomas *et al.,* 2011). *Rhodomicrobium vannielii* ATCC 17100, complete genome indicated the genes for aromatic hydrocarbon degradation. (NCBI Reference Sequence: NC_014664.1). *Rhodospirillum rubrum* ATCC 11170, *Rhodobacter sphaeroides* KD131, *Rhodobacter sphaeroides* ATCC 17025, *Rhodobacter sphaeroides* ATCC 17029 complete genome sequence showed the genes for 4-chlorobenzoate degradation. Fusaricacid resistance proteins, multidrug resistance protein coding genes were reported in *Rhodospirillum rubrum* ATCC 11170. Among *Rhodopseudomonas palustris* strains 70 % of the genes in each genome, shared by four or more strains. Between 10 % and 18 % of the genes in each genome are strain specific (Oda *et al.,* 2008). The complete genome sequence of *Rubrivivax benzoatilyticus* JA2 revealed many multidrug-resistant transporter, organic solvent resistance, and aromatic compound metabolizing genes (Mujahid *et al.,* 2011b). *Rhodopseudomonas palustris* CGA009, *Rhodopseudomonas palustris* TIE-1, *Rhodopseudomonas palustris* BisA53, *Rhodopseudomonas palustris* BisB18 and *Rhodopseudomonas palustris* BisB5 whole genome sequence analysis indicated the genes for benzoate degradation (Larimer *et al.,* 2004).

1.9 Definition of the problem

Aerobic metabolism of 4-toluenesulfonate is largely studied in chemotrophic bacteria and the enzymes, genes involved in 4-toluenesulfonate metabolism were well reported. Though there are reports on anaerobic desulfonation and demethylation of 4-toluenesulfonate, the work has not been extended for the elucidation of metabolic pathways, the enzymes and genes involved in anaerobic degradation of 4-toluenesulfonate were not studied. Though anoxygenic phototrophic bacteria are well known for aromatic hydrocarbon metabolism, the published information is only on purple non sulfur bacterial metabolism of aromatic hydrocarbons and alkylsulfonates. The present research work was initiated to explore the metabolic potential of purple sulfur bacteria by opting 4-toluenesulfonate as a test compound, with the following objectives.

OBJECTIVE

1. **Polyphasic taxonomic characterization of a 4-toluenesulfonate degrading anoxygenic purple sulfur bacterium**
2. **Catabolic studies of 4-toluenesulfonate by an isolated strain**

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 form 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 N_2 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$\pm$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$\pm$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$\pm$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 ºC until further use. The slants were sub cultured every 90 days and purity was checked periodically by streaking onto nutrient agar plates.

Ingredient	g l^{-1}
KH_2PO_4	0.25
$MgSO_4.7H_2O$	0.5
NaCl	20
NH_4Cl	0.34
$CaCl_2.2H_20$	0.25
Pyruvate	3.0
Yeast Extract	0.4
Ferric citrate solution (0.1 %, w/v)	1.5 ml
*Micronutrient solution (SL7)	1ml
**$Na_2S{\cdot}9H_2O/Na_2S_2O_3.5H_2O$	2 ml
***Vitamin B_{12} (2 mg/100 ml, w/v)	1ml

*SL7: (mg l^{-1}): HCl (25%, v/v) – 1 ml; $ZnCl_2$ (70); $MnCl_2.4H_2O$ (100); H_3BO_3 (60); $CoCl_2.6H_2O$ (200) $NiCl_2.6H_2O$ (20); $Na_2MoO_4.2H_2O$ (40); $CuCl_2.2H_2O$ (20)

**$Na_2S{\cdot}9H_2O$ solution $Na_2S{\cdot}9H_2O$ (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 B_{12} Vitamin B_{12} (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 C_{18} 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. Photoheterotrophically grown culture on agar slants was scraped with a loop to harvest about 40 mg of bacterial cells. The cells were placed in a clean 13 x 100 mm culture tube.

2.13.4 Endometabolome analysis through FT-IR analysis

For metabolome fingerprinting, exponentially growing photoheterotrophic 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^{0}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

triglycine sulfate) detector. Spectral data were processed using Spectrum One FT-IR software (Perkin Elmer).

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 $Na_2S.9H_2O$ (2 mM) / $Na_2S_2O_3$ .5 H_2O (5 mM), sulfite (2 mM) as the electron donor and $NaHCO_3$ (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 $Na_2S_2O_3.5H_2O$ (5 mM) as the electron donor and $NaHCO_3$ (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 × 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 NH_4Cl (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 (OD_{660}) 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, H_2 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 H_2 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 NH_4Cl, 0.07 %, w/v), organic substrates (glutamine, glutamate and urea, 0.07 %, w/v), sulfanilate, anthranilate (1 mM) and N_2 (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 NH_4Cl (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}], B_{12} [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 NH_4Cl (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].

↓

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 – 15ml
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^{o}C for 10 min to inactivate any DNAase present]

↓

Incubated for 30 min at 37°C

↓

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

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:

$$\text{mol \% G+C} = \frac{\text{G+C}}{\text{A+G+C+T}} \text{ x } 100$$

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′GAGTTTGATCCTGGCTCAG-3′) and Univ1492R (5′-GGTTACCTTGTTACGACT T-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

No. of cycles	Denaturation		Annealing		Elongation	
	Temp (°C)	Time	Temp (°C)	Time	Temp (°C)	Time
1	96	2 min				
32	96	40 s	50	40 s	72	1 min
1			42	1 min	72	5 min

(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′-TACGGGAGGCAGCAG-3′), 5′-790F(5′-GATACCCTGGTAGTCC-3′) and Univ1492R (5′-GGTTACCTTGTTACGACT 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).

Target loci	Function of gene/protein	Amplicon size(bp)	Primer Name	Primer sequence(5'-3')	Reference
16S-23S rRNA (ITS)	(16S-23SrRNA)	500	ITSf ITSr	GTGAAGTCGTAACAAGG CGTCACTTGACCATATCC	Garcia *et al.*, 1996,
PufM	Reaction center M	225	pufM.557F pufM.750R	CGCACCTGGACTGGAC CCCATGGTCCAGCGCCAGAA	Achenbach *et al.*, 2001
RecA	Recombinase A	450	recABDUP1 recABGDN2	CCCGAGTCCTCCGGNAARACNAC GCNACNACYTCRTCNGGRTT	Santos and Ochman 2004
DnaK	Chaperonin, HSP 60	850	INT1 INT2	CATCGGCATCATGGCNCAYTHGA CAGCATCGGCTGCAYNCCYTTRTT	Serrano *et al.*,2010
FusA	Translation elongation factor G	750	fusAF fusAR	CATCGGCATCATGGCNCAYATHGA CAGCATCGGCTGCAYNCCYTTRTT	Santos and Ochman 2004

Table 6: List of target loci and primers used for multilocus sequence analysis (MLSA) of strain JA121

Taxa	Genbank accession number (gene length)					
	16S rRNA	***fusA***	***ITS***	***pufM***	***recA***	***dnaK***
***Mch. littori* JA349^T**	AM947937 (1427 bp)	FN813506 (706 bp)	FN827327 (414 bp)	FN813510 (162 bp)	FN821505 (358 bp)	FN827323 (627 bp)
***Mch. chrysaorae* JA553^T**	FN813515 (1306 bp)	FN813508 (711 bp)	FN827326 (435 bp)	FN821502 (163 bp)	FN821504(3(35 8 bp)	FN827322 (659 bp)
***Mch. gracile* DSM 203^T**	EU850806 (1472 bp)	FJ376415 (330 bp)	FJ376574 (342 bp)	FJ376402 (225 bp)	FJ376426 (366 bp)	FJ376563 (741 bp)
***Mch. indicum* JA100^T**	AJ543328 (1435 bp)	FJ376420 (330 bp)	FJ376569 (342 bp)	FJ376402 (225 bp)	FJ376430 (366 bp)	FJ376560 (741 bp)
***Mch. bheemlicum* JA124^T**	AM180952 (1438 bp)	FJ376419 (330 bp)	FJ376568 (342 bp)	FJ376403 (225 bp)	FJ376428 (366 bp)	FJ376565 (741 bp)
***Mch. purpuratum* DSM 1591^T**	EU850807 (1467 bp)	FJ376421 (330 bp)	FJ376570 (342 bp)	FJ376395 (225 bp)	FJ376422 (366 bp)	FJ376559 (741 bp)
***Mch.fluminis* JA418^T**	FM210274 (1434 bp)	FN773063 (394 bp)	FN666580 (444 bp)	FN666585 (146 bp)	FN666588 (376 bp)	FN773064 (360 bp)
***Strain JA121**	AM179449 (1448 bp)	FN597063 (554 bp)	FN597062 (434 bp)	FN594741 (155 bp)	FN594744 (353 bp)	FN594743 (593 bp)

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

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/) and the restrict tion 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 ^oC 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.

Ingredient	Concentration
DNA	5 µl (200 – 300 ng)
10 X Nick translation buffer	5 µl (Supplied by BRIT, India)
dTTP	7 µl
dCTP	7 µl
dGTP	7 µl
α-^{32}P-dATP	5 µl (50 µCi)
H_2O	64 µl

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

After 2 h of incubation at 15 ^{o}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 ^{o}C for 1 h or over night at –20 ^{o}C and spin at 15000 rpm for 20 min at 4 ^{o}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 T_m 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 ^{o}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 [^{14}C] channel of the Tri-carb liquid scintillation counter (Model No. B1500, Zurich, Switzerland).

The percent hybridization was calculated as follows:

$$\% \text{ Hybridization} = \frac{\text{Counts obtained from heterologous hybridization}}{\text{Counts obtained from homologous hybridization}} \times 100$$

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.

$O.D_{660}$ of 0.1 = 0.3 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_2SO_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 10min) 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 0_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 0_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 0_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

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 appendorfs. 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 tolulica*], 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.

Primer sets	Sequence (5'–3')	Annealing temp (°C)
1) BSS FP1	ACTGGGTCATAGTGCTGTGCATGTCGCCCGG	60
2) BSS FP2	GTAGACGCAAGACCCAGAAGACCCGTTCGGAAG	60
3) BSS RP1	AATCGTGGTGATGGGGTTGTGCCAGCCGTTGGG	60
4) BSS RP2	GGTTCGCGAGCCCACTTCCATGTAAAGGCCGAC	60

Table 9: Primer sets designed from alignments of known *bss*A sequences

Steps	Temp (^{o}C)	Time (min)	cycles
Initial denaturation	94	4	
Denaturation	94	1	
Annealing	65	1	30
Elongation	72	1	
Final elongation	72	5	

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

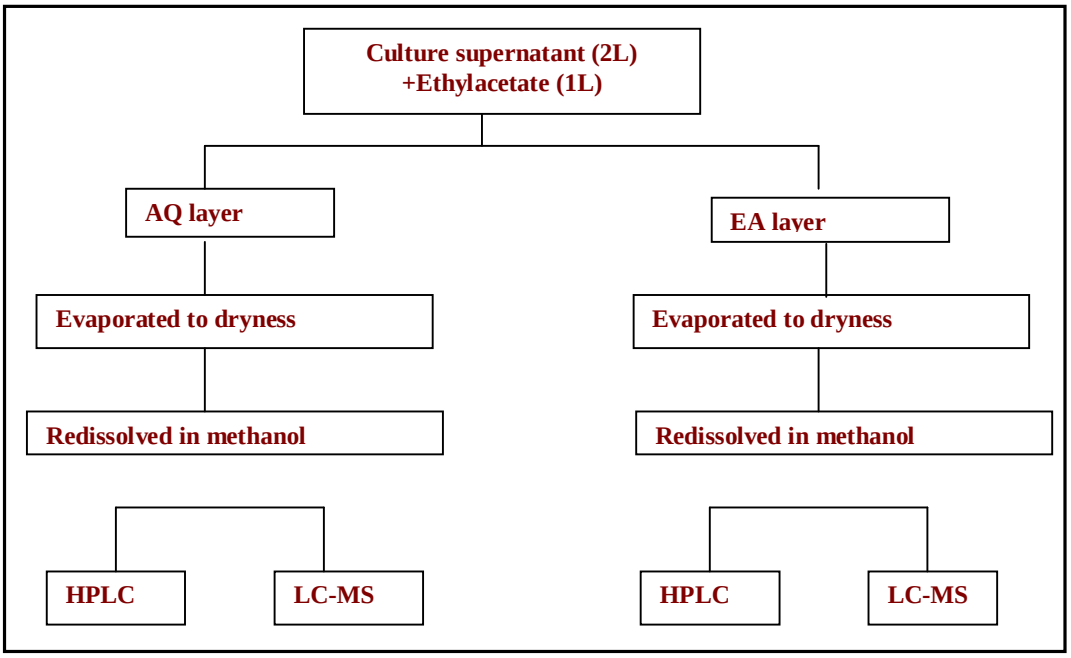
Cells of *Marichromatium* sp. JA121 grown in the presence and absence of 4-toluenesulfonate were harvested by centrifugation (16000 x g for 10 min) at 12, 24 and 66 h of phototropic assay was done in fully filled 250 ml reagent bottles under illumination (2400 lux) incubations. The culture supernatant 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 C_{18} 100A column (250 x 4.6mm) and the compounds were detected in UV-VIS detector at 222nm.The retention times (t_R in min) of pyruvate and 4-toluenesulfonate was 2.5 and 4.62 respectively.



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 ^{0}C (LC column oven) and 85 ^{0}C (MS ionization chamber). Methanol, water (1:1) was used as a solvent at 0.2 ml.min^{-1}, Luna 5µ C_{18} (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 Atmosphearic pressure chemical ionization (APCI) region under N_2 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 ºC (LC column oven) and 85 ºC (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.

Results

3.1 Isolation, purification and characterization of 4-toluenesulfonate degrading anoxygenic phototrophic purple sulfur bacterium

3.1.1 Sampling site

The sample (water on the rock in the sea shore) that yielded strain JA121 was collected on 30th March, 2004 from the fishing harbor of Visakhapatnam, India (GPS positioning of the sampling site is 17°41'42.40''N 83°18'08.80'' E). The sample had a pH of 7.0, 2 % (w/v) NaCl and temperature of 30 ^{o}C.

3.1.2 Enrichment and purification

Water sample collected form sea shore was kept for enrichment in three media compositions with 2 % NaCl, *viz* photoautotrophic medium with sulfanilate (3mM) as electron donor, the second with sulfide (3 mM) as electron donor and the third is the photolithoheterotrophic medium with sulfide (3 mM) and pyruvate (27 mM) as electron donor and carbon source respectively. Photosynthetic bacterial enrichment was observed in growth medium (Table 4) was purified by repeated streaking on selective media slants under anoxic conditions. This isolate Vdark is given with the strain number JA121, studied through polyphasic taxonomic characterization.

3.1.3 Polyphasic taxonomic characterization

3.1.3.1 Cultural characteristics

On agar slant, colonies of strain JA121 were round, convex, smooth and reddish brown colored (Fig. 6A). Size of the colony has reached 1-2 mm diameter after 7 days of incubation under fluorescent light (2,400 lx) at 30 ^{o}C.

3.1.3.2 Morphology and fine structure

Individual cells of strain JA121 were rod shaped 1.0–2.0 µm wide and 2.0–5.0 µm long, motile with a single monopolar flagellum, multiplied by binary fission and stained Gram negative. Each cell contained two to three elemental sulfur globules within the cells when grown photolithoheterotrophically using sulfide as electron donor (Fig 6C). Cells incubated photoorganoheterotrophically could not show sulfur deposition. Cell aggregates were observed when high sulfide (15 mM) was used in the medium (Fig 6D). Electron

microphotograph of ultrathin sections of the cells revealed vesicular type of internal membrane structures (Fig. 6E).

3.1.3.3 Pigment composition

Color of photosynthetically [anaerobically, in the light (2,400 lx)] grown cell suspension of strain JA121 was reddish brown (Fig 6B**).** The whole cell absorption spectrum of intact cells of the strain in sucrose showed maxima at 371, 461, 488, 521, 593, 800 and 851 nm, which corresponds to bacteriochlorophyll *a* and carotenoids (Fig 6G). The absorption spectrum for pigments extracted with acetone showed an absorption maxima at 445, 474 and 504 nm (Fig 6H), indicating the presence of carotenoids of the spirilloxanthin series. Critical analysis of carotenoids composition, after extracting the pigments into the solvent system (acetone and methanol [7:3]) through HPLC, indicated that strain JA121 has rhodopin (50 %) as the major pigment followed by spirilloxanthine (29 %), anhydrorhodovibrin (7 %), diapolycopene (6 %), rhodovibrin (4 %), lycopene (2 %) and tetrahydrolycopene (2 %) (Fig 6I).

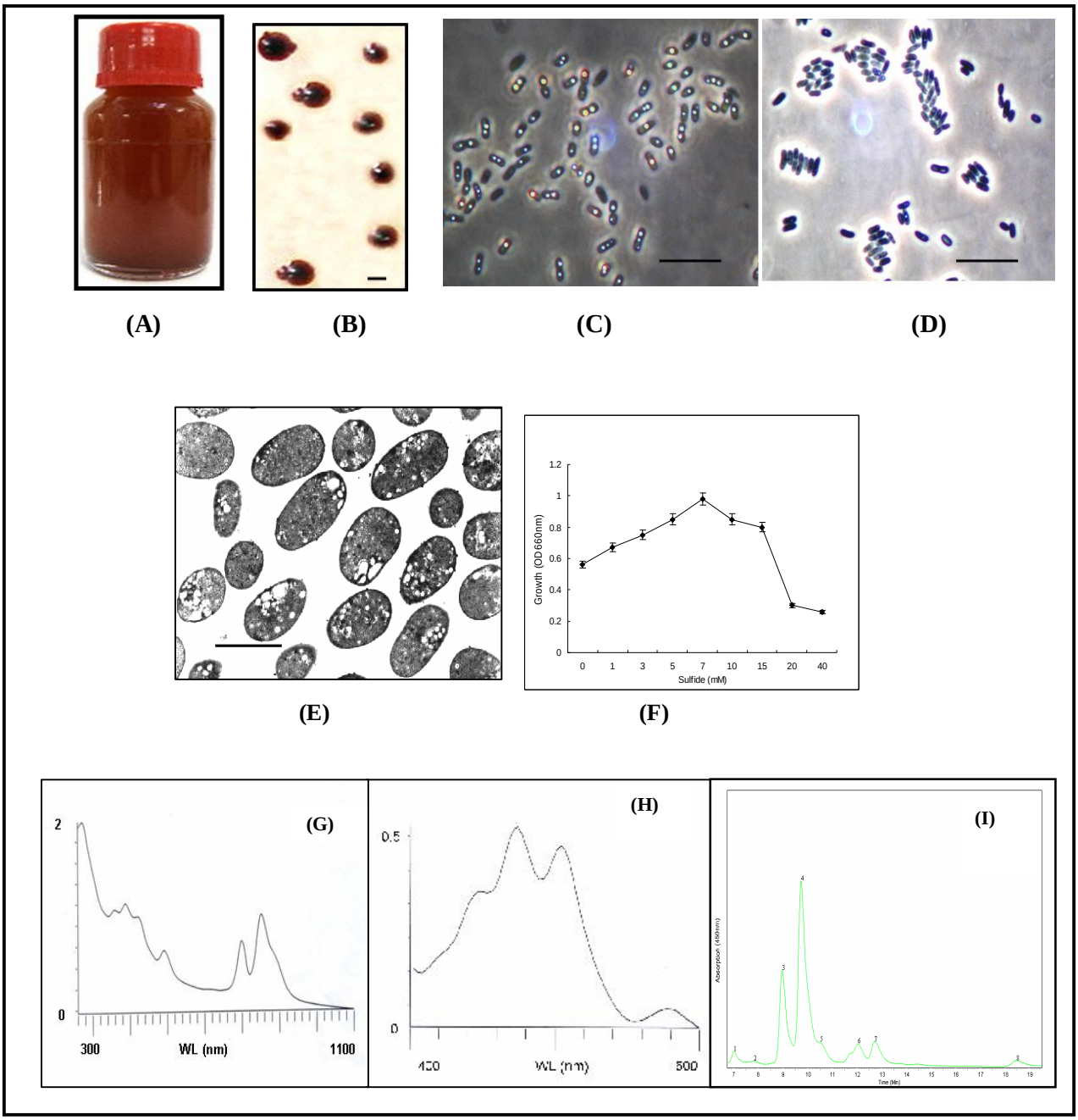


Fig 6: (A) Colony morphology [Bar, 2 mm], (B) broth culture (C) Phase-contrast micrograph of strain JA121 showing one to two sulfur granules inside the cell [Bar, 12.6 µm]. (D) Phase contrast microphotograph of strain JA121 grown at 15 mM sulfide [Bar, 12.6 µm].
(E) Electron micrograph of ultrathin sections of strain JA121 showing vesicular nature of photosynthetic membranes extending throughout the cell [Bar, 0.5 µm].
(F) Sulfide tolerance curve of strain JA121.
(G) Whole-cell absorption spectrum (H) acetone spectrum of extracted pigments of strain JA121.
(I) HPLC chromatogram showing the peaks of carotenoids extracted into acetone and methanol. 1, Diapolycopene; 2, Lycopene; 3, Spirilloxanthin; 4, Rhodopin; 5, Rhodovirbin; 7, Anhydrorhodovibrin; 8, Tetrahydrolycopene

3.1.3.4 Physiological characteristics

Strain JA121 grows photoorganoheterotrophically (anaerobically in the light [2400 lx] with pyruvate as carbon and electron source) and photolithoheterotrophically (anaerobically in the light [2400 lx] with $Na_2S.9H_2O$ [1 mM] or $Na_2S_2O_3.5H_2O$ [1 mM] as electron donors and organic substrates [0.3 % w/v] as carbon source). Photolithoautotrophy, chemolithoautotrophy, chemoorganoheterotrophy and fermentative mode of growth could not be demonstrated. The substrates, which were utilized as carbon sources and/e- donor under photolithoheterotrophic conditions by strain JA121, include acetate, propionate, pyruvate, malate, valerate and fumarate. Those, which could not be utilized by strain JA121, include butyrate, lactate, fructose, glucose, ethanol, propanol, glycerol, glycolate, crotonate, benzoate, citrate, tartrate, caprylate, caproate and casamino acids (Table 11). Thiosulfate, sulfite, sodium sulfide, cysteine, alkylsulfonates and arylsulfonates (Table 12) were utilized as sole sulfur sources by strain JA121 under photoheterotrophic conditions. Sulfide was obligately required for strain JA121 as a source of sulfur, and tolerated up to 15 mM (Fig. 6F). Ammonium chloride, sodium nitrate, glutamine, cysteine, tryptophan, sulfanilate and anthranilate were utilized as sole nitrogen sources, while molecular nitrogen, urea and nitrite could not support growth of strain JA121 (Table 13). Acetylene reduction activity for the enzyme nitrogenase was also not observed in strain JA121. Salt is obligatory for the growth of strain JA121 and growth occurs from 1.5-8.5 % (w/v) NaCl with an optima at 1.5-5.0 % (w/v). pH range and optima for strain JA121 were 6.5-8.5. Temperature range was from 25-35 °C and optimum was at 30-35 °C. Specific vitamins requirement was not observed for growth of strain JA121 (Table 14).

3.1.3.5 Utilization of arylsulfonates by *Marichromatium* spp.

Strain JA121 and other type strains of the genus *Marichromatium* viz., *Marichromatium gracile* DSM 203^T, *Marichromatium indicum* JA100^T, *Marichromatium purpuratum* DSM 1591^T, *Marichromatium bheemlicum* JA124^T and *Marichromatium flumins* JA418^T were screened for utilization of arylsulfonates (4-toluenesulfonate and benzenesulfonate). None of the Marichromatium species could utilize 4-toluenesulfonate, benzenesulfonate as sole source of carbon, however only *Mch. purpuratum* DSM 1591^T and strain JA121 could utilize 4-toluenesulfonate and benzenesulfonate as a sole source of

sulfur (Table 15). On repeated subcultures, *Mch*. *purpuratum* DSM 1591^T lost the utilization capability, where as strain JA121 could retain its capability to utilize 4-toluenesulfonate and benzenesulfonate.

3.1.3.6 Whole-cell fatty acids

Fatty acid methyl ester analysis was outsourced for strain JA121, revealed the predominance of $C_{16:0}$ (24.3 %); $C_{16:1}\omega7c/C_{16:1}\omega6c$ (32.3 %) and $C_{18:1}\omega7c$ (29.3 %); $C_{18:1}\omega6c$ (29.3 %); $C_{12:0}$ (6.1 %) $C_{14:0}$ (2.1 %) and other fatty acids were also identified in trace amounts (Table 16).

3.1.3.7 Quinones

Strain JA121 has ubiquinone-8 (Q-8) and menaquinone-7 (MK-7) as major quinone components and considerable amounts of Q-7 was also observed.

Carbon source/e- donor	Growth ($OD_{660\ nm}$)	Growth (+ / -)
Control (without carbon source)	0.00	-
Control (HCO_3^- 0.1 %, w/v)	0.02	-
Acetate	0.15	+
Propionate	0.24	+
Valerate	0.18	+
Pyruvate	0.65	+
Fumarate	0.48	+
Malate	0.41	+

Table 11: Photoheterotrophic growth of strain JA121 on different organic substrates as carbon sources/electron donors

Symbols: +, supported growth; $^-$, not supported growth

Strain JA121 was inoculated into mineral media with different organic substrates as carbon sources and NH_4Cl as nitrogen source. Results expressed are average values of an experiment done in triplicates after 72 h of light (2,400 lx) anaerobic incubation at 30 $\pm$ 2 °C. Organic substrates were utilized at a concentration of 0.3 % (w/v) except for fatty acids (propionate, butyrate, valerate and caproate), alcohols (ethanol, methanol) and glycerol, which were used at a concentration of 0.1 % (v/v).

Sulfur sources	Growth ($OD_{660\ nm}$)	Growth (+ /-)
Control (without sulfur source)	0.00	-
Magnesium sulfate (5 mM)	0.62	+
Sodium sulfite (5 mM)	0.40	+
Sodium sulfide (1 mM)	0.65	+
Sodium thiosulfate (5 mM)	0.60	+
Sodium thioglycolate (5 mM)	0.01	-
Cysteine (2 mM)	0.48	+
Methanesulfonate	0.4	+
Butanesulfonate	0.3	+
Taurine (2-aminoethanesulfonic acid)	0.3	+
Benzenesulfonate	0.3	+
4-Toluenesulfonate	0.4	+
4-Sulfobenzoate	0.4	+
5-Sulfosalicylate	0.24	+
Sulfanilate (4-Aminobenzenesulfonate)	0.4	+
Sodiumdodecylsulfate	0.0	-

Table 12: Photoheterotrophic growth of strain JA121 on various sulfur sources

Symbols: +, supported growth; ⁻, not supported growth

Experimental details as given in Table 4 except that pyruvate (0.3 %, w/v) was used as carbon source and sulfur source varied as indicated. Alkylsulfonates and arylsulfonates were used at the concentrations of 1 mM. Data values are the average of the experiment repeated thrice.

Nitrogen sources (0.07 %, w/v)	Growth ($OD_{660\ nm}$)	Growth (+ /-)
Control (without nitrogen source)	0.00	-
Ammonium chloride	0.65	+
Sodium nitrate	0.21	+
Sodium nitrite	0.01	-
Urea	0.03	-
N_2 (100 %, v/v gas phase)	0.02	-
Glutamate	0.01	-
Glutamine	0.68	+
Cysteine	0.21	+
Tryptophan	0.20	+
Sulfanilate	0.50	+
Anthranilate	0.40	+

Table 13: Photoheterotrophic growth of strain JA121 on various nitrogen sources

Symbols: +, supported growth; ⁻, not supported growth

Experimental details as given in Table 4, except that pyruvate (0.3 %, w/v) was used as carbon source and nitrogen source varied as indicated. Data values are the average of the experiment repeated thrice.

Growth factor	Growth ($OD_{660\ nm}$)	Growth (+ /-)
Control (without vitamin source)	0.00	NA
*Without biotin	0.60	+
*Without niacin	0.62	+
*Without thiamine	0.62	+
*Without *p*-aminobenzoate	0.65	+
*Without pyridoxal phosphate	0.62	+
*Without pantothenate	0.62	+
*Without Vitamin B_{12}	0.50	+
#All	0.7	+
Yeast extract	0.61	+

Table 14: Growth factor requirement by strain JA121

Experimental details as given in Table 4, except that pyruvate (0.3 %, w/v) was used as carbon source. *, other 7 vitamins were added; #, all 8 vitamins were added; NA, not applicable. Symbols: +, supported growth; ⁻, not supported growth

Marichromatium spp.	Arylsulfonates utilization (+/-)
Mch. purpuratum DSM 1591^T	+
Mch. gracile DSM 203^T	-
Mch. indicum JA100^T	-
Mch. bheemlicum JA124^T	-
Mch. fluminisi JA418^T	-
Strain JA121	+

Table 15: Utilization of arylsulfonates as a sole source of sulfur by *Marichromatium* spp.

Marichromatium species were grown photoheterotrophically in mineral medium (Lakshmi *et al.*, 2009) with pyruvate (22 mM), NH_4Cl (7 mM) as sole carbon and nitrogen sources respectively and arylsulfonates (4-toluenesulfonate and benzenesulfonate) as sole sulfur source (1.0 mM) listed in the table for 48 h and assayed for arylsulfonate utilization through HPLC.

Fatty acid profiles	mol %
$C_{12:0}$	6.1
$C_{14:0}$	2.1
$C_{16:0}$	24.3
$C_{16:1}$ ω7c	32.3
$C_{16:1}$ω7c alcohol	1.0
$C_{18:1}$ω5c	1.0
$C_{18:1}$ω6c	29.3
$C_{18:1}$ω7c	29.3

Table 16: Whole cell fatty acid composition of strain JA121.

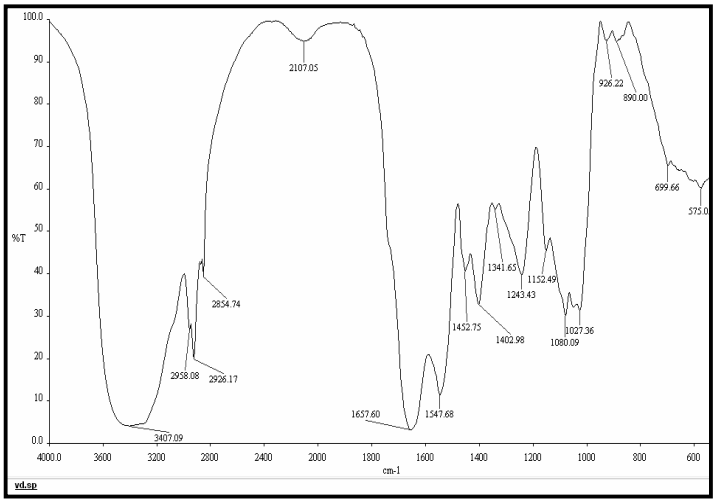


Fig 7: FTIR foot printing of strain JA121

Log phase culture of strain JA121 was harvested and pellet was lyophilized. Transmission spectrum of the lyophilized pellet was recorded using Schimadzu infrared spectrophotometer.

3.1.3.8 Molecular and Phylogenetic characterization

3.1.3.8.1 mol % G+C content of DNA

The genomic DNA base composition of strain JA121 was 64 mol % G+C (by HPLC).

3.1.3.8.2 16S rRNA gene sequence analysis of strain JA121

The phylogenetic relationship of strain JA121 to other purple sulfur bacteria was examined by 16S rRNA gene sequencing.

3.1.3.8.2.1 Phylogenetic analysis-Dendrogram

The 16S rRNA gene of the bacterial strain JA121 was amplified from the genomic DNA using the protocol mentioned in the material and methods and sequenced. The pair wise sequence similarities of strain JA121 with the nearest type strains were found using EzTaxon server. 16S rRNA gene sequence of strain JA121 showed 99.7 % similarity with the 16S rRNA gene of *Marichromatium gracile* DSM203^T, 99.3 % with *Marichromatium indicum* JA100^T, 99.3 % with *Marichromatium purpuratum* DSM1591^T, 98.2 % *Marichromatium bheemlicum* JA124^T, 98.9 % with *Marichromatium litoris* JA349^T, 98.8 % with *Marichromatium chrysaorae* JA553^T. 16S rRNA gene sequence of strain JA121 was deposited in the GenBank/EMBL/DDBJ with the accession number AM179449.

Dendrogram was constructed based on 16S rRNA gene sequences using the software as mentioned in materials and methods (Fig 8). 16S rRNA gene sequence of strain JA121 showed 98.34 % similarity with the 16S rRNA gene of *Marichromatium gracile* DSM203^T, 98.28 % with *Marichromatium indicum* JA100^T, 98.24 % with *Marichromatium purpuratum* DSM1591^T, 95 % *Marichromatium bheemlicum* JA124^T, 98 % with *Marichromatium litoris* JA349^T, 98.6 % with *Marichromatium chrysaorae* JA553^T.

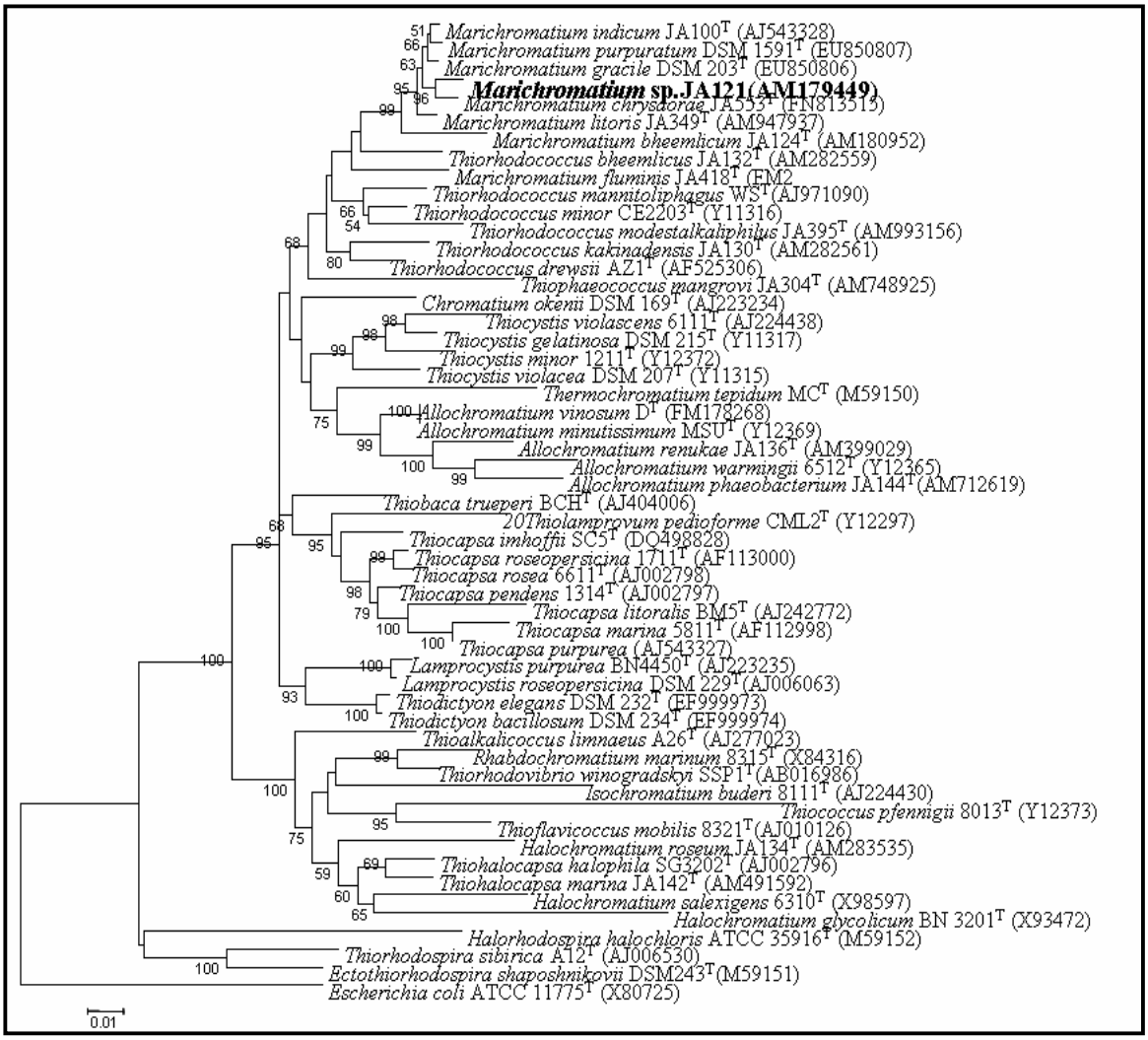


Fig 8: 16S rRNA gene-based dendrogram showing the phylogenetic position of strain JA121 within the order *chromatiales* of class *Gammaproteobacteria.* The tree was constructed by the neighbor-joining method using the MEGA4 software and rooted by using *E. coli* as the out group. Numbers at nodes represent bootstrap values (based on 100 resamplings). The GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar indicates nucleotide substitutions per 100 nucleotides.

3.1.3.8.3 Multilocus sequence analysis (MLSA)

3.1.3.8.3.1 Amplification and sequencing of the genes

The Phylogenetic relationship of strain JA121 to other purple sulfur bacteria was examined by amplification of four universally present protein coding and metabolic house keeping genes specific for purple sulfur 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 16 S rRNA and 16-23S internal transcriber spacer (*ITS*) was done. These genes were amplified using reported primer sets through TD-PCR as mentioned in methodology. The amplified genes were sequenced and deposited in the Gen Bank with accession numbers listed in the table 7. The individual and concatenated gene sequences of *recA, fusA, dnaK, pufM,* ITS and 16S rRNA of the strain JA121 were compared with the members of the genus *Marichromtium.*

3.1.8.3.2 Phylogenetic analysis of individual gene sequences

A. Phylogenetic analysis of ITS

The phylogenetic tree based on ITS indicated that strain JA121 showed similarity of 100 % with *Marichromatium fluminis* JA418^T, 53 % with *Marichromatium indicum* JA100^T and *Marichromatium bheemlicum* JA124^T, 51% with *Marichromatium chrysaorae* JA553^T, *Marichromatium gracile* DSM203^T, *Marichromatium purpuratum* DSM 1591^T and *Marichromatium litoris* JA349^T (Fig 9A).

B. Phylogenetic analysis of *fusA*

The phylogenetic tree based on *fusA* indicated that strain JA121 showed similarity of 98 % with *Marichromatium chrysoarae* JA553^T, 97 % with *Marichromatium gracile* DSM203^T, 95 % with *Marichromatium bheemlicum* JA124^T, 93 % with *Marichromatium purpuratum* DSM1591^T, *Marichromatium litoris* JA349^T, 92 % with *Marichromatium indicum* JA100^T and 66 % with *Marichromatium fluminis* JA418^T (Fig 9B).

C. Phylogenetic analysis of *recA*

The phylogenetic tree based on *recA* indicated that strain JA121 showed similarity of 99 % with *Marichromatium indicum* JA100^T, *Marichromatium chrysaorae* JA553^T 98 % with *Marichromatium gracile* DSM203^T, 97 % with *Marichromatium purpuratum*

DSM 1591^T, 96 % with *Marichromatium litoris* JA349^T, 95 % with *Marichromatium bheemlicum* JA124^T and 84 % with *Marichromatium fluminis* JA418^T (Fig 9C).

D. Phylogenetic analysis of *dnaK*

The phylogenetic tree based on *dnaK* indicated that strain JA121 showed similarity of 99 % with *Marichromatium indicum* JA100^T, 98 % with *Marichromatium purpuratum* DSM1591^T, *Marichromatium gracile* DSM203^T, *Marichromatium chrysaorae* JA553^T, *Marichromatium litoris* JA349^T, 97 % with *Marichromatium bheemlicum* JA124^T and 49 % with *Marichromatium fluminis* JA418^T (Fig 9D).

E. Phylogenetic analysis of *pufM*

The phylogenetic tree based on *pufM* indicated that strain JA121 showed similarity of 100 % with *Marichromatium gracile* DSM203^T, *Marichromatium indicum* JA100^T, *Marichromatium bheemlicum* JA124^T and *Marichromatium chrysaorae* JA553^T, 97 % with *Marichromatium litoris* JA349^T, 92 % with *Marichromatium purpuratum* DSM1591^T and 85 % with *Marichromatium fluminis* JA418^T (Fig 9E).

3.1.3.8.3.3 Phylogenetic analysis of concatenated gene sequences

Each gene was independently aligned using ClustalX and the alignment file was Gblocks edited before concatenation. Sequence length after Gblocks editing for the genes: 16S rRNA, *fusA*, ITS, *pufM, recA* and *dnaK* was 1159, 107, 189, 101, 252 and 176 bp, respectively. The similar length gene sequences were concatenated and further Gblocks edited to yield a uniform sequence length of 1869 bp for all strains of *Marichromatium*. After Gblocks improvements of the concatenated gene sequences of 16S rRNA, *ITS*, *pufM*, *dnaK*, *recA*, and *fusA*. the phylogenetic tree was constructed by the neighbor-joining method using the MEGA4 software (Fig 10). MLSA analysis of strain JA121 showed 99 % similarity with *Marichromatium gracile* DSM203^T, *Marichromatium indicum* JA100^T, 98% with *Marichromatium purpuratum* DSM1591^T, *Marichromatium litoris* JA349^T, *Marichromatium chrysaorae* JA553^T, *Marichromatium bheemlicum* JA124^T and 88% with *Marichromatium fluminis* JA418^T.

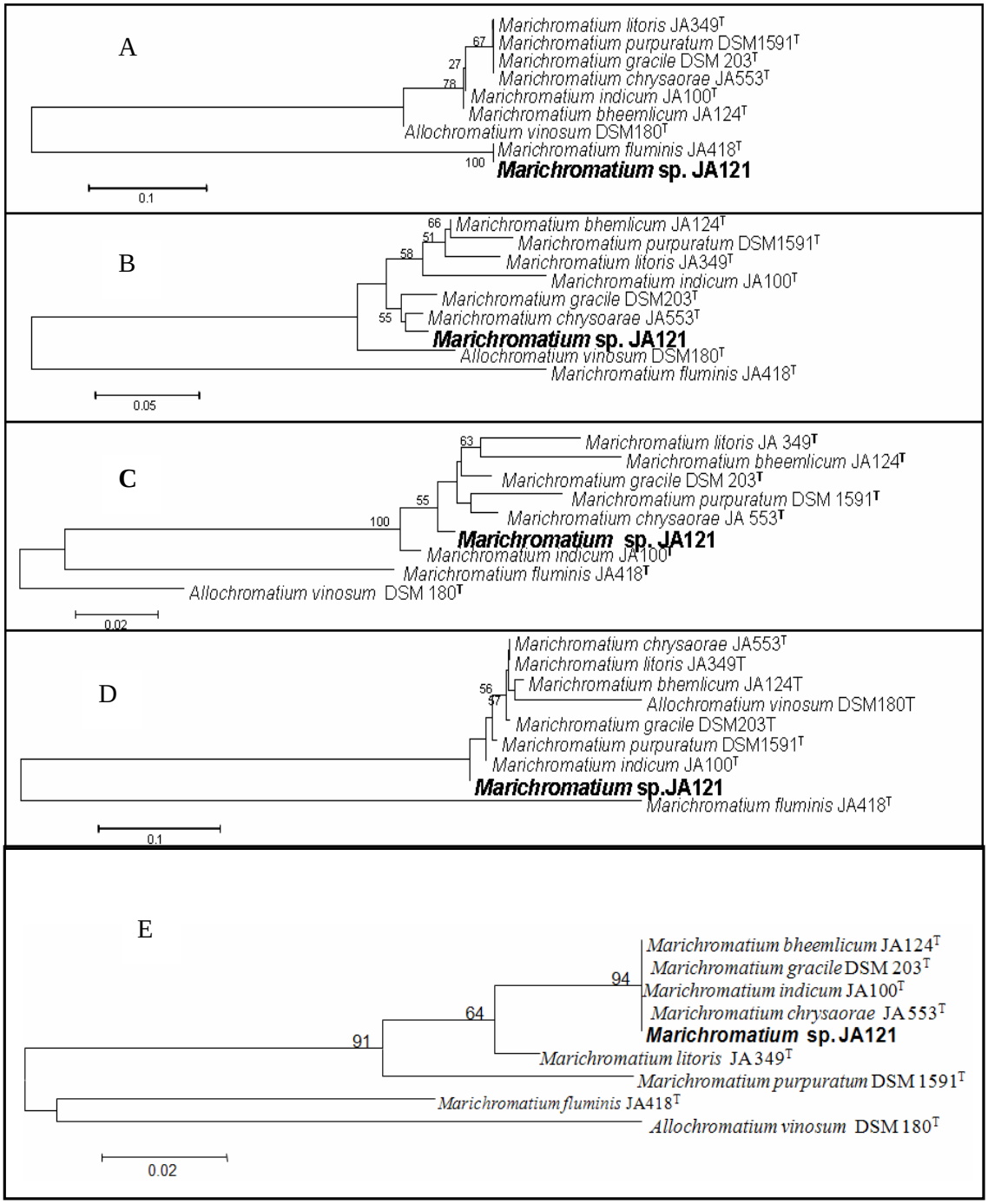


Fig 9: Phylogenetic trees based on gene sequences of A, ITS; B, *fusA*; C, *recA*; D, *dnaK* and E, *pufM*.

The sequences were regenerated by G blocks improvements and the phylogenetic trees were constructed by the neighbor-joining method using the MEGA4 software. Bar indicates % of sequence divergence. *Allochromatium vinosum* DSM 180^T was used as out group.

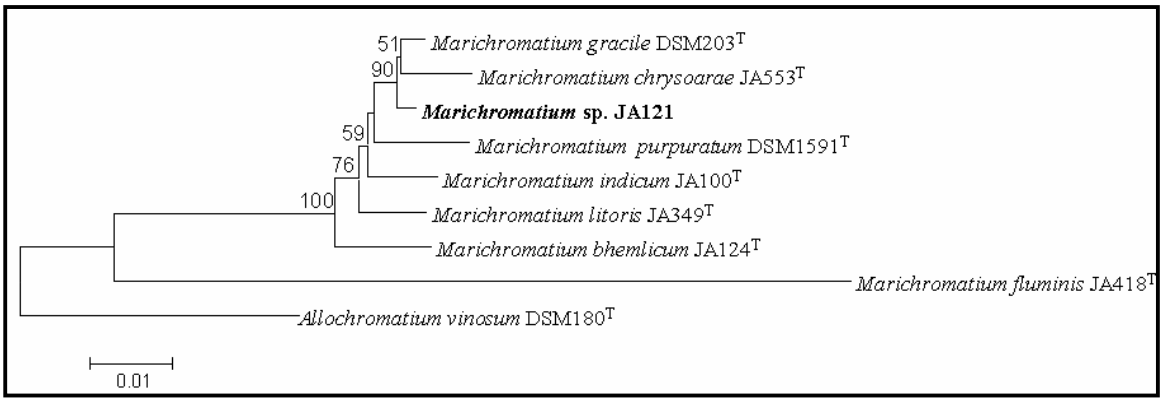


Fig 10: Phylogenetic tree based on concatenated gene sequences of 16S rRNA, ITS, *pufM*, *dnaK*, *recA*, and *fusA*. The sequence alignments were regenerated by G blocks improvements and the phylogenetic tree was constructed by the neighbor-joining method using the MEGA4 software. Bar indicates 1 % sequence divergence. *Allochromatium vinosum* DSM 180^T was used as out group.

3.1.3.8.4 MLSA barcoding

MLSA barcoding was done for strain JA121 and rest of the members of the genus *Marichromatium*. The Gblocks edited concatenated sequences of six genes 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/). The cleavage code given by the software in different colors were the restriction bands, used as the MLSA barcode. The barcodes obtained for type strains of *Marichromatium* were shown in Fig. 11, where variations in the bands observed for strain JA121 with the rest of the members of the genus *Marichromatium*.

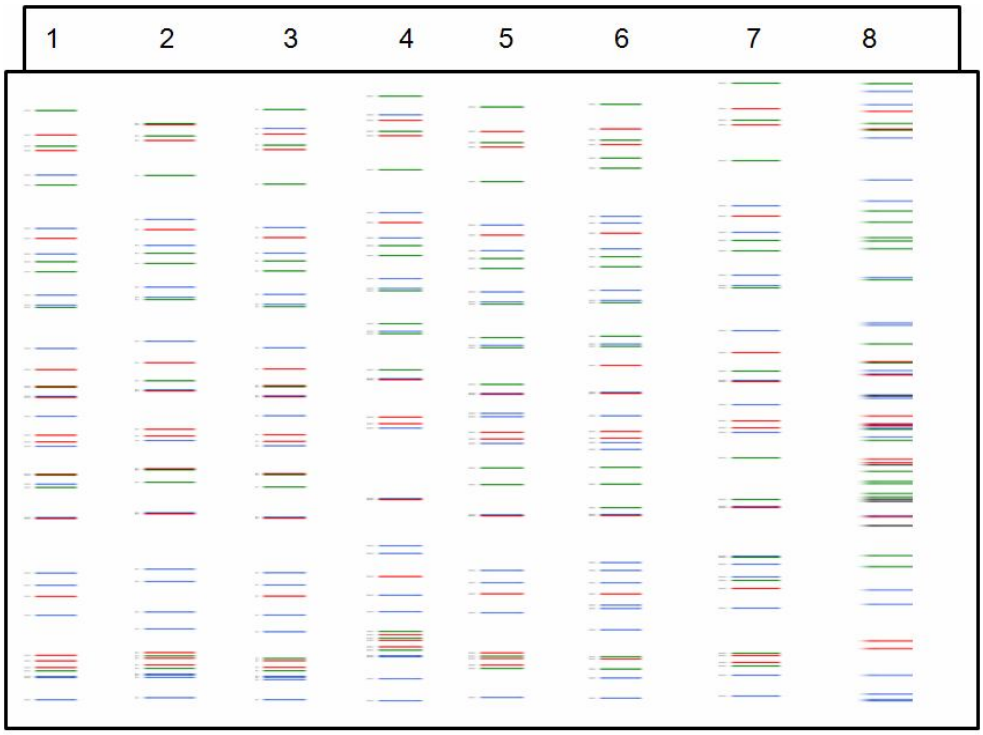


Fig 11: MLSA barcodes of 1, *Marichromatium* sp. JA121; 2, *Mch. chrysaorae* JA553^T 3, *Mch. gracile* DSM 203^T; 4, *Mch. purpuratum* DSM1591^T; 5, *Mch. indicum* JA100^T; 6, *Mch. bheemlicum* JA124^T 7, *Mch. litoris* JA349^T and 8, *Mch. fluminis* JA418^T

3.1.3.8.5 Plasmid detection

Plasmid was identified in strain JA121, which was absent in other type strains of *Marichromatium*.

3.1.3.8.6 DNA–DNA hybridization

DNA-DNA hybridization was done between strain JA121 and the type strains of *Marichromatium*. DDH relatedness values of strain JA121 with *Marichromatium gracile* DSM203^T are 60 %, 65 % with *Marichromatium purpuratum* DSM1591^T, 50 % with *Marichromatium indicum* JA100^T and 53 % with *Marichromatium bheemlicum* JA124^T (Table 17).

3.1.4 Culture deposition

Strain JA121 was deposited in two culture collection centers *viz.*, Korean collection for type cultures (KCTC) and NITE biological resource centre (NBRC) as *Marichromatium* sp. JA121 =KCTC 5816 =NBRC 106082.

	Mch. indicum JA100^T	Strain JA121	*Mch. bhemlicum* JA124^T	*Mch. gracile* DSM 203^T	*Mch. purpuratum* DSM 1591^T	*Mch. chrysoarae* JA553^T	*Mch. litoris* JA 349^T
Mch. indicum JA100^T	100						
Strain JA121	50	100					
Mch. bhemlicum JA124^T	61	53	100				
Mch. gracile DSM 203^T	81	60	65	100			
Mch. purpuratum DSM 1591^T	65	65	63	75	100		
Mch. chrysaorae JA553^T	42	ND	55	50	45	100	
Mch. litoris JA 349^T	40	ND	49	41	35	41	100

Table 17: DDH relatedness (%) among the *Marichromatium* spp.

The reassociation values are the mean of two independent experiments done in triplicates

ND=Not done

3.2. Utilization of alkylsulfonates, arylsulfonates and other aromatic hydrocarbons by *Marichromatium* sp. JA121

3.2.1. Utilization of alkylsulfonates and arylsulfonates by *Marichromatium* sp. JA121

Growth of *Marichromatium* sp. JA121 could not be demonstrated on alkylsulfonates (1 mM) when used as sole source of carbon or as electron donors, replacing pyruvate in the modified Biebl and Pfennig's medium. Among the alkylsulfonates tested, utilization of methanesulfonate, butane sulfonate and taurine (2-aminoethanesulfonate) was observed after 24 h of phototrophic incubation when added as sole sulfur source at a concentration of 1.0 mM (Table 18). Among the alkylsulfonates tested maximum utilization was observed with methanesulfonate (0.7 mM) by *Marichromatium* sp. JA121. Desulfonation of methanesulfonate, butane sulfonate and taurine was observed with the release of sulfite into the culture supernatant. The loss of compound in the culture supernatant could support growth of *Marichromatium* sp. JA121, since increase in the biomass (compared to control culture) could be demonstrated. Uninnoculated medium was used as control to check for possible photochemical reactions. Sodium dodecyl sulphate precipitate the initially added inoculums there by inhibited the growth of *Marichromatium* sp. JA121 even at a concentration of 0.5 mM.

Similarly growth of *Marichromatium* sp. JA121 could not be demonstrated on arylsulfonates when used as sole source of carbon or as electron donors, replacing pyruvate in the modified Biebl and Pfennig's medium. However growth and utilization of arylsulfonates was observed when added as sole sulfur source at a concentration of 1.0 mM (Table 18) replacing $MgSO_4$. $7H_2O$ in the modified Biebl and Pfennig's medium. Desulfonation of benzenesulfonate, 4-toluenesulfonate, 4-sulfobenzoate, 4-aminobenzenesulfonate, 5-sulfosalicylate was observed with the release of sulfite into the culture supernatant of *Marichromatium* sp. JA121. The increase in biomass yield of *Marichromatium* sp. JA121 was observed with all the arylsulfonates tested, when compared to control culture. Among the arylsulfonates tested, maximum utilization (0.7 mM) was observed with 4-toluenesulfonate. Uninnoculated medium was used as control to check for possible photochemical reactions.

The toxicity of arylsulfonates on growth of *Marichromatium* sp. JA121 was evaluated as minimum inhibitory concentration (MIC). The MIC values of benzenesulfonate, 4-toluenesulfonate, 4-sulfobenzoate, 5-sulfosalicylate and 4-aminobenzenesulfonate were 80, 180, 150, 80 and 150 mM, respectively.

3.2.2. Utilization of other aromatic hydrocarbons by *Marichromatium* sp. JA121

Growth of *Marichromatium* sp. JA121 could not be demonstrated on aromatic compounds when used as sole source of carbon or as electron donors, replacing pyruvate in the modified Biebl and Pfennig's medium. Among the various aromatic compounds tested, utilization of 2-aminobenzoate, *o*-xylene, *p*-xylene, *p*-cresol, toluene, L-phenylalanine, L-tryptophan, L-tyrosine, trans-cinnamate and salicylate was observed after 48 h of phototrophic incubation when added as supplement at a concentration of 0.5 mM (Table 19). The loss of compound from the culture supernatant could not support growth of *Marichromatium* sp. JA121, since increase in the biomass (compared to control culture) could not be demonstrated. Benzoate, 4-hydroxybenzoate, 3,4-dihydroxy benzoate, *m*-xylene, *o*-cresol and *m*-cresol levels in the supernatant remained same as the initial concentration (0.5 mM) and their utilization by *Marichromatium* sp. JA121 was not observed. Uninoculated medium was used as control to check for possible photochemical reactions. Indoles were observed in the L-tryptophan and 2-aminobenzoate induced culture supernatant.

Substrate (1 mM)	Biomass yield (mg dry wt.ml^{-1})	Utilization (mM)	Sulfite release (+/-)
Alkylsulfoantes			
Control (Without sulfur)	0.9	NA	NA
Methanesulfonate	1.2	0.7	+
Butanesulfonate	1.05	0.3	+
Taurine (2-aminoethanesulfonate)	1.05	0.3	+
Sodium dodecyl sulfate	0.0	0.0	-
Arylsulfonates			
Benzenesulfonate	1.2	0. 4	+
4-Toluenesulfonate	1.8	0.7	+
4-Sulfobenzoate	1.5	0.5	+
5-Sulfosalicylate	0.9	0.3	+
4-Aminobenzenesulfonate	1.5	0.6	+

Table 18: Utilization of organosulfonates (1.0 mM) by *Marichromatium* sp. JA121

Marichromatium sp. JA121 was grown photoheterotrophically in mineral medium (table 4) with pyruvate (27 mM) and NH_4Cl (6.4 mM) as sole carbon and nitrogen source respectively supplemented with different alkylsulfonates (1.0 mM), arylsulfonates (1.0 mM) as sole source of sulfur listed in the table for 48 h and assayed for utilization. Cells were harvested at regular intervals of time and levels of compounds were monitored through HPLC analysis, sulfite levels was estimated by acidfuchsin method (Denger *et al.*, 2001).

(NA: Not applicable)

Substrate (0.5 mM)	Utilization (+/-)
Benzoate	-
4-Hydroxy benzoate	_
3,4- Dihydroxybenzoate	_
2-Hydroxy benzoate	_
2-Aminobenzoate	+
L-Phenylalanine	+
L-Tryptophan	+
L-Tyrosine	+
trans-Cinnamate	+
Salicylate	+
o-Xylene	+
m-Xylene	_
p-Xylene	+
o-Cresol	_
m-Cresol	_
p-Cresol	+
Toluene	+
Control	NA

Table 19: Utilization of various aromatic compounds (0.5 mM) by whole cells of *Marichromatium* sp. JA121

Marichromatium sp. JA121 was grown photoheterotrophically in mineral medium (table 4) with pyruvate (27 mM) and NH_4Cl (6.4 mM) as sole carbon and as nitrogen source respectively supplemented with aromatic hydrocarbons (0.5 mM) listed in the table for 36 h and assayed for utilization. Cells were harvested at regular intervals of time and levels of compound were monitored through HPLC analysis.

[+ = utilized; - = Not utilized; NA= Not applicable]

3.3 Metabolism of 4- toluenesulfonate by *Marichromatium* sp. JA121

3.3.1 Growth and utilization of 4- toluenesulfonate by *Marichromatium* sp. JA121

Growth of *Marichromatium* sp. JA121 and 4-toluenesulfonate utilization could not be demonstrated when 4-toluenesulfonate was used as sole source of carbon, sole source of carbon and sulfur. However growth of *Marichromatium* sp. JA121 and simultaneous utilization of 4-toluenesulfonate (0.7 mM) was demonstrated when 4-toluenesulfonate was used as a sole source of sulfur when replaced with $MgSO_4.7H_2O$ for photoheterotrophic growth. 4-Toluenesulfonate utilization was extended to 96 h of phototrophic incubation of *Marichromatium* sp. JA121. *Marichromatium* sp. JA121 has the doubling time of 14 h compared to control culture (without 4-toluenesulfonate) that is having a doubling time of 28 h (Fig 12) and biomass yield was high when compared to control culture. Growth and utilization of 4- toluenesulfonate (0.7 mM) was not observed when the culture was incubated under dark anaerobic conditions even after 72 h of incubation. Presence of 4-toluenesulfonate was not altered the growth yield and biomass of *Marichromatium* sp. JA121 up to 120 mM (compared to control) and beyond 180 mM gradual decrease in growth was observed. Growth of *Marichromatium* sp. JA121 was inhibited at 180 mM. The 50 % inhibitory concentration (IC_{50}) of 4-toluenesulfonate on photoheterotrophic growth of *Marichromatium* sp. JA121 was ~120 mM.

4-Toluenesulfonate utilization was analyzed through HPLC, where 4-toluenesulfonate was resolved at 4.7 min (Fig 13A). Concomitant release of sulfite was observed in the culture supernatant with time (Fig 13B), however sulfite release was not in stoichiometry with 4-toluenesulfonate consumption in *Marichromatium* sp. JA121.

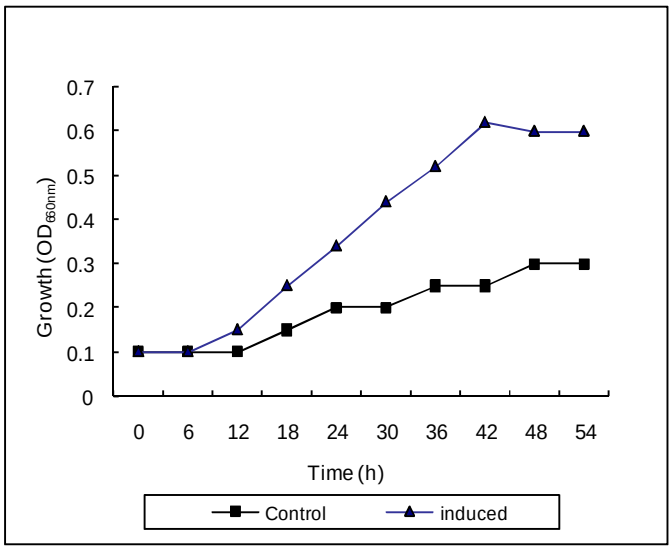
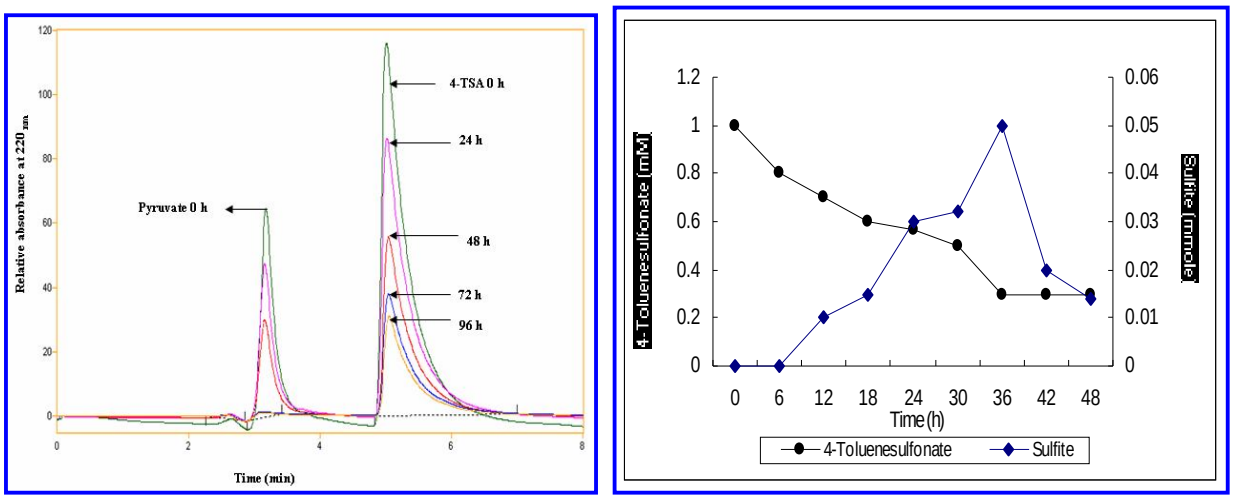


Fig 12: Growth of *Marichromatium* sp. JA121 in presence and absence of 4-toluenesulfonate

0.1 OD of 4-toluenesulfonate induced log phase culture of *Marichromatium* sp. JA121 was inoculated in mineral medium (Table 4) with pyruvate (27 mM) as sole carbon source and NH_4Cl (6.4 mM) as nitrogen source, 4-toluenesulfonate as source of sulfur (1.0 mM).

(Control = absence of 4-toluenesulfonate; Induced = presence of 4-toluenesulfonate)

.



(A) **(B)**

Fig 13A: Overlay of HPLC chromatograms of 4-toluenesulfonate induced culture supernatant of *Marichromatium* sp. JA121 drawn at different time intervals.

Growing cells of strain JA121 were pelleted out at different time intervals and 4-toluenesulfonate levels in the supernatant were analyzed through HPLC.

Fig 13B: Kinetics of 4-toluenesulfonate utilization and sulfite formation in the 4-toluenesulfonate induced culture supernatant of *Marichromatium* sp. JA121

4-Toluenesulfonate grown culture was pelleted at regular intervals of time and supernatant was analyzed for 4-toluenesulfonate utilization by HPLC and sulfite was assayed through colorimetric analysis

3.3.2 Influence of carbon sources, sulfur sources, NaCl on growth and 4-toluenesulfonate utilization by *Marichromatium* sp. JA121

Carbon sources

Influence of various organic substrates as carbon sources on the 4-toluenesulfonate utilization was studied by using the growing cells of *Marichromatium* sp. JA121. The carbon sources used include pyruvate, fumarate, succinate, malate, oxaloacetate and glucose. Maximum utilization of 4- toluenesulfonate was observed with pyruvate (0.7 mM), fumarate and malate (0.5 mM) as carbon source while other substrates like succinate, glucose inhibited the growth and 4- toluenesulfonate utilization by *Marichromatium* sp. JA121 (Table 20).

Sulfur sources

Influence of various sulfur sources on the 4- toluenesulfonate utilization was studied by using the growing cells of *Marichromatium* sp. JA121. The inorganic and organic sulfur sources include $MgSO_4$. $7H_2O$, Na_2S. $9H_2O$, $Na_2S_2O_3$, cysteine, methionine and cystine. In the presence of $Na_2S_2O_3$, cysteine, methionine and cystine 0.2 mM of 4-toluenesulfonate was utilized and 0.7 mM of utilization was observed in the control (4-toluenesulfonate as sole source of sulfur) (Table 21). *Marichromatium* sp. JA121 preferred to utilize simple inorganic and organic sulfur compounds instead of arylsulfonate 4-toluenesulfonate. In presence of sodium sulfide utilization of 4-toluenesulfonate was not observed.

Salt (sodium chloride)

Influence of sodium chloride on 4-toluenesulfonate utilization by *Marichromatium* sp. JA121 was studied at different concentrations of NaCl from 0-20 % (w/v). Sodium chloride influenced both biomass yield and 4-toluenesulfonate consumption. With increase in the sodium chloride concentration from 5-20 %, there is a decrease in the biomass yield of *Marichromatium* sp. JA121, while the concentration above 20 % of NaCl was growth inhibitory. Increase in salinity resulted in a decrease in the 4-toluenesulfonate consumption (Fig. 14).

Carbon source (0.3 %)	Biomass yield (mg dry wt . ml^{-1})	Utilization of 4-TSA (mM)
Control	0.0	0.0
Pyruvate	1.8	0.7
Fumarate	1.5	0.5
Succinate	NG	0.0
Malate	1.5	0.5
Glucose	NG	0.0
Oxaloacetate	1.2	0.2

Table 20: Effect of carbon substrates on growth and 4-toluenesulfonate utilization by *Marichromatium* sp. JA121

Experimental conditions are same as in fig 12 except for the different organic substrates used as carbon sources. Data pertains to the analysis of 4-toluenesulfonate levels in the culture supernatants after 72 h through HPLC.

(Control = without carbon substrate); NG= No growth; 4-TSA= 4-toluenesulfonate

Sulfur source+ 4-toluenesulfonate	Biomass yield (mg dry wt. ml^{-1})	4-toluenesulfonate utilization (mM)
Control	1.8	0.7
$MgSO_4$. $7H_2O$	1.8	0.4
Na_2S. $9H_2O$	1.8	0.0
$Na_2S_2O_4$. $7H_2O$	1.8	0.1
Cysteine	1.2	0.2
Methionine	1.2	0.2
Cystine	1.2	0.2

Table 21: Effect of sulfur sources on 4-toluenesulfonate utilization by *Marichromatium* sp. JA121

Experimental conditions are same as in fig 12 except for the different substrates used as sulfur sources. Data pertains to the analysis of 4-toluenesulfonate levels in the culture supernatants after 72 h of phototrophic incubation.

(Control =4-toluenesulfonate as sole source of sulfur);

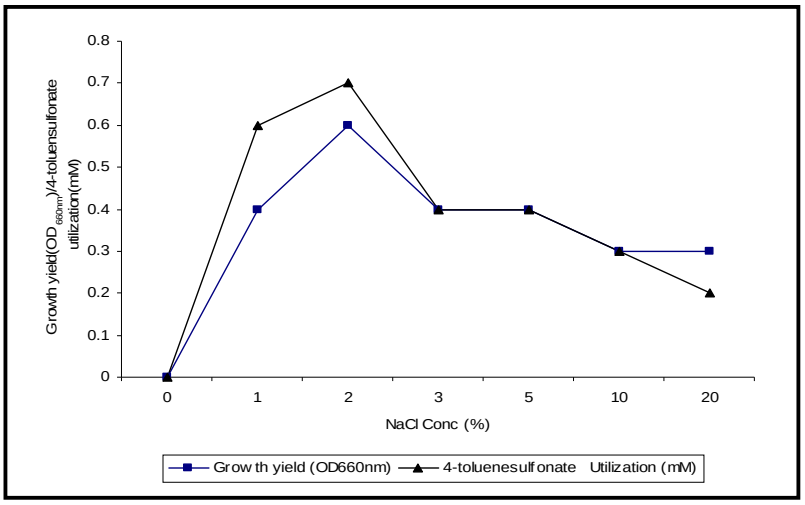


Fig 14: Effect of salt (sodium chloride) on growth of *Marichromatium* sp. JA121 and utilization of 4-toluenesulfonate

Experimental conditions are same as in fig 12 except for the different concentrations of NaCl used. Data pertains to the analysis of 4-toluenesulfonate levels in the culture supernatants after 72 h of phototrophic incubation.

3.3.3 Exo-metabolome analysis by Mass spectrometry

3.3.3.1 Profiling of exo-metabolome of uninduced and induced culture supernatant of *Marichromatium* sp. JA121 grown in the absence and presence of 4-toluenesulfonate

To compare the exometabolome of uninduced and induced culture of *Marichromatium* sp. JA121, the culture was grown without and with 4-toluenesulfonate and cells were harvested at two different time intervals of 12 h and 24 h. The culture supernatant was concentrated and analyzed through LCMS and metabolites were monitored. While comparing the metabolites of uninduced and induced culture of *Marichromatium* sp. JA121, the metabolites unique to 4-toluenesulfonate induced culture were encircled in the mass chromatograms. Twelve distinct metabolites were identified from the comparative LC-MS metabolite footprint (exometabolome) analysis (Fig 15). The m/z of the metabolites were 80, 93, 111, 124, 129, 153, 161, 171, 201, 209, 217 and 229. Five out of twelve of them were presumed as metabolites of 4-tolueneuslfonate based on their molecular masses (m/z) ; *viz* toluene (92 m/z), 4-methylcatechol (124 m/z), protocatechuaic acid (154 m/z), 4-sulfobenzoic aicd (202 m/z), 3-hydroxy, 4-sulfobenzoic acid (218 m/z).

3.3.3.2 Identification of the metabolites of 4-toluenesulfonate in the 4-toluenesulfonate induced bulk culture supernatant of *Marichromatium* sp. JA121

Metabolites of 4-toluenesulfonate were fractionated into aqueous and ethyl acetate fractions as per the protocol mentioned in methodology, from the 4-toluenesulfonate induced culture of *Marichromatium* sp. JA121, were analyzed through HPLC and mass spectrometry.

The HPLC analysis of methanolic extract of fractionated aqueous layer resolved two metabolites at R_T 2.9 min and 3.2 min. The metabolite with R_T 2.9 min was identified as 4-sulfobenzoate with the aid of authentic standard through co-elution chromatography (Fig 16A). The m/z of the metabolite 4-sulfobenzoate was confirmed through mass spectrometry recorded in ESI negative mode, where 4-sulfobenzoate corresponding mass (m/z) 203 was encircled in the mass chromatogram (Fig 16B).The metabolite with 3.2 was not identified due to the lack of authentic standards.

Similarly methanolic extract of fractionated ethyl acetate layer was analyzed by HPLC using the same solvent system. The metabolite with R_T 4.5 was identified as

protocatechuate using the standard and LCMS analysis of this fraction showed m/z 153 corresponds to protocatechuate Fig 17A and B).

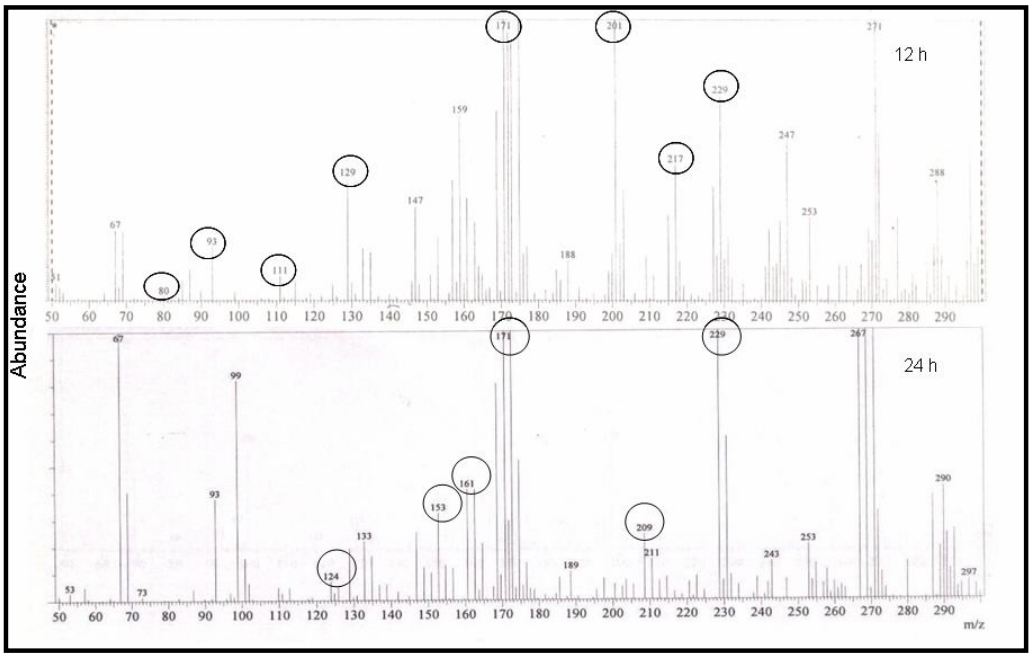
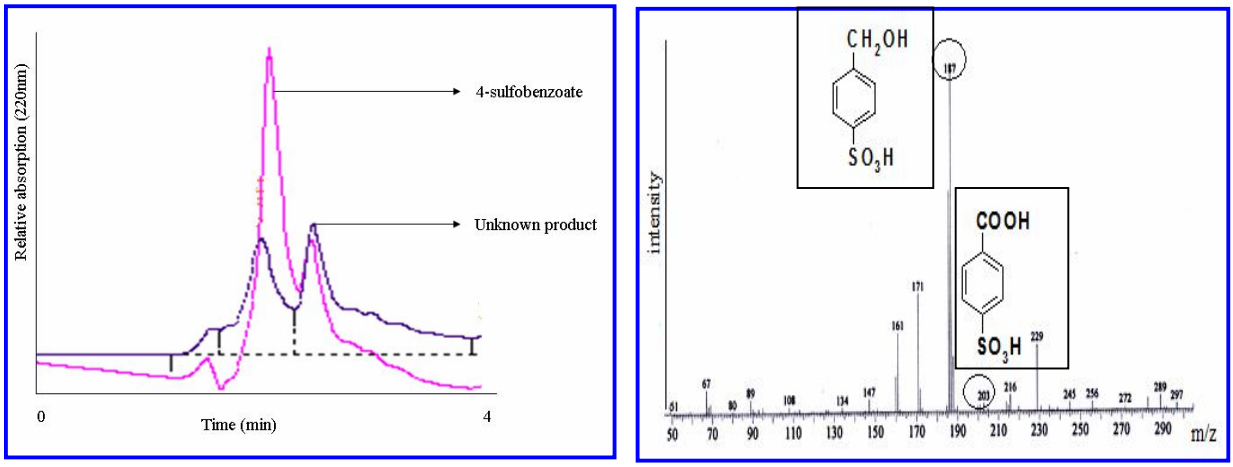


Fig 15: Comparative LC-MS metabolite foot printing of 4-toluenesulfonate induced culture supernatant of *Marichromatium* sp. JA121 drawn at 12 h and 24 h.

The overlaid mass spectra recorded in ESI negative mode, where the encircled masses were not found in control and uncircled masses are there in both control and induced culture supernatant.



(A) **(B)**

Fig 16: Metabolite analysis of fractionated aqueous sample of 4-toluenesulfonate induced culture supernatant of *Marichromatium* sp. JA121

(A) Overlay of HPLC chromatograms of fractionated aqueous sample and standard 4-sulfobenzoate added fractionated aqueous sample

(B) Mass spectrum of fractionated aqueous sample, encircled masses (m/z) 203 corresponds to 4-sulfobenzoate and 187 corresponds to 4-sulfobenzylalcohol. Inserts are the structures of 4-sulfobenzylalcohol and 4-sulfobenzoic acid.

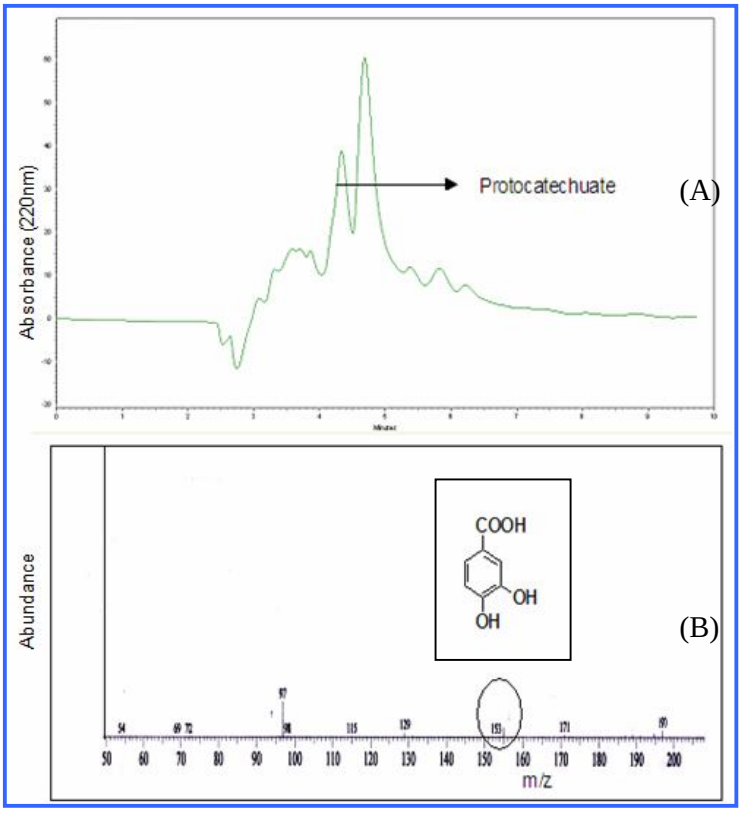


Fig 17: Metabolite analysis of fractionated ethyl acetate sample of 4-toluenesulfonate induced culture supernatant of *Marichromatium* sp. JA121

A: HPLC chromatogram of methanolic extract of fractionated ethyl acetate sample of 4-toluenesulfonate induced culture supernatant.

B: Mass spectrum of methanolic extract of fractionated ethyl acetate sample of 4-toluenesulfonate induced culture supernatant, recorded in ESI negative mode.

The encircled ion mass (m/z) 153 corresponds to protocatechuate. Insert is the structure of protocatechuate.

3.3.4 Enzyme assays with cell free extracts of *Marichromatium* sp. JA121

Studies with the whole cells of *Marichromatium* sp. JA121 indicated the consumption of 4-toluenesulfonate and formation of the metabolites like 4-sulfobenzoate and protocatechuate into 4-toluenesulfonate induced culture supernatant and the same was studied using cell free extracts of *Marichromatium.* sp. JA121. 4-Toluenesulfonate methylmonooxygenase, 4-sulfobenzoate 3, 4- dioxygenase and protocatechuate oxidase enzyme activities were carried out with the cell free extracts of 4-toluenesulfonate induced culture of *Marichromatium.* sp. JA121.

3.3.4.1 4-Toluenesulfonate methylmonooxygenase (EC 1.12.4)

4-Toluenesulfonate methyl monoxygenase catalyzes the conversion of 4-toluenesulfonate to 4-sulfobenzylalcohol. Cell free extracts of 4-toluenesulfonate adapted strain JA121 was used for 4-toluenesulfonate methylmonooxygenase assay. The enzyme assay samples drawn at different time intervals were protein precipitated and analyzed through HPLC and LCMS.

The enzyme assay mixture containing 15 µmoles of 4-toluenesulfonate as initial concentration. The HPLC analysis of the enzyme assay mixture drawn after 15 min of incubation has shown 3 µmoles of 4-toluenesulfonate (R_T 4.5 min) consumption with concomitant formation of the products 4-sulfobenzoate (R_T 2.9 min) and protocatechuate (R_T 4.5 min)(Fig. 18A). In the enzyme assay mixture 4-toluenesulfonate consumption started after 5 min and completed by 30 min of incubation. While 4-sulfobenzoate formation started after 10 min and reached a maximum of 2 µmoles at the end of 30 min of enzyme assay (Fig. 19) and protocatechuate (0.15 µmoles) formation was observed after 20 min with the release of sulfite. The masses were confirmed by LC-MS analysis, where the encircled masses (m/z) 201 and 154 correspond to 4-sulfobenzoate and protocatechuate respectively (Fig. 18B). In addition to these products two metabolites were observed with R_T 3.3 min (UK1) and 3.8 min (UK2) in the assay mixture, due to the lack of standards these metabolites were not confirmed. However the metabolite UK1 is having the absorption maxima of 260, 255 and 239 nm and the metabolite UK2 is having the absorption maxima of 220, 260 and 210nm. In the enzyme assay mixture sulfite release was also observed.

To check for 4-toluenesulfonate methylmonooxygenase activity, the same enzyme assay mixture was analyzed through oxygen electrode for oxygen consumption with time. During the course of reaction the increase in oxygen uptake with time was observed compared to control enzyme assay sample (Fig. 20). Fifty nmoles of oxygen. min^{-1} . mg $protein^{-1}$ was observed for 3 µmoles of 4-toluenesulfonate consumption.

3.3.4.2 4-sulfobenzoate 3, 4- dioxygenase (EC 1.14.12.8)

4-Sulfobenzoate 3, 4- dioxygenase catalyzing the conversion of 4-sulfobenzoate to protocatechuate and sulfite. Cell free extracts of 4-toluenesulfonate adapted *Marichromatium* sp. JA121 was used for assay. 4-sulfobenzoate 3, 4- dioxygenase was confirmed with increase in oxygen uptake with time after adding the substrate 4-sulfobenzoate (Fig. 20) and simultaneous formation of the product protocatechuate and sulfite (Fig. 19), which were identified through HPLC and masses were confirmed through LCMS analyses. Thirty nmoles of oxygen.min^{-1}.mg $protein^{-1}$ was observed for 1 µmole of 4-sulfobenzoate consumption.

3.3.4.3 Protocatechuate oxygenase (EC 1.13.11.3)

Protocatechuate 3, 4-dioxygenase catalyzing the conversion of protocatechuate to ring-opened product 3-carboxy-*cis, cis*- muconate. This enzyme activity was assayed with cell free extracts of 4-toluenesulfonate induced culture of *Marichromatium* sp. JA121, where the consumption of protocatechuate was not observed till 45 min of enzyme assay.

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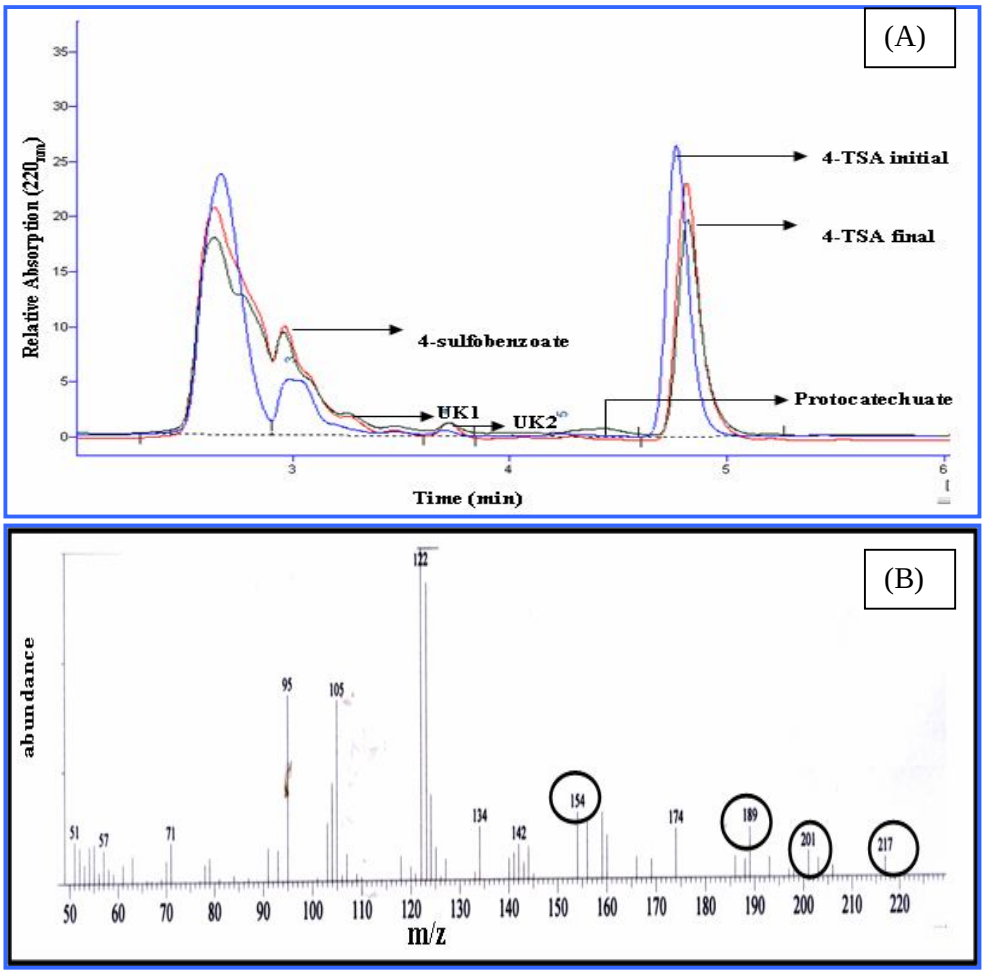

Fig 18: HPLC and LCMS analyses of the metabolites of 4-toluenesulfonate in the cell free extracts enzyme assayed samples.

(A) Overlay of HPLC chromatograms of 4-toluenesulfonate induced cell free extracts enzyme assayed samples drawn at different time intervals.
UK1= Unknown 1; UK 2= Unknown 2; 4-TSA = 4-toluenesulfonate
4-TSA initial=the predenatured protein, obtained from the 4-toluenesulfonate induced cell free extracts, added enzyme assay sample after protein precipitation was analyzed through HPLC
4-TSA final= The active protein added enzyme assayed sample drawn after 20min

(B)Mass chromatogram of the 20 min enzyme assayed sample recorded in ESI negative mode, where the masses encircled were not found in control.

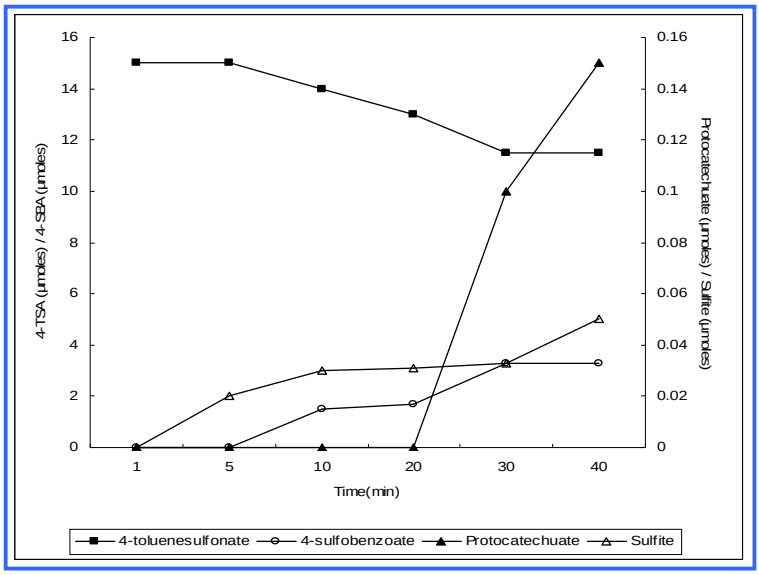

Fig 19: Kinetics of 4-toluenesulfonate utilization and metabolites 4-sulfobenzoate, protocatechuate and sulfite formation in the cell free extracts enzyme-assayed sample of *Marichromatium* sp. JA121

4-TSA=4-Toluenesulfonate; 4-SBA=4-Sulfobenzoate

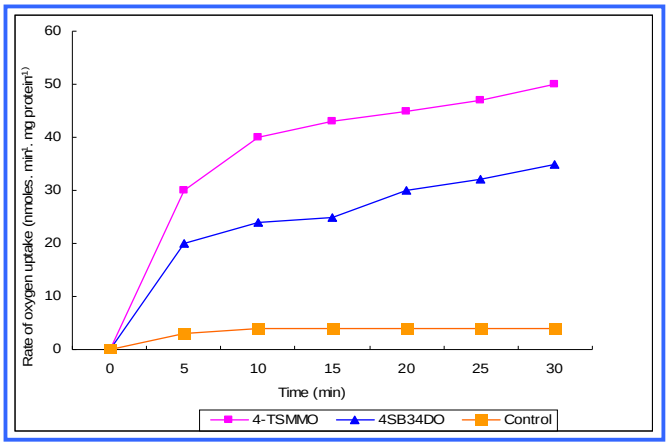

Fig 20: Oxygen uptake profile of the enzyme assays with cell free extracts of 4-toluenesulfonate induced culture of *Marichromatium* sp. JA121

4-TSMMO= 4- toluenesulfonate-methyl monoxygenase
4-SB3, 4DO= 4-sulfobenzoate, 3, 4 dioxygenase
Rate of oxygen uptake with time in cell free extracts enzyme assays viz (4-TSMMO) 4-toluenesulfonate-methyl monoxygenase and (4-SBDO) 4-sulfobenzoate, 3,4 dioxygenase

3.4 Conjugative metabolism of 4-toluenesulfonate by *Marichromatium* sp. JA121

In the catabolism of 4-toluenesulfonate by *Marichromatium* sp. JA121, metabolic foot printing analysis indicated twelve unique masses in the exo-metabolome of 4-toluenesulfonate induced culture. Among which four masses were identified as 4-sulfobenzylalcohol (188 m/z), protocatechuaic acid (154 m/z), 4-sulfobenzoic acid (202 m/z), 3-hydroxy, 4-sulfobenzoic acid (218 m/z). The remaining masses were not identified. To identify the remaining metabolites, experimental parameters are changed by following the parameters of anaerobic toluene (structural analogue of 4-toluenesulfoante) catabolism by phototrophic bacteria.

In the phototrophic purple bacteria *Blastochloris sulfoviridis* TOP1 toluene, the structural analogue of 4-toluenesulfonate, was metabolized under complete anaerobic conditions through succinate or fumarate conjugation by benzylsuccinate synthase enzyme forming benzylsuccinate as a product (Zengler *et al.*, 1999). Succinate or fumarate conjugation was also reported for anaerobic degradation of isomers of cresol and xylene in denitrifying and sulfate reducing bacteria *Thauera aromatica* (Beller *et al.*, 1997), *Azoarcus* (Beller and Spormamm 1999) and *Desulfobacculum* (Rabus and Heider 1998) etc. We have studied the utilization of 4-toluenesulfonate in presence of succinate and fumarate by *Marichromatium* sp. JA121.

3.4.1 Influence of succinate on utilization of 4-toluenesulfonate by *Marichromatium* sp. JA121

The effect of succinate on growth and utilization of 4-toluenesulfonate by *Marichromatium* sp. JA121 was studied by replacing pyruvate (0.3 % [w/v]) with succinate (0.3 % [w/v]) as a carbon source in the modified Biebl and Pfennig's medium. Similarly fumarate was also tested. Growth and utilization of 4-toluenesulfonate could not be demonstrated on succinate (25 mM) by *Marichromatium* sp. JA121 under photoheterotrophic conditions (Table 22). However, in addition to succinate (8 mM) when pyruvate (18 mM) was given as a carbon source, growth and 4-toluenesulfonate utilization was observed with concomitant utilization of succinate in the 4-toluenesulfonate grown culture of *Marichromatium* sp. JA121 (Fig. 21). Based on HPLC analysis of the time course samples, by the end of 48 h of incubation 0.5 mM of 4-toluenesulfonate, 3.6 mM of succinate and complete utilization of pyruvate was observed (Fig. 22). In the presence of fumarate 0.5 mM of 4-toluenesulfonate was utilized.

Carbon source + 4-TSA	Biomass yield (mg dry wt.ml^{-1})	Pyruvate/Succinate utilization	4-TSA utilization (mM)
Succinate	0.0	-	0.0
Pyruvate + Succinate	1.5	+	0.5
Pyruvate	1.8	+	0.7
Fumarate	1.5	+	0.5

Table 22: Influence of succinate on growth and utilization of 4-toluenesulfonate by *Marichromatium* sp. JA121

Experimental conditions are same as in fig 12 except for the different organic substrates used as carbon sources. Data pertains to the analysis of 4-toluenesulfonate, succinate and pyruvate levels in the culture supernatants after 48 h of phototrophic incubation.

4-TSA= 4-Toluenesulfonate

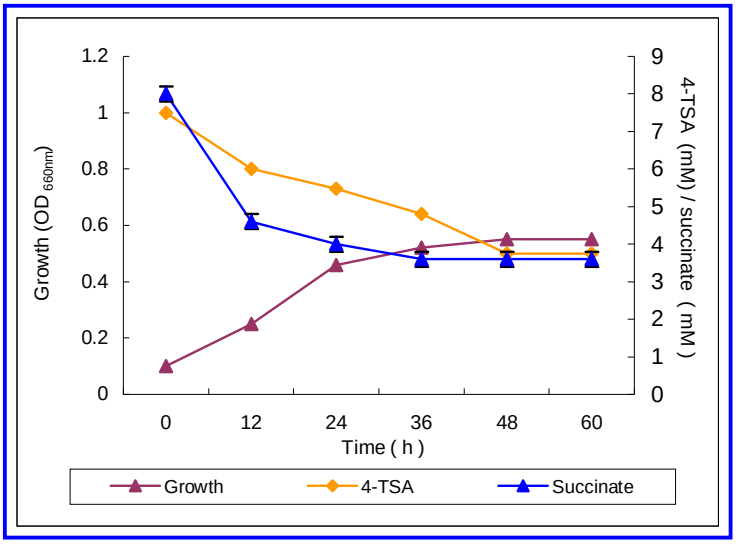

Fig 21: Growth, succinate and 4-toluenesulfonate utilization by *Marichromatium* sp. JA121

Experimental conditions are same as in fig 12 except that succinate (8 mM) was used as additional carbon source. Data pertains to the analysis of 4-toluenesulfonate and succinate levels in the culture supernatants at regular intervals of time through HPLC analysis.

4-TSA= 4-Toluenesulfonate

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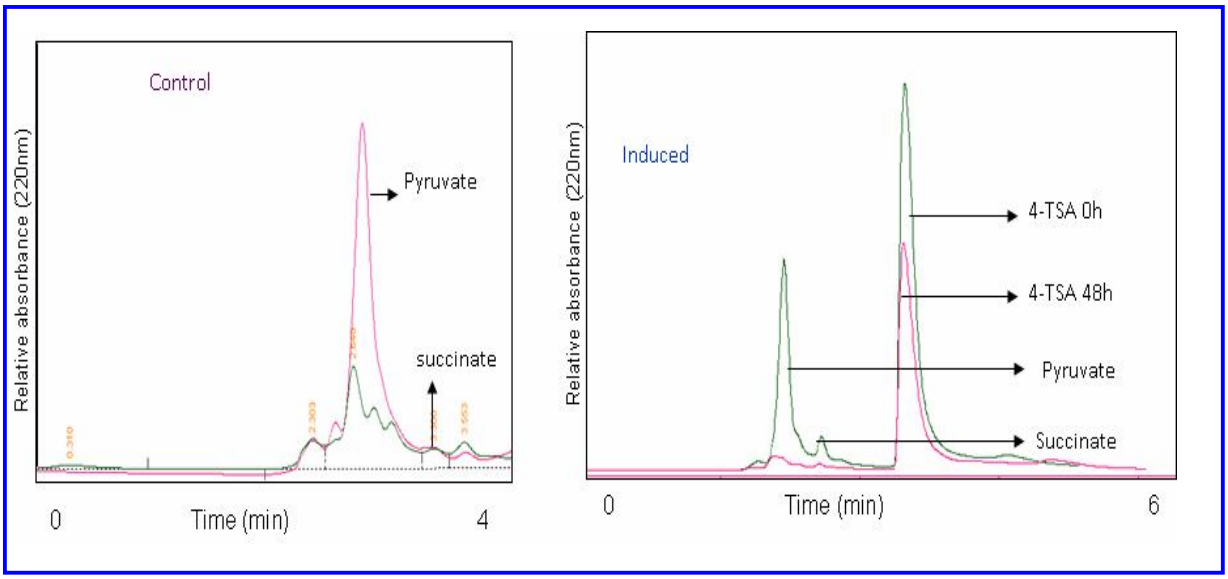


Fig 22: HPLC chromatogram depicting the consumption of pyruvate and succinate in control and 4-toluenesulfonate induced culture of *Marichromatium* sp. JA121

Control: Represents the overlay of HPLC chromatograms of control culture supernatant of *Marichromatium* sp. JA121 grown in absence of 4-toluenesulfonate, drawn at 0 h and 48 h of incubation.

Induced: Represents the overlay of HPLC chromatograms of 4-toluenesulfonate induced culture supernatant of *Marichromatium* sp. JA121 drawn at 0 h and 48 h of incubation.

4-TSA = 4-toluenesulfonate

3.4.2 Exometabolome analysis

3.4.2.1 Metabolite foot printing of the induced and uninduced culture of *Marichromatium* sp. JA121 grown in presence and absence of 4-toluenesulfonate (pyruvate as carbon substrate and succinate as co substrate)

To compare the exometabolome in presence and absence of 4-toluenesulfonate, the cells of *Marichromatium* sp. JA121 grown with and without 4-toluenesulfonate were harvested, culture supernatant was concentrated and analyzed using LC-MS.

Nine distinct metabolites were identified from the comparative (cells grown in the presence and absence of 4-toluenesulfonate) LC-MS metabolite footprint (exometabolome) analysis done at two stages of growth (24 h and 48 h) (Fig 23). The m/z of the metabolites are 147, 161, 171, 187, 209 and 229. Among the nine metabolites two metabolites with m/z 187 was identified as oxidative pathway metabolite *viz* 4-sulfobenzylalcohol and the other metabolite with m/z 209 was presumed to be succinate conjugated metabolite benzylsuccinate.

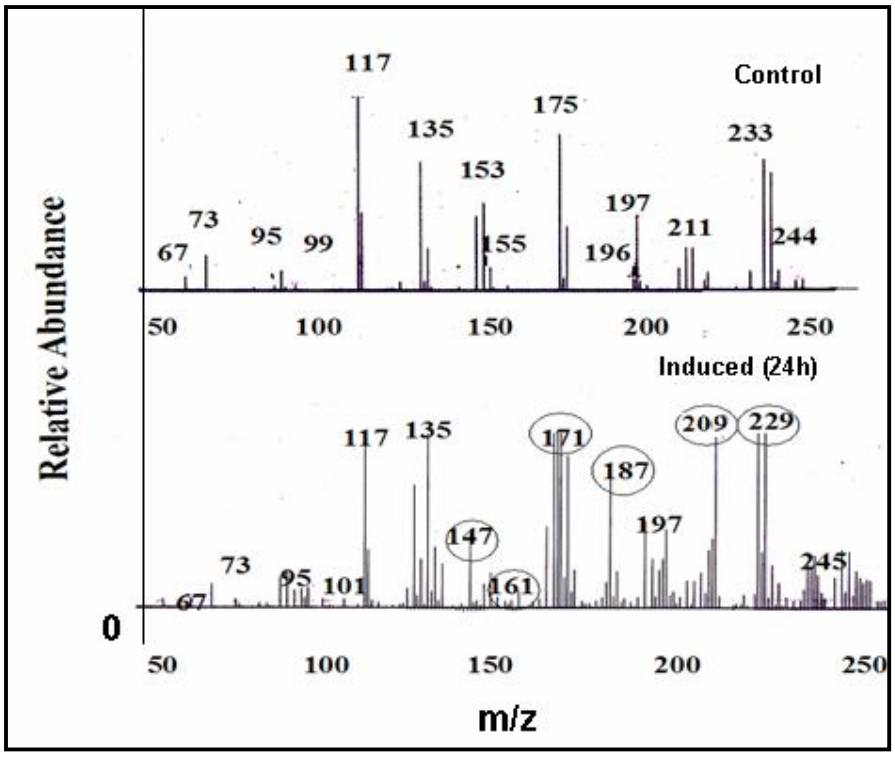


Fig 23: Comparative LCMS metabolite foot printing of culture supernatant of *Marichromatium* sp. JA121 grown with and without 4-toluenesulfonate

Control: Represents the mass chromatogram of control culture supernatant of *Marichromatium* sp. JA121 grown without 4-toluenesulfonate.

Induced 24 h: Represents the mass chromatogram of induced culture supernatant of *Marichromatium* sp. JA121 grown with 4-toluenesulfonate drawn after 24 h of incubation. The encircled masses were absent in control supernatant.

3.4.3 Enzyme assays with cell free extracts

Studies with whole cells of *Marichromatium* sp. JA121 indicated the consumption of 4-toluenesulfonate and formation of metabolites like 4-sulfobenzylalcohol, 3-hydroxy, 4-sulohobenzylalcohol and benzylsuccinate in the 4-toluenesulfonate induced culture and the same was studied using cell free extracts of *Marichromatium* sp. JA121 grown with and without 4-toluenesulfonate. However, maximum consumption was observed with 4-toluenesulfonate induced cell free extracts. Requirement of coenzymes and cofactors for consumption of 4-toluenesulfonate could not be demonstrated by the cell free extracts of *Marichromatium* sp. JA121.

Based on earlier reports, benzylsuccinate synthase was a key enzyme involved in succinate or fumarate conjugation to toluene, isomers of cresol and xylene under anaerobic conditions and formation of metabolites like hydroxybenzylsuccinate and benzylsuccinate.

3.4.3.1 Benzylsuccinate synthase assay under anaerobic conditions

Cell free extracts of 4-toluenesulfonate induced *Marichromatium* sp. JA121 was used for assay under anaerobic conditions. In the 30 min enzyme assayed sample the product with t_R 22 min was identified as benzylsuccinate through HPLC analysis (Fig. 24) using authentic standard. The initial concentration of 4-toluenesulfonate is 5 µmoles, after 30 min 2.5 µmoles of 4-toluenesulfonate was consumed with the formation of 1.8 µmoles of benzylsuccinate in the cell free extracts enzyme assay sample. The masses of the metabolites were analyzed through LC-MS analysis (Fig. 25). The product benzylsuccinate corresponding mass (m/z) 208 was identified in the 30 min enzyme assayed sample.

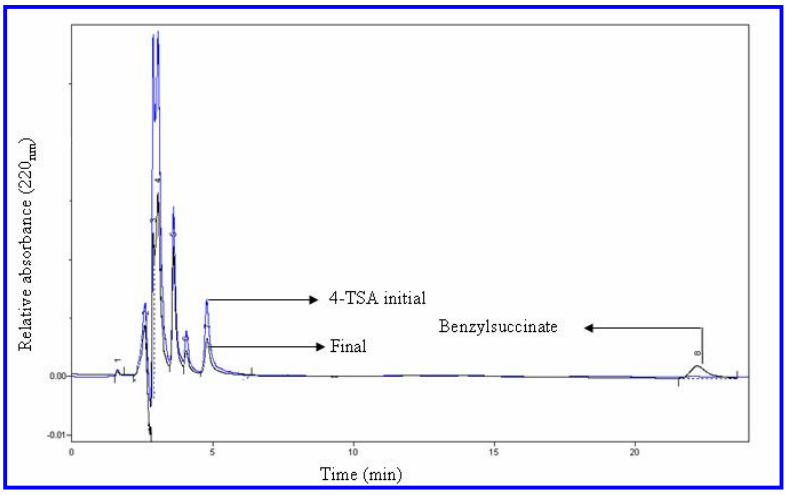


Fig 24: HPLC chromatogram showing the utilization of 4-toluenesulfonate in cell free extracts enzyme assay mixture under anaerobic conditions.

Experimental conditions are mentioned in methodology. Aliquots were drawn from the assay mixture at different time intervals and assay was stopped by adding 5 µl of 1N HCl and 4-Toluenesulfonate levels of the sample were analyzed using HPLC.
4-TSA=4-Toluenesulfonate

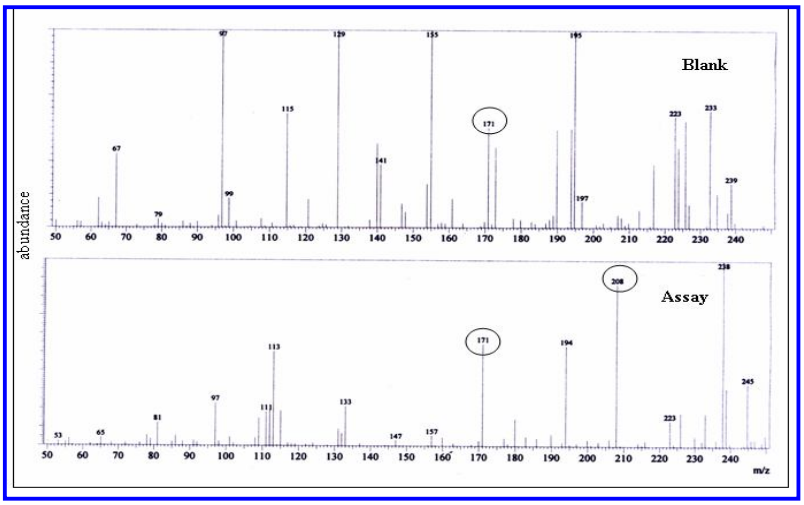


Fig 25: Comparative LCMS metabolite profiling of the cell free extracts enzyme (benzylsuccinate synthase) assayed samples at 0 min and 30 min.

Blank: Mass chromatogram showing the ion masses of the metabolites of the cell free extracts enzyme assay sample at 0 min. The encircled mass (m/z) 171 corresponds to 4-toluenesulfonate.

Assay: Represents the ion masses of the metabolites of 4-toluenesulfonate in cell free extracts enzyme assay sample after 30 min

3.4.3.2 Benzylsuccinate synthase assay under aerobic conditions

Cell free extracts of 4-toluenesulfonate induced *Marichromatium* sp. JA121 was used for enzyme assay under aerobic conditions. In the 30 min enzyme assayed sample benzylsuccinate (t_R 22) and an unknown product with t_R 27.8 min were identified through HPLC analysis (Fig. 26). The initial concentration of 4-toluenesulfonate is 5 µmoles, after 30 min of enzyme assay. 1.5 µmoles of 4-toluenesulfonate was consumed with the formation of 0.6 µmoles of benzylsuccinate in the cell free extracts enzyme assay sample.

The masses of the metabolites were analyzed through LCMS. Metabolite profiling of the control sample and enzyme assayed sample indicated that two metabolites with m/z 208 and 225 were unique to enzyme assayed sample. Among these metabolites benzylsuccinate corresponding mass was identified as 208 (Fig. 27).

The metabolite with m/z 225 was subjected to mass fragmentation, indicating the fragments with m/z 104, 122 and 199 (Fig. 28). Based on the mass fragmentation the metabolite was presumed as succinate conjugated product 4-hydroxybenzylsucciate.

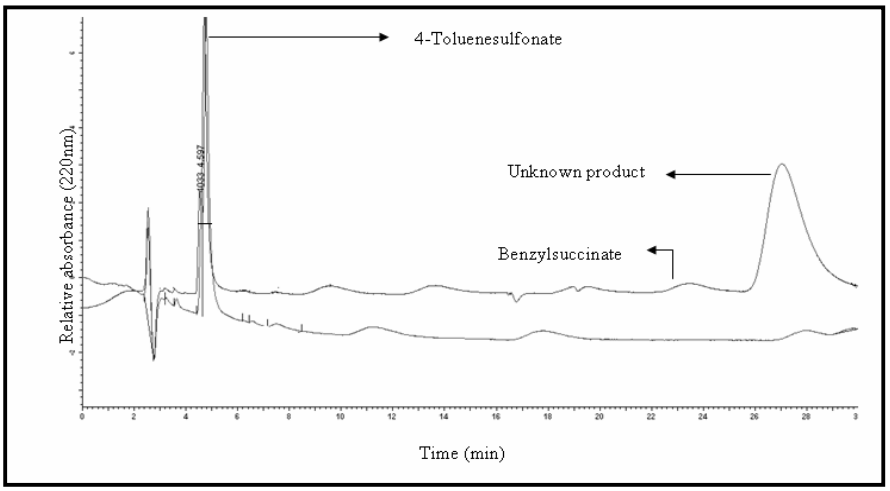


Fig 26: HPLC chromatogram showing the metabolites of 4-toluenesulfonate in cell free extracts enzyme (benzylsuccinate synthase assay) assay sample under aerobic conditions.

Experimental conditions are mentioned in methodology. Aliquots were drawn from the assay mixture at different time intervals and assay was stopped by adding 5 µl of 1N HCl and 4-toluenesulfonate levels of the sample were analyzed using HPLC.

4-TSA=4-Toluenesulfonate

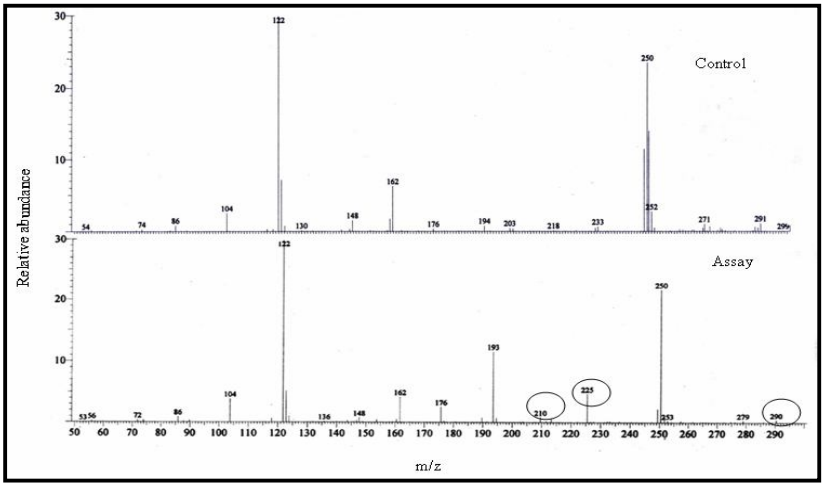


Fig 27: Comparative LCMS metabolite profiling of the benzylsuccinate synthase assayed samples with cell free extracts of *Marichromatium* sp. JA121.

Control: Mass chromatogram of the benzylsuccinate synthase enzyme assayed sample at 0 min,

Assay: Mass chromatogram of the benzylsuccinate synthase enzyme assayed sample after 30 min. Masses encircled were not found in control.

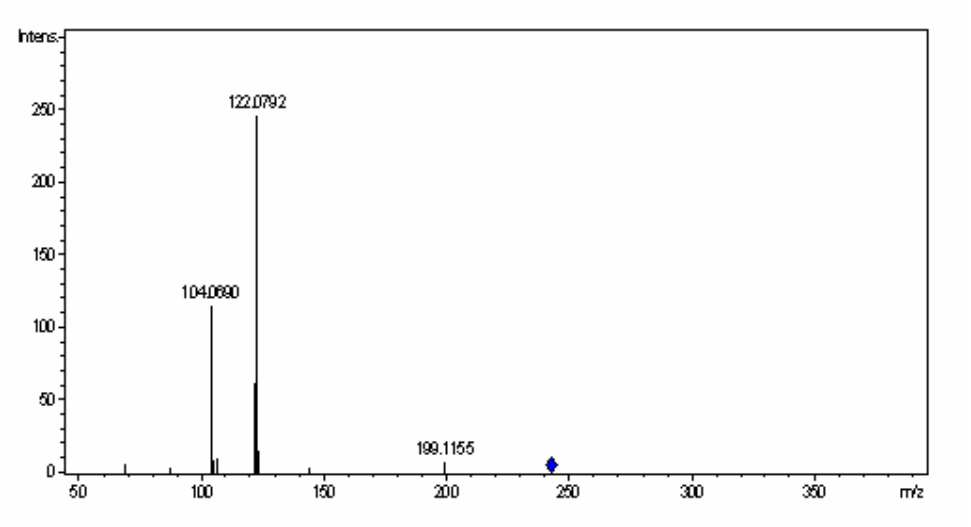


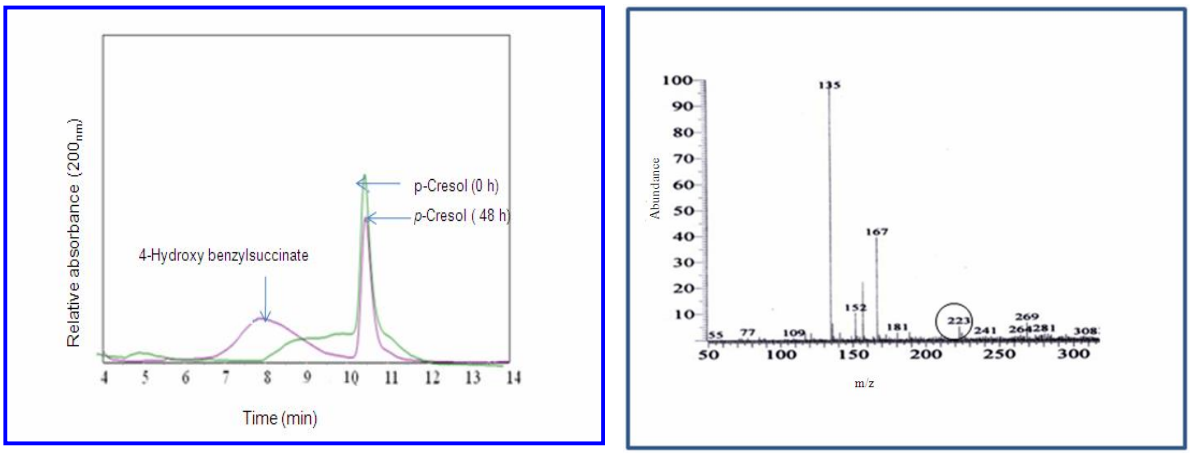
Fig 28: MS/MS chromatogram of unidentified metabolite with m/z 225 observed in benzylsuccinate synthase enzyme assay (under aerobic conditions) sample drawn after 30 min.

3.4.4 Growth and utilization of *p*-cresol by *Marichromatium* sp. JA121

As 4-hydroxybenzylsuccinate was reported as a metabolite of *p*-cresol and succinate conjugation, utilization of *p*-cresol was studied by *Marichromatium* sp. JA121. Growth of *Marichromatium* sp. JA121could not be demonstrated as when cresol was given as a carbon source. *Marichromatium* sp. JA121 could utilize *p*-cresol and *o*-cresol but not *m*-cresol as a supplement. 0.4 mM of *p*-cresol was utilized by the end of 48 h of phototrophic incubation. In the culture supernatant product with t_R 8 min was identified as 4-hydroxybenzylsuccinate through HPLC analysis (Fig. 29A).

3.4.4.1 Metabolite confirmation by LCMS/ MS

The mass of the metabolite 4-hydroxybenzylsuccinate was confirmed by LCMS analysis and this metabolite has shown a molecular ion mass of 225 (m/z) (Fig. 29B) and further mass fragmented (Fig. 30). In presence of 4-toluenesulfonate we observed an unknown metabolite with t_R 27.8 in cell free extracts assay mixture. The mass analysis of this metabolite and fragmentation pattern was identical to that of 4-hydroxybenzylsucciante (Table 23). Based on these observations the unidentified metabolite was confirmed as 4-hydroxybenzylsuccinate, which was observed in *p*-cresol metabolism by *Marichromatium* sp. JA121.



(A) **(B)**

Fig 29: HPLC and LCMS analyses of metabolites of *p*-cresol in the culture supernatant of *Marichromatium* sp. JA121

(A) HPLC chromatogram showing the utilization of *p*-cresol with time in the culture supernatant of *Marichromatium* sp. JA121

(B) Represents the ion masses identified in the culture supernatant of *Marichromatium* sp. JA121 grown with *p*-cresol as supplement. The encircled mass corresponds to 4-hydroxybenzylsuccinate.

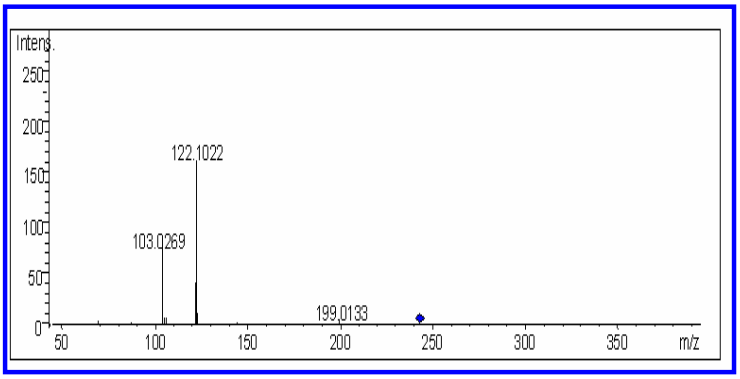


Fig 30: MS/MS chromatogram of 4-hydroxybenzylsuccinate with m/z 225 observed in the *p*-cresol induced culture of *Marichromatium* sp. JA121

4-Hydroxybenzylsuccinate (m/z 225)	Unknown product (m/z 223)
199	199
122	122
103	104

Table 23: Fragments observed in MS/MS chromatograms of 4-hydroxybenzylsuccinate (product of *p*-cresol) and unidentified product of 4-toluenesulfonate in the benzylsuccinate synthase enzyme assayed sample.

3.4.5 Amplification of benzylsuccinate synthase A (*bssA*) gene

In *Marichromatium* sp. JA121 the product benzylsuccinate and the enzyme benzylsuccinate synthase were identified during the metabolism of the 4-toluenesulfonate. Hence the gene coding for benzylsuccinate synthase was amplified. In *Thaura aromatica*, three benzylsuccinate synthase structural genes were reported *viz bssA*, *bssB* and *bssC*. The *bssA* gene, coding for the large α subunit of benzylsuccinate synthase, is highly conserved among the anaerobes degrading toluene and its derivatives (Leuthner *et al.*, 1998).

Hence *bssA* gene was amplified from the genomic DNA in *Marichromatium* sp. JA121 using set of designed primers (bssAF2 and bssAR1) and amplification was not obtained with other primer sets (Table 9 as given in methodology). The expected length of amplicon of size ~365bp was observed on the 2 % agarose gel along with some nonspecific bands. The positive amplicon was gel eluted, purified and sequenced (Fig. 31). The putative and partial *bssA* gene sequence has been deposited in Genbank under accession number GQ923885. The dendrogram was constructed for partial *bssA* gene encoding protein using the software as mentioned in materials and methods. BLAST analysis showed % of similarity between 46-57 with the reported *bssA* gene sequences.

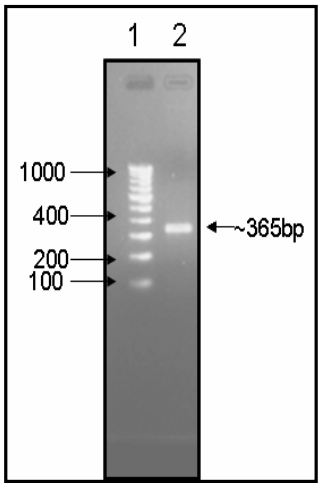


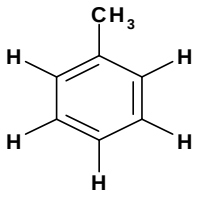
Fig 31: 2 % agarose gel showing amplicon from PCR. Expected amplicon length was ~365 bp

Lane 1:100bp DNA ladder

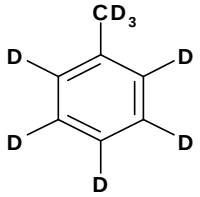
Lane 2: Gel purified amplicon

3.5 Ratification of catabolites of 4-toluenesulfonate in *Marichromatium* sp. JA121 by stable isotope feeding studies

In *Marichromatium* sp. JA121 4-toluenesulfonate metabolism was channeled through two different pathways *viz*-oxidative pathway and succinate conjugative pathway. To confirm the catabolic pathways of 4-toluenesulfonate, its structural analogue toluene (deuteriated), a suitable metabolite probe (d_8 98 %) was used for studies. Culture was supplemented with unlabelled (d_0-) toluene (m/z 92), labeled (d_8-) toluene (m/z 100) separately and along with succinate.



d_0-Toluene



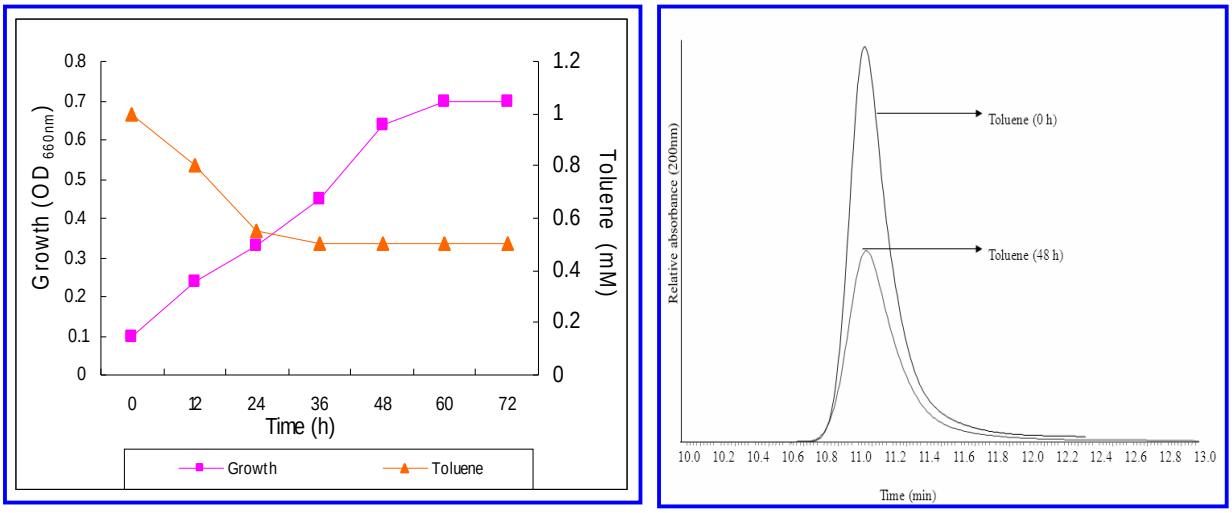
d_8-toluene

3.5.1 Growth and utilization of toluene by *Marichromatium* sp. JA121

Growth of *Marichromatium* sp. JA121 could not be demonstrated on toluene as sole source of carbon, in place of pyruvate. Utilization of toluene (0.5 mM) from the culture supernatant was observed after 48 h of phototrophic incubation when added as supplement at a concentration of 1.0 mM (Fig. 32A). The loss of compound in the supernatant could not support growth of *Marichromatium* sp. JA121. Since increase in the biomass (compared to control culture) could not be demonstrated.

3.5.2. Effect of toluene on growth of *Marichromatium* sp. JA121

Effect of different concentrations of toluene (0.5-10.0 mM) on photoheterotrophic growth of *Marichromatium* sp. JA121 was studied. Presence of toluene did not alter the growth yield and biomass of *Marichromatium* sp. JA121 up to 2.5 mM (compared to control) and beyond 2.5 mM gradual decrease in growth was observed. Growth of *Marichromatium* sp. JA121 was inhibited at 7 mM. The 50 % inhibitory concentration (IC_{50}) of toluene on photoheterotrophic growth of *Marichromatium* sp. JA121 was 5.0 mM.



A **B**

Fig 32A: Kinetics of growth and toluene utilization by *Marichromatium* sp. JA121.

B:Overlay of HPLC chromatograms of the toluene induced culture supernatant of *Marichromatium* sp. JA121

Experimental conditions are same as in fig 12 except for toluene (1 mM) used as supplement. Data pertains to the analysis of toluene levels in the culture supernatants through HPLC at 0 h and 48 h.

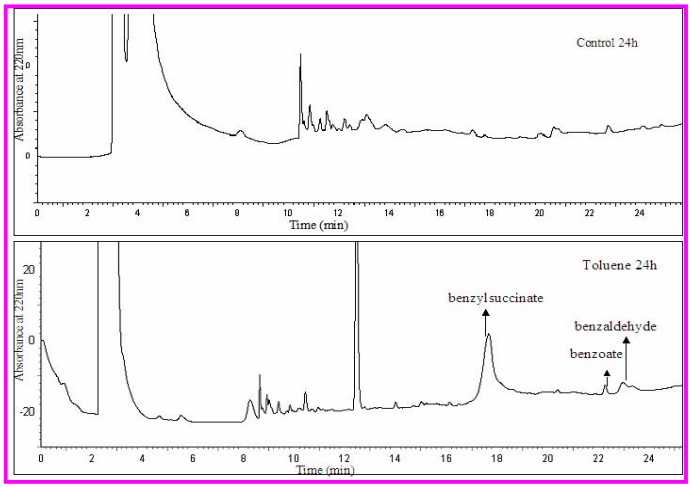


Fig 33: HPLC chromatograms of the control and toluene induced culture supernatant drawn after 24 h of incubation. The compounds with t_R 17.6 min and 22.2 min were identified as benzylsuccinate and benzoate respectively

3.5.3. Metabolite identification through HPLC and LCMS analyses

HPLC analysis of time course samples indicated that 0.5 mM of toluene was consumed by the end of 48 h of phototrophic incubation (Fig. 32B). The products were identified as benzylsuccinate (t_R 17.6 min) and benzoate (t_R 22.2 min) and benzaldehyde (t_R 23 min) using authentic standards, in both unlabelled and labeled (d_8) toluene fed cultures of *Marichromatium* sp. JA121 (Fig. 33). The masses of the metabolites were analyzed through LCMS indicated benzylsuccinate, benzoate corresponding masses with m/z 207 and 123 respectively, in the unlabelled toluene fed culture of *Marichromatium* sp. JA121 and in d_8 toluene fed culture, benzylsuccinate, benzoate corresponding masses with m/z 213 and 127 respectively (Fig. 34). When m/z of the products compared, the increase in m/z of +6 units, m/z of +4 units was observed for benzylsuccinate and benzoate. LC-MS analysis of the culture supernatant samples drawn at different time intervals indicated the isotopic enrichment signals (increase in m/z of metabolite) for two metabolites benzoate and benzylsuccinate (Table 24). This result indicated that the provided precursor (d_8 toluene) is incorporated into the metabolites.

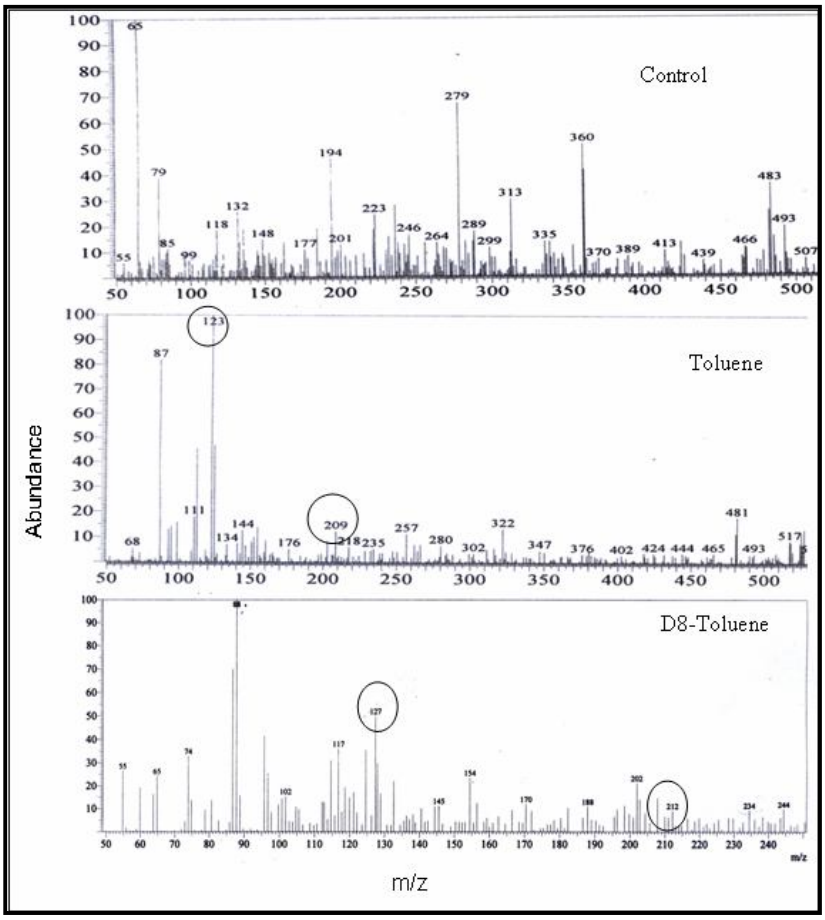


Fig 34: Comparative LCMS metabolite profiling of the culture supernatant of *Marichromatium* sp. JA121 grown without toluene, with toluene and d_8-toluene.

Control: Represents the ion masses of the culture supernatant of *Marichromatium* sp. JA121 grown without toluene.

Toluene: Represents the ion masses of the culture supernatant of *Marichromatium* sp. JA121 grown with toluene drawn after 48 h of incubation. The masses encircled were not identified in the control culture supernatant.

d_8-Toluene: Represents the ion masses of the culture supernatant of *Marichromatium* sp. JA12**1** grown with d_8-toluene drawn after 48 h of incubation. The masses encircled were not identified in the control culture supernatant

Metabolite	Unlabelled molecular mass (m/z)	Labelled molecular mass (m/z)
Benzoate	122	127
Benzylsuccinate	208	213

Table 24: Molecular ion mass (m/z) of benzoate and benzylsuccinate obtained from LC-MS analysis of toluene induced culture supernatant fractions of *Marichromatium* sp. JA121 supplemented with unlabelled and labeled d_8-toluene.

Discussion

4.1 Discussion

Organosulfonates are wide spread, occurring naturally and anthropogenic. Of which, the natural organosulfonates includes those generated from archea, bacteria, spiders, vertebrates, algae and palnts (Cook *et al.,* 2007). The next class of organosulfonates comprises anthropogenic sulfonates includes arylsulfonates or synthetic detergents. These synthetic detergents were the first synthetic organic chemicals creating environmental problems characterized by having covalently linked carbon sulfur bond. Owing to their high water solubility anthropogenic sulfonates were not removed completely from water at wastewater treatment plants and subsequently accumulated in the aquatic and terrestrial environments (Dudley *et al.,* 1994; Zerbinati *et al.,* 1997; Reidiker *et al.,* 2000). The presence of thermodynamically stable C-S bond makes arylsulfonates more stable and earlier literature sited arylsulfonates as nonbiodegradable (Bretcher *et al.,* 1981) or rarely biodegradable (Schwitzguebel *et al.,* 2002).

Among the anthropogenic sulfonates 4-toluenesulfonate served as a model compound in the biodegradation of anthropogenic sulfonates (Locher *et al.,* 1989). In chemotrophic bacteria aerobic degradation of 4-toluenesulfonate through methyl group oxidation involving oxygenases is wide spread (Cain and Farr, 1968; Focht andWilliams 1970; Locher *et al.,* 1989; Kertesz *et al.,* 1994; Dudly *et al.,* 1994; David *et al.,* 2003 and Tralau *et al.,* 2011). However, the metabolic pathway elucidation and enzymology of anaerobic biodegradation of 4-toluenesulfonate by chemotrophic bacteria was less studied. Though chemotrophic bacterial degradation of arylsulfonates was well studied, the knowledge on the degradation of arylsulfonates by phototrophic bacteria was still not understood.

Anoxygenic phototrophic bacteria are metabolically versatile, among which purple non sulfur bacteria are known to metabolize various aromatic hydrocarbons (Blasco and Castillo 1992; Sasikala *et al.,* 1994; Zengler *et al.,* 1999; Usha *et al.,* 2007; Kusalatha *et al.,* 2010; Ranjith *et al.,* 2011; Mujahid *et al.,* 2011, Lakshmi prasuna *et al.,* 2012). Anoxygenic phototrophic bacteria metabolizing organosulfur compounds include alkylsulfonates (Novak *et al.,* 2004). However, to date there are no reports on arylsulfonate metabolism by anoxygenic phototrophic bacteria. Thus in the present study

attention has been focused on the isolation, characterization of an anoxygenic phototrophic purple sulfur bacterium and its role in metabolizing arylsulfonates.

4.2 Taxonomic status of the purified bacterial isolate degrading 4-toluenesulfonate

Since marine environments constantly receive sulfur containing organic compounds, which are both natural (Baxter *et al.,* 2002) and anthropogenic (Gonza´ lez- Mazo *et al.,* 1997), an attempt was made to isolate an anoxygenic phototrophic bacterial strain from a marine sediment sample collected from Ramakrishna beach of Visakhapatnam by selective enrichment culture technique using sulfanilate. Anil et al (2007a) reported a novel selective enrichment method for phototrophic green sulfur bacteria even in the presence of purple sulfur and purple non sulfur bacteria, to selectively isolate sulfanilate-metabolizing anoxygenic phototrophic bacteria from marine habitats. However, the isolated pure cultures of green sulfur bacteria were incapable of utilizing sulfanilate as sole source of sulfur. The purified purple sulfur bacterial isolate designated as Vdark given with strain number JA121 and its taxonomic identity was studied through polyphasic taxonomic approach.

16S rRNA gene sequence based analysis indicated that strain JA121 belongs to the genus *Marichromatium* in class *Gammaproteobacteria*. The genus *Marichromatium* is a taxonomically challenging group, was first established by Imhoff et al (1998) to separate the true marine species of the genus *Chromatium* from their freshwater counterparts. At present, the genus *Marichromatium* comprises four species, *Marichromatium gracile* DSM203^T (Imhoff *et al.,* 1998) (originally described as *Chromatium gracile*; Strzeszewski, 1913), *Marichromatium purpuratum* DSM1591^T (Imhoff *et al.,* 1998) (originally described as *Chromatium purpuratum*; Imhoff and Truper, 1980) and *Marichromatium indicum* JA100^T (Arunasri *et al.,* 2005), *Marichromatium bheemlicum* JA124^T (Anil *et al.,* 2007b). In addition to the above validly described species names, one biotype and two novel species of *Marichromatium* were effectively published, which include *Marichromatium gracile* biotype *thermosulfidiphilum* SW26 (Serrano *et al.,* 2009), *Marichromatium litoris* JA349^T and *Marichromatium chrysaorae* JA553^T (Shivali *et al.,* 2011). *Marichromatium fluminis* JA418^T (Sucharita *et al.,* 2010) was reclassified as *Phaeochromatium fluminis* JA418^T (Shivali *et al.,* 2012).

Cells of strain JA121 are rod shaped, motile by mono polar flagellum, stained Gram-negative, multiplied by binary fission and having two to three intracellular sulfur globules. Phototrophic culture is reddish brown. I*n vivo* absorption spectra of intact cells indicated the presence of bacteriochlorophyll-*a* and carotenoids of spirilloxanthin series. Strain JA121 showed similarity in spirilloxanthine series of carotenoids (Except *Marichromatium purpuratum* DSM 1591^T okenone is the carotenoid), quinones (Menaquinone and ubiquinone) and saline requirement with rest of the members of the genus *Marichromatium*. Obligate vitamin requirement was not observed for strain JA121, however growth enhancement was observed in the presence of vitamin B_{12}. Strain JA121 differed with other *Marichromatium* spp. by showing the photolithoheterotrophic, photoorganoheterotrophy growth modes. Photoautotrophy, chemolithoautotrophy, chemolithoheterotrophy, chemoorganoheterotrophy and fermentative mode of growth could not be demonstrated.

The substrates, which were utilized as carbon/e- donor under photolithoheterotrophic conditions by strain JA121, include acetate, propionate, pyruvate, malate, valerate and fumarate. Those, which could not be utilized, include butyrate, succinate, lactate, fructose, glucose, ethanol, propanol, glycerol, glycolate, crotonate, benzoate, citrate, tartarate, caprylate, caproate and casamino acids (Table 11). Sulfate, sulfite, thiosulfate, sodium sulfide, cysteine, alkylsulfonates and arylsulfonates (Table 12) were utilized as sulfur sources (1 mM) under photoheterotrophic conditions. However none of the reported strains of the genus *Marichromatium* utilize arylsulfonates as sole source of sulfur. Since *Marichromatium chrysaorae* $JA553^T$ and *Marichromatium litoris* $JA349^T$ were recently published, arylsulfonates utilization was not screened. Growth supporting nitrogen sources include ammonium chloride, sodium nitrate, glutamine, tryptophan, anthranilate and sulfanilate, while molecular nitrogen and nitrite did not support growth of strain JA121 (Table 13). However none of the reported strains of *Marichromatium* could utilize sulfanilate as sole nitrogen source. Predominant component of cellular fatty acids include $C_{16:0}$, $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{18:1}\omega6c$ and $C_{18:1}\omega7c$, minor fatty acids include $C_{12:0}$, $C_{14:0}$, $C_{18:1}\omega5c$ and $C_{16:1}\omega7c$ OH.

Important phenotypic traits that distinguish strain 121 from other members of the genus *Marichromatium* include high sulfide tolerance 15 mM, where cells were clustered (Fig 6D). Such high sulfide tolerance was reported for green sulfur bacteria (Pfennig

1975) and for the purple sulfur bacteria *Marichromatium indicum* JA100^T (8 mM) (Arunasri *et al.,* 2005) and *Thiorhodococcus drewsii* (11mM) (Zaar *et al.,* 2003), *Marichromatium gracile* biotype *thermosulfidiphilum* SW26 [16 mM] (Serrano *et al.,* 2009).

Though strain JA121 has a 16S rRNA gene sequence similarity >99 % to those of *Marichromatium* species differed in some of the genotypic traits include plasmid presence (Except *Marichromatium gracile* biotype *thermosulfidiphilum* SW26). Since 16S rRNA gene sequence similarity is high, whole genomic DNA-DNA hybridization study was made which has been a cornerstone of bacterial species determination. The DDH reassociation values are between 56-65 % with the type strains of *Marichromatium* (Table 17). As *Marichromatium chrysaorae* JA553^T, *Marichromatium litoris* JA318^T were published recently DDH study was not made. In contrast to this, two type strains of *Marichromatium* such as *Marichromatium gracile* DSM203^T and *Marichromatium purpuratum* DSM1591^T were defined as heterotypic synonyms due to the high whole genomic similarity (DDH relatedness of 71 %) (Serrano *et al.,* 2009).

As an alternative to DDH, the comparative sequence analysis of protein-encoding genes was explored, by which the phylogenetic relationship of 19 *Mycobacterium* species (Takewaki *et al.,* 1994) and *Bacillus subtilis* (Wang *et al.,* 2007) was determined by using the *dnaJ* gene and *gyrB* gene respectively. However using single gene analysis is debatable, due to the horizontal gene transfer, duplication and homologous recombination (Feil *et al.,* 2001; Fracer *et al.,* 2007; Ochman *et al.,* 2000) of the genes in bacterial chromosomes. In this context multi gene analysis such as multilocus sequence analysis (MLSA) was explored for species circumscription with in the genus *Marichromatium* (Serrano *et al.,* 2010). The concatenated MLSA analysis of four protein-coding genes (*recA. FusA, dnaK, pufM*) and together with ITS and 16S rRNA genes similarity is high (>97.5 %) and is closely related to the type strain of *Marichromatium gracile* DSM203^T (Fig 10). In addition to MLSA analysis, MLSA barcodes (concatenated MLSA sequence based restriction bands) obtained by the *in silico* analyses were reported for the identification of the *Marichromatium* spp. (Shivali *et al.,* 2012). Similarly strain JA121 showed difference in MLSA barcodes with respect to the members of the genus *Marichromatium* (Fig 11). Based on MLSA analysis, MLSA barcoding and phenotypic traits differences *Marichromatium fluminis* JA418^T was reclassified into new genus

Phaeochromatium and as new type species *Phaeochromatium fluminis* JA418^T (Shivali *et al.*, 2012).

Strain JA121 showed high genomic similarity but differ in growth modes, saline tolerance, carotenoids composition, sulfide tolerance, arylsulfonate utilization, organic substrate utilization, with respect to *Marichromatium gracile* DSM203^T (Table 25). Though the commonality among all the members of the genus *Marichromatium* is that they represent the true marine species with wide growth capabilities none of the reported species of this genus *Marichromatium* are known for aromatic hydrocarbon metabolism. While comparing the arylsulfonate metabolic potentialities of *Marichromatium spp.,* strain JA121 only has the potential to metabolize arylsulfonates as a sole source of sulfur. Strain JA121 was deposited in two culture collection centers *viz.*, Korean collection for type cultures (KCTC) and NITE biological resource centre (NBRC) as *Marichromatium* sp. JA121 =KCTC 5816 =NBRC 106082. Since strain JA121 has the potential to describe it as a new species, the incomplete DDH study keeps the taxonomic species affiliation pending for strain JA121.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Cell size (W x L) (μm)	1-2 x 2-5	1-1.3 x 2-6	0.8-1 x 2-7	0.8-1 x 2-4	1.2-1.7 x 3-4	1-1.2 x 2-4	1-1.3 x 2-5
Rosette formation	Absent	Absent	Present	Absent	Absent	Absent	Absent
Number of sulfur granules/cell	2 and more	2 or more	Single	2 or more	2 or more	Single	2 or more
NaCl range (optimum %)	1.5-8.5 (1.5-5)	0.5 – 8 (2-3)	0.05-13 (1-4)	1.5-11 (1.5-8.5)	2 -7 (5)	2-5 (2-4)	2-5 (2-6)
Optimum growth temperature (°C)	30-35	25 – 30	30 – 35	30 – 35	30 – 35	25-30	25-35
Optimum pH	6.5-8.5	6.5 – 7.6	6.0–7.5	6.5-8.5	7.2 – 7.6	6.5-7.5	7.0-8.0
Growth modes	PLH, POH	POH, PLA, COH, CLA, CLH	PLH, PLA, CLH, POH (with cystine)	PLH, PLA, POH	POH, PLA, COH, CLA, CLH	POH, PLH	POH, PLH, COH, CLH
Carotenoids composition (mol%)	Rp (50), Sp (29), Rv (4), Ahrv (7),Daly (6), Ly (2) Thly (2)	Rp (75), Sp (9), Rv (7), Ahrv (4), Ly (5)	Rp (50), Sp (35), Rv(1), Ahrv (13), Ly (1)	Rp (58), Sp (30), Ahrv (10), Ly (1)	OK (100)	Rp (65), Sp (9), Rv (11), Ahrv (5), Ly (10)	Rp (58), Sp (20), Rv (7), Ahrv (6), Ly (4)
Vitamin requirement	None	None	None	Pyridoxal phosphate	None	None	None
$^{\$}$N sources utilized	NH4Cl, NO_3^-, glutamine, sulfanilate,anthranilate, tryptophan	NH_4Cl, glutamate, N_2	NH_4Cl, urea, N_2	NH_4Cl, Glutamine, Glutamate.	NH_4Cl, Glutamate	Glutamate, Glutamine NH_4Cl,	Glutamine NH_4Cl,
Sulfide tolerance (mM)	15	4	4	8	4	6.5	7.0
Utilization of 4-toluene sulfonate as sulfur source	+	-	-	-	-	ND	ND
Utilization of sulfanilate as nitrogen source	+	-	-	-	-	ND	ND
Major [>70 mol%] quinones (ratio)	Q8, MK7 (8:2)	Q8, MK7 (8:2)	Q8, MK7 (8:2)	Q8, MK7 (9:1)	Q8, MK7 (8:2)	Q8, MK7 (8:2)	Q8, MK7 (8:2)
$^{\$}$Growth supporting organic substrates/e- donors							
Propionate	+	+	+	-	+	+	+
Butyrate	-	+	+	-	+	w	W
Valerate	+	-	-	+	+	w	w
Succinate	-	+	-	+	+	+	+
Fumarate	+	+	-	+	+	+	+
Fructose	-	-	+	-	-	-	-
Lactate	-	+	+	-	+	+	-
Methanol	-	-	+	+	-	-	-
Whole cell fatty acids						-	-
$C_{12:0}$	6.1	9.2	3.0	12.5	1.8	5.0	3.7
$C_{14:0}$	2.1	--	1.8	--	--	2.0	1.1
$C_{14:0}$2OH	--	--	2.1	--	--	3.2	--
$C_{16:0}$	24.3	15.6	2.7	--	20.5	18.7	21.3
$C_{16:0}$ n-OH	--	--	1.7	--	--	2.6	--
$C_{18:0}$	1.5	4.2	--	--	--	--	1.3
$C_{18:3}$ω6,9,12c	--	--	2.7	33.4	--	--	--
$C_{16:1}$ω7c/$C_{16:1}$ ω6c	32.3	10.5	--	--	29.4	11.2	21.4
$C_{16:1}$ω7c alcohol	1.0	2.1	--	--	--	--	--
$C_{18:1}$ω7c	29.3	49.2	1.8	--	40.4	52	37.5
$C_{18:1}$ω5c	1.0	--	68.5	54.0	--	--	--

Molecular Features							
DNA G+C content (mol%)	64	71.2	66.7	67	68.2	68.4	70.5
16S rRNA sequence similarity (%)	100	98.34	98.28	95	98.24	98	98.6
DDH (%) relatedness	100	60	50	53	65	NT	NT
MLSA sequence similariy (%)	100	99	99	98	98	98	98
Plasmid presence	+	-	-	-	-	NT	NT
Benzylsuccinate synthase gene (*bssA*)	+	-	-	-	-	NT	NT

Table 25: Distinguishing characteristics of Strain JA121 with the members of the genus *Marichromatium*

- Sources: Except for strain JA121 remaining comparative data was taken from Shivali et al [2011].
- 1, Strain JA121; 2, *Mch. gracile* DSM 203^{T}; 3, *Mch. indicum* JA100^{T}; 4,*Mch. bheemlicum*; JA124^{T} 5, *Mch. Purpuratum*; 1591^{T} 6 *Mch. litoris* JA 349^{T}; 7, *Mch. Chrysaorae* JA553^{T}
- CLA, Chemolithoautotrophy; CLH, Chemolithoheterotrophy; COH, Chemo-organoheterotrophy; PLA, Photolithoautotrophy; PLH, Photolithoheterotrophy; POH, Photo-organoheterotrophy.
- Symbols: +, substrate utilized; −, substrate not utilized; w, growth turbidity was very weak. NT, not tested
- Rp, rhodopin; Sp, spirilloxanthin; Rv, rhodovibrin; Ahrv, anhydrorhodovibrin; Ly, lycopene; Thly, Tetrahydro lycopene; Daly; Diapolycopene; Ok, okenone ; Q8, quinone; MK, menequinone.

4.3 Oxidative catabolism of 4-toluenesulfonate by *Marichromatium* sp. JA121

While screening the arylsulfonates utilization by *Marichromatium* sp. JA121 growth was not demonstrated when used as sole source of carbon, however these compounds served as sole source of sulfur (Table 18). Since 4-toluenesulfonate has served as a model compound for arylsulfonate biodegradation studies and utilization of 4-toluenesulfonate was maximum by *Marichromatium* sp. JA121, this compound was opted for catabolic studies. 4-Toluenesulfonate utilization by *Marichromatium* sp. JA121 adds to the list of microbial utilization of different arylsulfonates. Of which utilization of 4-toluenesulfonate as a carbon source was reported in *Pseudomonas aeruginosa* A (Cain and Farr, 1968), *Comamonas testosteroni* H-8 (Ripin *et al.,* 1971), *Comamonas testosteroni* T-2 (Locher *et al.,* 1989a), *Achromobacter xylosoxidans* TA12-A and *Ensifer adhaerens* TA12-B (Tralau *et al.,* 2011). Microbial utilization of 4-toluenesulfonate as a sole sulfur source was reported in *Pseudomonas putida* S-313, *Klebsiella oxytoca* KS3D (Kertesz *et al.,* 1994; Dudly *et al.,* 1994), *Rhodococcus opacus* ISO-5 (David *et al.,* 2003), *Clostridium pasteurianum* DSM 12136 (Chih-Ching Chien 2005), *Cupriavidus metallidurans* and *Variovoraxparadoxus* T (Schmalenberger 2007). In the present study, 0.7 mM of 4-toluenesulfonate was utilized by *Marichromatium* sp. JA121 as sole source of sulfur (Fig 13B).

The parameters influencing 4-toluenesulfonate utilization was studied in *Marichromatium* sp. JA121. Carbon sources influenced 4-toluenesulfonate utilization and pyruvate was the appropriate carbon source at which maximum utilization of 4-toluenesulfonate was observed (Table 20). In presence of other sulfur sources (organic and inorganic), 4-toluenesulfonate utilization by *Marichromatium* sp. JA121 was reduced and completely inhibited in the presence of sodium sulfide (Table 21). Such an inhibition of arylsulfonates utilization in presence of other sulfur sources was explained in *Pseudomonas putida* S-313, where the expression of arylsulfatase enzyme and *asf* genes (arylsulfonate degrading genes) were maximal during growth with benzenesulfonate or toluenesulfonate, and not expressed during growth with either sulfate or pentanesulfonate. The induction of the *asf* operon required the presence of aromatic sulfonates as co-inducers. During growth with a combination of sulfate and toluenesulfonate, *asf* expression was down regulated (Paul *et al.,* 1999; Schmalenberger and Kertesz 2007). Sodium chloride concentration also influenced 4-toluenesulfonate utilization and optimum

is 2 % (Fig 14). The plausible explanation is, since *Marichromatium* sp. JA121 is a marine isolate for which salt is obligate for its growth, concomitantly effecting 4-toluenesulfonate utilization. Saline dependent toluene degradation was reported in *Pseudomonas aeruginosa* (Zahra *et al.,* 2012) and Shiaris (1989) reported a positive correlation between salinity and the rate of mineralization of phenanthrene in estuarine sediments. In oil contaminated soils, polyaromatic hydrocarbons degradation was saline dependent and >1% saline decreases the rate of biodegradation of poly aromatic hydrocarbons (Dariush and Saeed 2009), where high salinity dehydrates the soil concomitantly affecting the growth and degradation of aromatic hydrocarbons.

Exometabolome of 4-toluenesulfonate induced and uninduced cultures was initially compared for metabolic footprints from different growth phases. The analysis of metabolites in both induced and uninduced cultures led to the identification of five masses, which were reported earlier as metabolites of 4-toluenesulfonate in chemotrophic bacteria. These masses of the metabolites include m/z 157 (mass corresponds to benzenesulfonate reported in *Methanosarcina mazei* MM by Shcherbakova *et al.,* 2003), m/z 124 (mass corresponds to 4-methylcatechol reported in *Alcaligens* sp. O-1 by Junker *et al.,* 1994), m/z 154, 202, 218 (masses corresponds to protocatechuate, 4-sulfobenzoate, 3-hydroxy 4-sulfobenzoate respectively reported in *Comamonas testosteroni* T-2 by Locher *et al.,* 1989a). However 4-sulfobenzoate and protocatechuate were identified by metabolite fractionation from the exometabolome, HPLC and LCMS analyses (Fig. 16, 17). The same products were reported in the oxidative pathway of 4-toluenesulfonate degradation by *Comamonas testosteroni* T-2 (Locher *et al.,* 1989a), *Achromobacter xylosoxidans* TA12-A and *Ensifer adhaerens* TA12-B (Tralau *et al.,* 2011). In *Comamonas testosteroni* T-2, 4-toluenesulfonate methylmonooxygenase system is a 152 kDa protein having component B (reductase) and component M (oxygenase) catalyzing the conversion of 4-toluenesulofonate to 4-sulfobenzylalcohol (Locher *et al.,* 1991) and 4-sulfobenzoate dioxygenase system is a 132 kDa protein having component A (oxygenase) and component B (Reductase) catalyses the conversion of 4-sulfobenzoate to protocatechuate and sulfite (Locher *et al.,* 1991). These two enzymes encoding plasmid (72 $\pm$ 4 kb) was fully sequenced in *Comamonas testosteroni* T-2 (Tralau *et al.,* 2001). While studying the enzymology of degradation of 4-toluenesulfonate with cell free extracts of 4-toluenesulfonate induced culture, 4-toluenesulfonate methyl monoxygense,

4-sulfobenzoate 3,4-dioxygenase activities were also identified by oxygen uptake studies and product formation in *Marichromatium* sp. JA121.

From the experimental results, a pathway was proposed for catabolism of 4-toluenesulfonate by *Marichromatium* sp. JA121 by demonstrating each enzymatic reaction and by identifying intermediates (Fig. 35). According to the proposed pathway, methyl group of 4-toluenesulofonate was first oxidized to 4-sulfobenzylalcohol, and 4-sulfobenzoate followed by ring hydroxylation giving 3-hydroxy, 4-sulfobenzoate then desulfonated to protocatechuate. In the proposed pathway intermediate (4-sulfobenzylalcohol) in the parentheses have absorption maxima similar to the 4-sulfobenzylalcohol (Locher *et al.,* 1989a), and the other intermediate (3-hydroxy, 4-sulfobenzoate) was considered from mass spectral analysis, which were not able to be confirmed through HPLC due to the lack of authentic standards. Further degradation of protocatechuate (ring cleavage) was not observed in *Marichromatium* sp. JA121.

In *Comamonas testosteroni* T-2 the enzymes involved in 4-toluenesulfonate catabolism were plasmid encoded and protocatechuate ring degrading enzymes were chromosomal encoded (Locher *et al.,* 1989a). The enzymes involved in 4-toluenesulfonate catabolism may be plasmid encoded in *Marichromatium* sp. JA121, however in depth analysis is required for further confirmation. In *Pseudomonas putida* S-313 arylsulfonate degrading proteins were chromosomally encoded (Paul *et al.,* 1999), where 4-toluenesufonate was desulfonated to 4-hydroxy toluene.

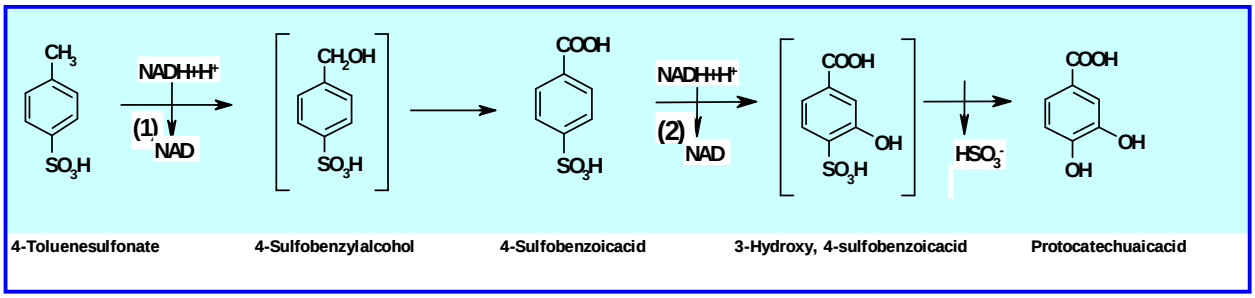


Fig 35: The proposed catabolic pathway of 4-toluenesulfonate by *Marichromatium* sp. JA121.

The numbers represent; 1, 4-toluenesulfonate methylmonoxygenase

2, 4-sulfobenzoate 3,4 dioxygenase

Metabolites in the parentheses were confirmed through mass spectral analysis

4.4 Conjugative metabolism of 4-toluenesulfonate by *Marichromatium* sp. JA121

Toluene is anoxically degraded to CO_2 by an anaerobic denitrifying bacterium, *Thauera aromatica* K172 by adopting conjugative mechanism involving fumarate/succinate as conjugate (Biegert *et al.,* 1996). The oxygen sensitive, redox-active flavin cofactor (without iron centers) containing benzylsuccinate synthase (EC 4.1.99.11) is a $\alpha_2\beta_2\gamma_2$ enzyme coded by at least four genes organized as a single operon (Leuthner *et al.,* 1998). Based on the metabolic intermediates of toluene in *Thauera aromatica* K172 (Biegert *et al.,* 1996) and phototrophic bacterium *Blastochloris sulfoviridis* Top1 (Zengler *et al.,* 1999), conjugative metabolite was identified the in *Marichromatium* sp. JA121 during the exometabolome analysis. In order to augment these metabolites, *Marichromatium* sp. JA121 was grown on pyruvate and succinate (as an additional carbon source). Though growth of *Marichromatium* sp. JA121 was not supported by succinate as sole source of carbon, its consumption was observed only in the presence of 4-toluenesulfonate.

In *Marichromatium* sp. JA121, the formation of product benzylsuccinate indicates the presence of the enzyme benzylsuccinate synthase. While screening the oxygen sensitivity of the benzylsuccinate synthase enzyme, assay was studied with cell free extracts under both aerobic and anaerobic conditions. During the enzyme analysis under anaerobic conditions the product benzylsuccinate was identified based on HPLC and LCMS analyses (Fig. 24, 25) and identified as a dead end metabolite in the enzyme analysis (Further transformation of the product benzylsuccinate was not observed). Similarly under anaerobic conditions benzylsuccinate and one unidentified product were observed. After mass fragmentation analysis the unidentified metabolite was presumed as hydroxyl benzyl succinate. In *Desulfobacterium cetonicum* 480 anaerobic degradation of *p*-cresol was reported with the formation of 4-hydroxybenzylsuccinate as a product (Muller *et al.,* 2001). In a similar manner *Marichromatium* sp. JA121 could utilize *p*-cresol forming the product 4-hydroxybenzylsuccinate. The mass fragmentation of the *p*-cresol metabolite 4-hydroxybenzylsuccinate was similar to the mass fragmentation of the unidentified product formed in the benzylsuccinate synthase assay under aerobic conditions (Table 23).

Benzylsuccinate synthase was detected in bacteria degrading toluene anaerobically, includes denitrifying bacteria (*Thauera aromatica* T1 [Evans *et al.,* 1991a, 1991b], *Thauera aromatica* K172 [Schocher *et al.,* 1991]), *Azoarcus sp*. strain T [Achong *et al.,* 2001], *Azoarcus sp*. strain EbN1 [Rabus *et al.,* 1995]), iron(III) reducing (*Geobacter grbiciae* [Winderl *et al.,* 2007], *Geobacter metallireducens* [Lovely and Lonergan. 1990; Lovely *et al.,* 1993], sulfate-reducing bacteria (Strain PRTOL1 [Beller *et al.,* 1992] , *Desulfobacula toluolica* [Rabus *et al.,* 1993], *Desulfobacterium cetonicum* [Muller *et al.,* 1999], *Desulfosarcina cetonica* [Winderl *et al.,* 2007], *Desulfotomaculum* sp. [Winderl *et al.,* 2007]), a phototrophic bacterium (*Blastochloris sulfoviridis* strain ToP1 [Zengler *et al.,* 1999]) and toluene-degrading methanogenic consortia (*Methanospirillum* genera [Edwards and Grbic 1994]). The oxygen sensitivity of benzylsuccinate synthase was explained by the oxygen mediated polypeptide cleavage due to the formation of a peroxy radical at Cα of the glycyl residue, elimination of the super oxide radical and subsequent hydrolysis of the generated imino bond to an amide bond and a glyoxylyl group (Leuthner *et al.,* 1998). In *Thauera aromatica* three benzyl succinate synthase structural genes were reported *viz bssA, bssB and bssC*. The *bssA* gene, coding for the large α-subunit of benzylsuccinate synthase, is highly conserved among the anaerobic bacteria degrading toluene and its derivatives (Leuthner *et al.,* 1998). In *Marichromatium* sp. JA121 *bssA* gene was amplified using two sets of designed primers, of which positive amplification was observed with one set of primers (bssAF2 and bssAR1). The amplicon ~365bp was observed and the putative and partial *bssA* gene sequence has been deposited in Genbank under accession number GQ923885.

From the identified metabolites and enzymic activities, conjugative pathway of 4-toluenesulfonate was proposed in *Marichromatium* sp. JA121 (Fig 36). In the presence of succinate/fumarate, 4-toluenesulfonate was desulfonated to benzylsuccinate and then hydroxylated to a chimeric metabolite 4-hydroxybenzylsuccinate. Similar fumarate-addition reactions were discovered to initiate anaerobic degradation of *p*-, *m*-, and *o*-xylene (Beller & Spormann, 1997, Krieger, *et al.*, 1999), 2-methylnaphthalene (Annweiler, *et al.*, 2000), *m*- and *p*-cresol (Müller, *et al.*, 1999, Müller, *et al.*, 2001), and ethylbenzene (Kniemeyer, *et al.*, 2003). However, this is the first report on catabolism of 4-toluenesulfonate through succinate/ fumarate conjugation by a phototrophic purple sulfur bacterium, *Marichromatium* sp. JA121. In this context *Marichromatium* sp. JA121

differs with other reported chemotrophic bacteria, by degrading 4-toluenesulfonate through succinate or fumarate conjugation.

Stable isotope analysis was reported for elucidation of biodegradation pathways , transformation studies (Martin *et al.*, 2005) and in microbial ecology (Boschker *et al.*, 2002). Since stable isotope of 4-toluenesulfonate was not available, in the present study, metabolite based stable isotope probing using d_8 toluene was employed for oxidative and conjugative pathways confirmation in *Marichromatium* sp. JA121. Stable isotope probing of metabolites viz, benzylsuccinate and benzoate were observed in toluene metabolism by *Marichromatium* sp. JA121. Since the mechanism of methyl group oxidation and conjugation was observed for toluene metabolism by *Marichromatium* sp. JA121, in similar manner *Marichromatium* sp. JA121 may be mimicking the same mechanism for catabolism of 4-toluenesulfonate. The existence of multiple pathways for degradation/transformations of phenol under both aerobic and anaerobic conditions was reported in microbial populations of *Rhodopseudomonas palustris* straddling between oxic to anoxic transition zones (Larimer *et al.*, 2004). A denitrifying bacterium, *Thauera* sp. DNT1 also modulates the expression of two different pathways (aerobic and anaerobic pathways) for toluene degradation according to the oxygen availability in the environment (Shinoda *et al.*, 2004). This is the first evidence for 4-toluenesulfonate catabolism by a purple sulfur bacterium *Marichromatium* sp. JA121, modulating both oxidative and conjugative pathways.

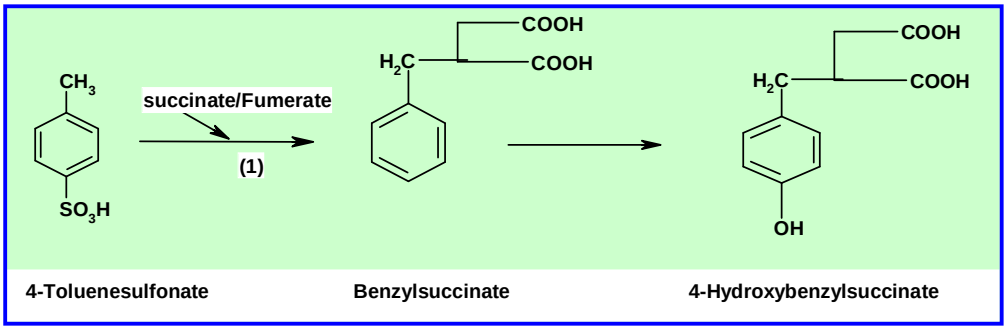


Fig 36: The proposed chimeric pathway of 4-toluenesulfonate catabolism by *Marichromatium* sp. JA121
1= Benzylsuccinate synthase

Conclusions

5.0 Conclusions

- An anoxygenic phototrophic purple sulfur bacterium isolated from a marine sediment was characterized through polyphasic taxonomic approach, identified as a member of the genus *Marichromatium* and designated as strain JA121. However, species affiliation needs more critical analysis to conclude strain JA121 as a new species.
- *Marichromatium* sp. JA121 utilizes a wide range of alkylsulfonates, arylsulfonates as sole source of sulfur and photo-bio-transform several other aromatic hydrocarbons.
- 4-Toluenesulfonate was utilized as sole source of sulfur by strain JA121, 4-sulfobenzoate and protocatechuate were identified as products. This oxidative metabolism of 4-toluenesulfonate was supported by the presence of 4-toluenesulfonate methylmonooxygenase and 4-sulfobenzoate 3,4 –dioxygenases.
- A conjugative pathway of 4-toluenesulfonate also exists in *Marichromatium* sp. JA121. The products benzylsuccinate and 4-hydroxybenzylsuccinate is catalyzed by a novel oxygen insensitive, succinate/fumarate dependent *bssA* gene (NCBI: GQ923885) encoded benzylsuccinate synthase.
- Multitasking of 4-toluenesulfonate probably helps strain JA121 to function under oxic, anoxic and transit zones in the process of degradation of arylsulfonates.

References

Achenbach, L. A., Carey, J. and Madigan, M. T. (2001). Photosynthetic and phylogenetic primers for detection of anoxygenic phototrophs in natural environments. Appl. Environ. Microbiol. 67: 2922–2926.

Achong, G. R., Rodriguez, A. M. and Spormann, A. M. (2001). Benzylsuccinate synthase of *Azoarcus sp*. strain T: cloning, sequencing, transcriptional organization, and its role in anaerobic toluene and *m*-xylene mineralization.183: 6763-6770.

Alonso, M. C. and Barcelo, D. (2000). Stability of sulfonated derivatives of benzene and naphthalene on disposable solid-phase extraction pre-columns and in an aqueous matrix. Journal of Chromatography A, 889: 231–244.

Altenbach, B. and Giger, W. (1995). Determination of Benzene sulfonates and Naphthalenesulfonates in Waste-Water by Solid-Phase Extraction with Graphitized Carbon-Black and Ion-Pair Liquid-Chromatography with UV Detection. Analytical Chemistry, 67: 2325-2333.

Anil Kumar, P., Srinivas T. N. R. Sasikala, Ch. Ramana, Ch. V., Süling, J. and Imhoff, J. F. (2007a). Selective enrichment of green sulfur bacteria in the presence of 4-aminobenzenesulfonate (sulfanilate) World Journal of Microbiology & Biotechnology, 23(3): 393-399.

Anil Kumar, P., Sasi Jyothsna, T., Srinivas, T. N. R., Sasikala, C., Ramana, C. V. and Imhoff, J. F. (2007b). *Marichromatium bheemlicum* sp. nov., a non-diazotrophic, gammaproteobacterium from a marine aquaculture pond. Int. J. Syst. Evol. Microbiol. 57: 1261–1265.

Anliker, R. (1977). Color chemistry and the environment. Ecotoxicol. Environ. Safety, 1:211–237.

Annweiler, E., Materna, A., Safinowski, M., Kappler, A., Richnow, H., Michaelis, W. and Meckenstock, R. (2000). Anaerobic degradation of 2-methylnaphthalene by a sulfate reducing enrichment culture. Applied Environmental Microbiology, 66: 5329–5333.

Anuradha, K., Jagtap, N., Umesh, B., Jadhav, Jyoti, P., Bapat, Vishwas A., Govindwar, and Sanjay, P. (2009). Biotechnological strategies for phytoremediation of the sulfonated azo dye Direct Red 5B using Blumea malcolmii Hook. Bioresource Technology, 100: 4104-4110.

Arunasri., Sasikala, Ch., Ramana, V. C., Suling, J. and Imhoff, J.F. (2005). *Marichromatium indicum* sp. nov., a new purple sulfur Gammaproteobacterium from mangrove soil of Goa India. Int.J.Syst.Evol.Microbiol.55: 673–679.

Autry, A. R. and Fitzgerald, J. W. (1990). Sulfonate S: a major form of forest soil organic sulfur. Biology and Fertility of Soils 10: 50– 56.

Awasthi, S.K., Ashfaq, M. and Singh, S. (2009). Effect of glucose and chloramphenicol on ABS biodegradation by a bacterial consortium. Biology and Medicine 15-19.

Baker, S.C., Kelly D. P. and Murrell, J. C. (1991). Microbial degradation of methanesulphonic acid: a missing link in the biogeochemical sulphur cycle. Nature, 350:627–628.

Baxter, N., J., Scanlan, J. and De Marco, P. (2002). Duplicate copies of genes encoding methane sulfonate monooxygenase in *Marinosulfonomonas methylotropha* strain TR3 and detection of methanesulfonate utilizers in the environment. Appl Environ Microbiol, 68:289–296.

Behret, H., Ahlers, J., Ettel, S., Feicht, E., Futterer, E., Mangelsdorf, I., Pohlenz-Michel, C., Rob, H., Sterzl-Eckert, H., Vogel, D., Weis, L. and Widmann, K. (1991). p-Toluolsulfonsaeure. Beratergremium fu¨r umweltrelevante Altstoffe (BUA)– Stoffberichte, vol. 63. Verlag Chemie, Weinheim, Germany.

Beller, H.R., Reinhard, M. and Grbic-Galic, D. (1992). Metabolic by-products of anaerobic toluene degradation by sulfate-reducing enrichment cultures. Appl Environ Microbiol, 58: 3192–3195.

Beller, H. R. and Spormann, A. M. (1997). Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. Appl Environ Microbiol, 63: 3729–3731.

Beller, H., R., Spormann, A., M., (1997). Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J Bacteriol.;179:670–676.

Beller, H. R. and E. A. Edwards. 2000. Anaerobic toluene activation by benzylsuccinate synthase in a highly enriched methanogenic culture. Appl. Environ. Microbiol. 66:5503-5505.

Benning, C. (1998). Membrane lipids in anoxygenic photosynthetic bacteria. In: Lipids in photosynthesis: structure, function and genetics. Advances in photosynthesis 6: 83-101.

Biedlingmaier, J.J. and Schmidt, A. (1983). Arylsulfonic acids and some S-containing detergents as sulfur sources for growth of *Chlorella fusca*. Arch Microbiol., .136:124–130.

Biegert, T., Fuchs, G. and Heider, J. (1996). Evidence that anaerobic oxidation of toluene in the denitryfing bacterium *Thaurea aromatica* is initiated by formation of benzylscuccinate from toluene and fumarate. Eur. J. Biochem, 238: 661-668.

Blasco, R. and Castillo, F. (1992). Light-dependent degradation of nitrophenols by the phototrophic bacterium *Rhodobacter capsulatus* E1F1. Appl. Environ. Microbiol. 58:690–695.

Bonsen, P. P. M., Spudich, J, A, Nelson, D, L, and Kornberg, A. (1969). Biochemical studies of bacterial sporulation and germination. A sulfonic acid as a major sulfur compound of Bacillus subtilis spores. J. Bacteriol., 98: 62–68.

Boschker, H.T.S. and Middelburg, J. J. (2002). Stable isotopes and biomarkers in miocrobial ecology. FEMS Microbiol. Ecol. 40:85-95.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Bretscher, H. (1981). Waste disposal in the chemical industry. In: Microbial Metabolism of Xenobiotics and Recalcitrant Compounds (Leisinger, T., Cook, A.M., Huëtter, R. and Nuëesch, J., Eds.) 65-74. Academic Press, London.

Brilon, C., Beckmann, W., Hellwig, M. and Knackmuss, H.J. (1981) Catabolism of naphthalenic acids by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22. Appl Environ Microbiol 42:44-45.

Bryant, D.A., Garcia Costas, A. M, Maresca, J., A., Chew, A., G., M., Klatt, C., G., Bateson, M., M., Tallon, L., J., Hostetler, J., Nelson, W., C., Heidelberg J., F., and Ward, D., M., (2007). Candidatus Chloracidobacterium thermophilum: An Aerobic Phototrophic Acidobacterium. Science. 317 (5837), 523-526.

Budzikiewicz, H., Fuchs, R., Taraz, K., Marek-Kozaczuk, M. and Skorupska, A. (1998). Dihydropyoverdin- 7-sulfonic acids – unusual bacterial metabolites. Nat Prod Lett. 12:125–130.

Burkhard, J., Feigel, and Knackmuss, H.J. (1988). Bacterial catabolism of sulfanilic acid via catechol-4-sulfonic acid FEMS Microbiology Letters 55:113-117.

Burkhard, J., Feigel, Hans-Joachim Knackmuss(1993). Syntrophic interactions during degradation of 4-aminobenzenesulfonic acid by a two species bacterial culture. Arch Microbiol. 159:124-130.

Cain, R., B. and Farr, D. R. (1968). Metabolism of arylsulphonates by micro-organisms. Biochemical Journal 106: 859-877.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17: 540-552.

Chassagnole, C., Noisommit-Rizzi, N., Schmid, J. W., Mauch, K. and Reuss, M. (2002). Dynamic modeling of the central carbon metabolism of *Escherichia coli*. Biotechnol Bioeng. 79: 53–73.

Chih-ching Chien (2005). Arylsulfonates as sole source of sulfur for *Clostridium pasteurianum* DSM 12136. Basic Microbiol. 45: 274–278.

Chivukula, M., Spadaro, J.T. and Renganathan, V. (1995). Lignin peroxidase-catalyzed oxidation of sulfonated azo dyes generates novel sulfophenyl hydroperoxides. Biochemistry.34:7765-72.

Choorit, W., Thanahoset, P., Thongpradistha, J., Sasaki, K. and Noparatnaraporn, N. (2002). Identification and cultivation of photosynthetic bacteria in wastewater from a concentrated latex processing factory. Biotechnol. Lett. 24: 1055-1058.

Collins, M.D., Pirouz, T., Goodfellow, M. and Minnikin, D.E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. J. Gen. Microbiol. 100, 221-230.

Contzen, M., Wittich, R.M., Knackmuss, H.J. and Stolz, A. (1996). Degradation of benzene 1,3-disulfonate by a mixed bacterial culture. FEMS Microbiol Lett.136:45-50.

Contzen, M. Edward, R., Moore, B., Blomeli, S., Stolz, A. and Peter K. (2000). *Hydrogenophaga intermedia* sp. nov., a 4-aminobenzenesulfonate degrading organism system. Appl. Microbiol. 23, 487-493.

Cook, A.M., Heike Laue, A. and Frank Junker, A (1999). Microbial desulfonation FEMS Microbiology Reviews 22: 399-419.

Cook, A., M. (1998). Sulfonated surfactants and related compounds: facets of their desulfonation by aerobic and anaerobic bacteria. Tenside Surfact. Deterg. 35: 52-56.

Cook, A. M., Laue, H. and Junker, F. (1999). Microbial desulfonation. FEMS Microbiol. Rev. 22:399-419.

Cook, A.M., Denger, K. and Smits T. H. M. (2006). Dissimilation of C3-sulfonates. Arch Microbiol 185:83–90.

Cook, A. M., Smits, T. H. M. and Denger, K. (2007). Sulfonates and organotrophic sulfite metabolism. In *Microbial Sulfur Metabolism*, pp. 170-183. Edited by C. Dahl & C. G. Friedrich (ed.): Springer Verlag, Berlin.

Cripps, C., Bumpus, J.A. and Aust, S. D.(1990). Biodegradation of azo and heterocyclic dyes by *Phanerochaete chrysosporium.* Appl Environ Microbiol. 56: 1114–1118.

Dangmann, E., Stolz, A., Kuhm, A. E., Hammer, A., Feigel, B., Noisommit-Rizzi, N., Rizzi, M., Reuss, M.and Knackmuss, H. J. (1996). Degradation of 4-aminobenzenesulfonate by a two-species bacterial coculture. Physiological interactions between *Hydrogenophaga palleronii* S1 and *Agrobacterium radiobacter* S2. Biodegradation.7:223-239.

Dariush, M.T., Saeed M. and Ali, H. (2009). Effect of salinity on biodegradation of polycyclic aromatic hydrocarbons (pahs) of heavy crude oil in soil bull. Environ Contam Toxicol. 82:179–184.

David, S., Melanie, L., Schonenberger, R., Marc, J.F., Suter and Cook A.M. (2003). Desulfonation and degradation of the disulfo diphenyl ethercarboxylates from linear alkyl diphenyl etherdisulfonate surfactants Applied and Environmental Microbiology. 69: 938-944.

Denger, K., Kertesz, M., A., Vock, E.H., Schoën, R., Maëgli, A., and Cook, A.M. (1996). Anaerobic desulfonation of 4-tolylsulfonate and 2-(4-sulfophenyl) butyrate by a *Clostridium* sp. Appl. Environ. Microbiol. 62:1526 -1530.

Denger, K., Ruff, J., Rein, U. and Cook, A.M. (2001). Sulphoacetaldehyde sulpho-lyase (EC 4.4.1.12) from *Desulfonispora thiosulfatigenes* : purification, properties and primary sequence Biochem. J. 357: 581-586.

Difco Manual. (1998). 11th edition, Difco Laboratories, Sparks, Maryland, USA.

Dodgson, K.S., White, G.F. and Fitzgerald, J.W. (1982). Sulfatases of Microbial Origin. CRC Press, Boca Raton, FL.

Dudley, M.W. and Frost, J. W. (1994). Biocatalytic desulfurization of arylsulfonates. Bioorg. Med. Chem. 2: 681-690.

Edwards, E. A. and Grbic-Galic, D. (1994). Anaerobic degradation of toluene and o-xylene by a methanogenic consortium. Appl Environ Microbiol. 60:313–322.

Elvers, B., Hawkins, S., and Russey, W., (1994). Ullmann's encyclopedia of industrial chemistry. VCH Verlagsgesellschaft mbH, Weinheim, Germany.

Endo, K., Kondo, H. and Ishimoto, M. (1977). Degradation of benzenesulphonate to sulphite in bacterial extract. Journal of Biochemistry. 82: 1397- 1402.

Evans, P. J., D. T. Mang, and L. Y. Young. (1991a). Degradation of toluene and *m*xylene and transformation of *o*-xylene by denitrifying enrichment cultures. Appl. Environ. Microbiol. 57:450-454.

Evans, P. J., D. T. Mang, K. S. Kim, and L. Y. Young. (1991b). Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57:1139- 1145.

Feigel, B. and Knackmushs, J. (1988). Bacterial catabolism of sulfanilic acid via catechol-Csulfonic acid. FEMS Microbiology Letters 55: 113-1 18.

Feigel, B.J. and Knackmuss, H. J. (1993). Syntrophic interactions during degradation of 4-aminobenzenesulfonic acid by a two species bacterial culture. Arch. Microbiol. 159:124-130.

Feil, E. J., Holmes, E. C., Bessen, D. E., Chan, M. S., Day, N. P., Enright, M. C., Goldstein, R., Hood, D. W., Kalia, A., Moore, C. E., Zhou, J. and Spratt, B. G. (2001). Recombination with in natural populations of pathogenic bacteria: short-term empirical estimate sandlong-termphylogenetic consequences. Proc. Natl. Acad. Sci. 98:182–187.

Focht, D. D.and Williams, F. D. (1970). The degradation of p-toluenesulphonate by a *Pseudomonas.* Canadian Journal of Microbiology 16: 309- 316.

Fraser, C., Hanage, W. P. and Spratt, B.G. (2007). Recombination and the nature of bacterial speciation. Science 315: 476–480.

Garcia-Martinez, J., Martinez-Murcia, A., Anton, A.I. and Rodriguez-Valera, F. (1996). Comparison of the small 16S to 23S intergenic spacer region (ISR) of the rRNA operons of some *Escherichia coli* strains of the ECOR collection and *E. coli* K-12. J. Bacteriol. 21: 6374–6377.

Gatti, G. and Casu, B. (1979). Studies on the Conformation of Heparin by lH and 13C NMR Spectroscopy. Macromolecules. 12: 1001–1007.

Gonzalez-Mazo, E., Barcelo, H.M and Gomez-Parra, A. (1997). Monitoring long-chain intermediate products from the degradation of linear alkylbenzene sulfonates in the marine environment by solid-phase extraction followed by liquid chromatography/ionspray mass spectrometry. Environ Sci Technol 31:504–510.

Goszczynski, S., Paszczynski, A., Pasti-grigsby, M. B., Crawford, R. L. and Crawford, D.L. (1994). New pathway for degradation of sulfonated azo dyes by microbial peroxidases of *Phanerochaete chrysosporium* and *streptomyces chromofuscus*. Journal of bacreriology. 176:1339-1347.

Harwood, C. S. and Gibson.J. (1988). Anaerobic and aerobic metabolism of diverse aromatic compounds by the photosynthetic bacterium *Rhodopseudomonas palustris*. Appl. Environ. Microbiol. 54:712–717.

Heising, S. and Schink, B. (1998). Phototrophic oxidation of ferrous iron by a *Rhodomicrobium vannielii* strain. Microbiol. 144: 2263-2269.

Heising, S., Richter, L., Wolfgang, L. and Schink, B. (1999). *Chlorobium ferroxidans* sp. nov., a phototrophic green sulfur bacterium that oxidises ferrous iron in cocultures with a "Geospirillum" sp. Strain. Arch. Microbiol 172: 116-124.

Herbert and Holliman F.G. (1964). Aeruginosin B, a naturally occurring phenazine sulphonicacid. Proc.chem.soc.19.

Hickford, S.J.H., Küpper, F.C., Zhang, G., Carrano, C.J. Blunt, J.W. and Butler, A. (2004). Petrobactin sulfonate, a new siderophore produced by the marine bacterium Marinobacter hydrocarbonoclasticus. J Nat Prod 2004:1897–1899.

Hilberg, M., Pierik, A.J., Bill, E., Friedrich, T., Lippert, M.L. and Heider, J. (2012). Identification of FeS clusters in the glycyl-radical enzyme benzylsuccinate synthase via EPR and Mössbauer spectroscopy. J Biol Inorg Chem. 17:49-56.

Hiraishi, A., Hoshino, Y. and Kitamura, H. (1984). Isoprenoid quinone composition in the classification of Rhodospirillaceae. J. Gen. Appl. Microbiol. 30: 197-210.

Hiraishi, A., J. L. Shi, and H. Kitamura. 1989. Effects of organic nutrient strength on the purple nonsulfur bacterial content and metabolic activity of photosynthetic sludge for wastewater treatment. J. Ferment. Bioeng. 68:269-276.

Hooper, SW. (1991). Biodegradation of sulfonated aromatics. In: Chaudhry GR (ed) Biological Degradation and Bioremediation of Toxic Chemicals. 169-182. Chapman & Hall, London.

Huxtable, R.J. (1992) .Physiological actions of taurine. Physiol Rev 72:101–163.

Imhoff, J. F. (1984). Reassignment of the genus Ectothiorhodospira a new family Ectothiorhodospiraceae fam. nov., and emended description of the *Chromatiaceae Bavendamm* 1924. Int. J. Syst. Bacteriol. 34: 338-339.

Imhoff, J. F. (1995). Taxonomy and physiology of phototrophic purple bacteria and green sulfur bacteria. In "Anoxygenic photosynthetic bacteria". (Blankenship, R. E., Madigan, M. T., and Bauer, C. E. (eds)), p. 1-15. Kluwer Academic ublishers, The Netherlands.

Imhoff, J.F. (2001a). True marine and halophilic anoxygenic phototrophic bacteria. Arch. Microbiol. 176: 243-254.

Imhoff, J.F. (2001b). Transfer of *Rhodopseudomonas acidophila* to the new genus *Rhodoblastus* as *Rhodoblastus acidophilus* gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol., 51: 1863-1866.

Imhoff, J. F. and Trüper, H. G. (1989). The purple nonsulfur bacteria. In "Bergey's Manual of Systematic Bacteriology", Vol. 3, p. 1658-1661. (Staley, J. T., Bryant, M. P., Pfennig, N. and Holt, J. G. (eds)). Williams & Wilkins, Baltimore.

Imhoff, J.F. and Truper, H.G. (1980). *Chromatium purpuratum* sp. nov., a new species of the Chromatiaceae. Zentbl Bakteriol IAbt Orig C l , 61-69.

Imhoff, J.F., Suling, J. and Petri,R. (1998). Phylogenetic relationships among *Chromatiaceae*, their taxonomic reclassification and description of the new genera *Allochromatium, Halochromatium, Isochromatium, Marichromatium, Thiococcus, Thiohalocapsa and Thermochromatium*. Int.J.Syst.Bacteriol.48:1129–1143.

Imhoff, J.F. and Caumette, P. (2004). Recommended standards for the description of new species of anoxygenic phototrophic bacteria.

Imhoff, J.F., Petri, R. and Süling, J. (1998). Reclassification of species of the spiral-shaped phototrophic purple non-sulfur bacteria of the α-Proteobacteria: description of the new genera *Phaeospirillum* gen. nov., *Rhodovibrio* gen. nov., *Rhodothalassium* gen. nov. and *Roseospira* gen. nov. as well as transfer of *Rhodospirillum fulvum* to *Phaeospirillum fulvum* comb. nov., of *Rhodospirillum molischianum* to *Phaeospirillum molischianum* comb. nov., of *Rhodospirillum salinarum* to *Rhodovibrio salinarum* comb. nov., of *Rhodospirillum sodomense* to *Rhodovibrio sodomensis* comb. nov., of *Rhodospirillum salexigen*s to *Rhodothalassium salexigens* comb. nov. and of *Rhodospirillum mediosalinum* to *Roseospira mediosalina* comb. nov. Int. J. Syst. Bacteriol. 48: 793-798.

Jagjit. S. Y., Lawrence, D.L., Nuck, B.A., Federle, T.W. and Adinarayana C.R. (2001). Biotransformation of linear alkylbenzene sulfonate (LAS) by *Phanerochaete chrysosporium* : oxidation of alkyl side-chainBiodegradation 12: 443–453.

Johnston, J. B., Murray, K. and Cain, R.B. (1975). Microbial metabolism of aryl sulphonates. A reassessment of colorimetric methods for the determination of sulphite and their use in measuring desulphonation of aryl and alkylbenzene sulphonates. Antonie van Leeuwenhoek 41: 493-511.

Junker, F. and Cook, A.M. (1997). Characterization of the *p*-Toluenesulfonate Operon *tsaMBCD* and *tsaR* in *Comamonas testosteroni* T-2 Journal Of Bacteriology. 919–927.

Junker, F., Leisinger, T. and Cook, A.M. (1994). 3-Sulphocatechol 2,3-dioxygenase and other dioxygenases (EC 1.13.11.2 and EC 1.14.12.-) in the degradative pathways of 2-aminobenzenesulphonic, benzenesulphonic and 4-toluenesulphonic acids in

Alcaligenes sp. strain O-1.Microbiology. 140:1713-22.

Kamal, V. S., and R. C. Wyndham., (1990). Anaerobic phototrophic metabolism of 3-chlorobenzoate by *Rhodopseudomonas palustris* WS17. Appl. Environ. Microbiol. 56:3871–3873.

Kantachote, D., Salwa, T. and Kamontam, U. (2005). The potential use of anoxygenic photosynthetic bacteria for treating latex rubber sheet wastewater. Electron. J. Biotech. 8: 314-323.

Kasomu, I. B. M. and Obst, M. (2009). The influence of picocyanobacterial photosynthesis on calcite precipitation. Int. J. Environ. Sci. Tech. 6: 557-562.

Keck, A., Conradt, D., Mahler, A., Stolz, A., Mattes, R. and Klein, J. (2006). Identification and functional analysis of the genes for naphthalenesulfonate catabolism by *Sphingomonas xenophaga* BN6 Microbiology. 152: 1929-1940.

Kertesz , M.A., Cook A.M. and Leisinger. T. (1994). Microbial metabolism of sulfur and phosphorus-containing xenobiotics. FEMS Microbiology Reviews 15:195-215.

Kertesz, M. A. (2000). Riding the sulfur cycle—metabolism of sulfonates and sulfate esters in Gram-negative bacteria. FEMS Microbiol. Rev. 24:135-175.

Khanna, P., Rajkumar, B. and. Jothikumar, N. (1992). Anoxygenic degradation of aromatic substances by Rhodopseudomonas palustris. Curr. Microbiol. 26:1–9.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111-120.

Knappe, J., Neugebauer, F.A., Blaschkowski, H.P., Gänzler, M. (1984). Post-translational activation introduces a free radical into pyruvate formate-lyase. Proc Natl Acad Sci U S A. 81:1332-5.

Kneifel, H., Elmendorff, K., Eberhard H. and Soeder, C.J. (1997). Biotransformation of 1-naphthalenesulfonic acid by the green alga Scenedesmus obliquus. Arch Microbiol. 167 : 32–37.

Kniemeyer,O., Thomas, F.,2 Heinz, W.,Frank O.G. and Widdel.F.(2003).Anaerobic Degradation of Ethylbenzene by a New Type of Marine Sulfate-Reducing Bacterium Appl Environ Microbiol.69: 760–768.

Koechlin, B.A. (1954). The isolation and identification of the major anion fraction of the axoplasm of squid giant nerve fibers. Proc Natl Acad Sci USA 40:60–62.

Kondo, H., Yazawa, M., Enami, H. and Ishimoto, M. (1982). Sulphite production from benzenesulphonate by bacterial enzyme. (In Japanese) *Ganryu Aminosan* 5: 237-242.

Krieger, C. J., Roseboom, W.,. Albracht, S. P. and A. M. Spormann. (2001). A stable organic free radical in anaerobic benzylsuccinate synthase of *Azoarcus sp*. strain T. J. Biol. Chem. 276:12924-12927.

Krieger, C.J., Beller, H.R., Reinhard, M. and Spormann, A. M. (1999). Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus* sp. strain T. J Bacteriol. 181:6403–6410.

Krueger, C.J., Radakovich, K.M., Sawyer, T.E., Barber, L.B., Smith, R.L. and Field, J.A. (1998) Biodegradation of the surfactant linear alkylbenzenesulfonate in sewage contaminated groundwater: A comparison of column experiments and field tracer tests. Environ. Sci. Technol. 32: 3954-3961.

Krueger, C.J., Barber, L.B., Metge, D.W., and Field, J.A., 1998, Fate and transport of linear alkylbenzenesulfonate in a sewage-contaminated aquifer: A comparison of natural gradient tracer tests: Environ. Sci. Technol. 323: 1134-1142.

Kushalatha, M., Vidya, G. and Chandrakant, K. (2010). Photobiodegradation of halogenated aromatic pollutants Advances in Bioscience and Biotechnology. 1: 238-240.

Lakshmi, K. V. N. S., Sasikala, Ch. and Ramana Ch. V., (2009). *Rhodoplanes pokkaliisoli* sp. nov., a phototrophic alphaproteobacterium isolated from a waterlogged brackish paddy soil. Int J Syst Evol Microbiol 59, 2153-2157.

Lakshmi Prasuna, M., Mujahid, Md., Sasikala, Ch. and Ramana, Ch.V. (2012). l-Phenylalanine catabolism and l-phenyllactic acid production by a phototrophic bacterium, *Rubrivivax benzoatilyticus* JA2 Microbiological Research. 167:526–531.

Larimer, F.W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do,L., Miriam, L. L., Pelletier, D.A., Beatty, T., Lang, A.S., Tabita, F.R., Gibson, J.L., Hanson, T.E., Bobst, C., Torres, J.L.T., Peres, C., Harrison, F.H., Gibson, J. and Harwood, C.S. (2004). Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris* Nature Biotechnology 22: 55.

Leuthner, B., Leutwein, C., Schulz, H., Hörth, P., Haehnel, W., Schiltz, E., Schägger, H. and Heider J. (1998). Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol Microbiol. 28:615–628.

Leuthner, B. and J. Heider. (2000). Anaerobic toluene catabolism of *Thauera aromatica*: the *bbs* operon codes for enzymes of beta oxidation of the intermediate benzylsuccinate. J. Bacteriol. 182:272-277.

Lewis, M.A.,. Pittinger, C.A., Davidson D.H. and Ritchie. C.J. (1993). In Situ Response of natural periphyton to an anionic surfactant and an environmental risk assessment for phytotoxic effects. environmental toxicology and chemistry. Vol. 12(10): 1803-1812. Symposium on Surfactants and Their Environmental Safety, 11th Annual Meeting, Society of Environm Toxicology and Chemistry, Arlington, VA, Nov. 11-15, 1990.

Li, L., Patterson, D.P., Fox, C.C., Lin, B., Coschigano, P.W. and Marsh, E.N. (2009). Subunit structure of benzylsuccinate synthase. Biochemistry.17;48:1284-92.

Lie, T.L., Leadbetter, J.R. and Leadbetter, E.R. (1998) Metabolism of sulfonic acids and other organosulfur compounds by sulfate-reducing bacteria. Geomicrobiol. J. 15: 135- 149.

Lie, T.J., Pitta, T., Leadbetter, E.R., Godchaux, W. and Leadbetter, J.R. (1996). Sulfonates: novel electron acceptors in anaerobic respiration. Arch. Microbiol. 166: 204 210.

Locher,H.H., Leisinger, T. and Cook, A.M. (1989a) Degradation of p-Toluenesulphonic acid via sidechain oxidation, desulphonation and meta ring cleavage in *Pseudomonas* (*Comamonas*) *testosteroni* T-2 Journal of General Microbiology 135: 1969-1978.

Locher, H.H., Thurnheer, T., Leisinger, T., cook, A.M. (1989b). 3-Nitrobenzenesulfonic acid, 3- aminobenzenesulfonic acid and 4-aminobenzenesulfonic acid as sole carbon sources for bacteria. Applied and Environmental Microbiology. 55: 492-494.

Locher,H.H., Leisinger, T. and Cook, A.M. (1991). 4-Toluenesulfonate methyl-monooxygenase from *Comamonas testosteroni* T-2: purification and some properties of the oxygenase component Journal of bacteriology. 173: 3741-3748.

Locher, H. H., Leisinger, T. and Cook., A.M. (1991). 4-Sulphobenzoate 3,4-dioxygenase: purification and properties of a desulphonative two- component enzyme system from *Comamonas testosteroni* T-2. Biochem. J. 274:833-842.

Locher, H.H., Poolman, B., Cook, A.M. and Konings, W. (1993). Uptake of 4-Toluene Sulfonate by *Comamonas testosteroni* T-2 . journal of bacteriology. 175:1075-1080,

Lovely, D. R. and D. J. Lonergan. 1990. Anaerobic oxidation of toluene, phenol and *p*-cresol by dissimilatory iron-reducing organism GS-15. Appl. Environ. Microbiol. 56: 1858-1864.

Lovley, D. R., Giovannoni, S. J., White, D. C., Champine, J. E., Phillips, E. J., Gorby, Y. A. and. Goodwin. S. (1993). *Geobacter metallireducens gen. nov. sp. nov.*, a

microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Arch. Microbiol. 159:336-344.

Luther, M., Soeder, C.J. (1991). 1-Naphthalenesulfonic acid and sulfate as sulfur sources for the green alga *Scenedesmus obliquus*. Water Res. 25:299–307.

Madigan, M. T. and Gest, H. (1988). Selective enrichment and isolation of *Rhodopseudomonas palustris* using transcinnam.

Madukasi, E. I., Dai, X., He, C. and Zhou, J. (2010). Winter Potentials of phototrophic bacteria in treating pharmaceutical wastewater. Int. J. Environ. Sci. Tech., 7: 165-174.

Mampel, J., Ruff, J., Junker, F. and Cook, A.M. (1999). The oxygenase component of the 2-aminobenzenesulfonate dioxygenase system from *Alcaligenes* sp. strain O-1. Microbiology.145: 3255–3264.

Mampel, J. Maier, E., Tralau, T., Ruff, J., Benz, R. and Cook, A.M. (2004). A novel outer-membrane anion channel (porin) as part of a putatively two-component transport system for 4-toluenesulphonate in *Comamonas testosteroni* T-2. Biochem J. 383: 91-9.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3, 208-218.

Martin, E., Luc, Z., Daniel, H. and Schwarzenbach,R.P. (2005). A New Concept Linking Observable Stable Isotope Fractionation to Transformation Pathways of Organic PollutantsEnviron. Sci. Technol.39:6896–6916.

Mars, A. E., Houwing, J., Dolfing, J and Janssen, D.B. (1996). Degradation of Toluene and Trichloroethylene by *Burkholderia cepacia* G4 in Growth-Limited Fed-Batch Culture. Applied And Environmental Microbiology. 62:886–891.

Martelli, H.L, Benson, A.A. (1964). Sulfocarbohydrate metabolism. 1. Bacterial production and utilization of sulfoacetate. Biochim Biophys Acta 93:169–171.

McAvoy, D.C., Eckho, W.S. and Rapaport, R.A.., (1993). Fate of linear alkylbenzene sulfonate in the environment. Environ. Toxicol. Chem. 12: 977-987.

McGrath, J.E. and Harfoot, C. G. (1997). Reductive dehalogenation of halocarboxylic acids by phototrophic genera *Rhodospirillum* and *Rhodopseudomonas*. Appl. Environ. Microbiol. 63:3333–3335.

Mesbah,M., Premachandran, U. and Whitman, W.B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. Int. J.Syst. Bacteriol. 39:159–167.

Montgomery, L. and Vogel, T. M. (1992). Dechlorination of 2,3,5,6-tetrachlorobiphenyl by a phototrophic enrichment culture. FEMS Microbiol. Lett. 94:247–250.

Morris, R. M., Rappe, M. S., Urbach, E., Connon, S. A. and Giovannoni, S. J. (2004). Prevalence of the Chloroflexi-related SAR202 bacterioplankton cluster throughout the mesopelagic zone and deep ocean. Appl. Environ. Microbiol. 70: 2836-2842.

Mujahid, Md., Sasikala, Ch. and Ramana Ch. V. (2010). Aniline-Induced Tryptophan Production and Identification of Indole Derivatives from Three Purple Bacteria Curr Microbiol. 61:285–290.

Mujahid, Md., Sasikala, Ch. and. Ramana, Ch. V. (2011a). Production of indole-3-acetic acid and related indole derivatives from L-tryptophan by *Rubrivivax benzoatilyticus* JA2 Appl Microbiol Biotechnol 89:1001–1008.

Mujahid, Md., Arvind, I., Lakshmi Prasuna, M., Rama Prasad E.V.V., Sasikala, Ch. and Venkata Ramana, Ch. (2011b). Genome Sequence of the Phototrophic Betaproteobacterium *Rubrivivax benzoatilyticus* Strain JA2^{T} J. Bacteriol.193:2898.

Müller, J. A., Galushko, A. S., Kappler, A. and Schink, B. (1999). Anaerobic degradation of m-cresol by *Desulfobacterium cetonicum* is initiated by formation of 3-hydroxybenzylsuccinate. Arch Microbiol.172:287–294.

Müller. J., Galushko, A., Kappler, A. and Schink, B. (2001). Initiation of anaerobic degradation of pcresol by formation of 4-Hydroxybenzylsuccinate in *Desulfobacterium cetonicum*. J. Bac. 183: 752-757.

Muralikrishna, C. and Renganathan, V. (1993). Peroxidase-catalyzed desulfonation of 3,5-dimethyl-4-hydroxy and 3,5-dimethyl-4- aminobenzenesulfonic acids. Biochem Biophys Res Commun., 15;197:798-804.

Myung, K. K., Choi, K. M., Yin, C. R., Lee, K. Y., Im, W. T., Lim, J. H., Lee, S. T. (2004). Odorous swine wastewater treatment by purple non-sulfur bacteria, Rhodopseupdomonas Pulustris, isolated from eutrophicated ponds. Biotech Lett., 26: 819-822.

Nanda Devi, P., Sasikala, C. and Ramana, C.V. (2000). Light-dependent transformation of anthranilate to indole by *Rhodobacter sphaeroides* OU5 Journal of Industrial Microbiology & Biotechnology 24: 219–221.

Neil, M.J. (2001). International nonproprietary names (INN) for radicals and groups

proposed for pharmaceutical substances by the World Health Organization. In: The Merck index. Merck, Whitehorse Station.

Nortemann, B., Baumgartej, N., Rast, H. G. and Knackmushs, J. (1986). Bacterial communities degrading amino- and naphthalene-2-sulfonates. Appl. and Environ. Microbiol. 52: 195- 1202.

Novak, R.T.,. Gritzer, R. F. Edward, E.R., Leadbetter, R. and Godchaux. W. (2004). Phototrophic utilization of taurine by the purple nonsulfur bacteria Rhodopseudomonas palustris and *Rhodobacter sphaeroides.* Microbiology 150:1881–1891.

Ochman, H., Lawrence, J.G. and Groisman, E.A. (2000). Lateral gene transfer and the nature of bacterial innovation. Nature 405: 299–305.

Oda, Y., Larimer, B.F.W., Patrick S. G., Chainc., Malfattic, S., Maria V. S., Lisa M. V., Land, M.L., Braatsch, F.S., Beattyf, J. T., Pelletierb, D.A., Schaefera, A.L. and Harwooda,C.S. (2008). Multiple genome sequences reveal adaptations of a phototrophic bacterium to sediment microenvironments PNAS. 105: 18543–18548.

Ovenden, S.P.B. and Capon, R.J. (1999). Echinosulfonic acids A-C and echinosulfone A: novel bromoindole sulfonic acids and a sulfone from a southern Australian marine sponge, Echinodictyum. J Nat Prod 62:1246–1249.

Paszczynski, A., Pasti-Grigsby, M.B., Goszczynski, S., Crawford, R.L. and Crawford, D.L. (1992). Mineralization of sulfonated azo dyes and sulfanilic acid by Phanerochaete chrysosporium and Streptomyces chromofuscus. Appl Environ Microbiol. 58:3598-604.

Paul, V., Wietek, C., Kahnert, A., WuÈest, T. and Kertesz, A.M. (1999). Genetic organization of sulphur-controlled aryl desulphonation in Pseudomonas putida S-313 Molecular Microbiology 32:913-926.

Pfennig, N. (1975). The phototrophic bacteria and their role in the sulfur cycle, Plant Soil 43: 1–16.

Pfennig, N.,. Eimhjellen, N. E. and. Jensen, S. L. (1965). A new isolate of the *Rhodosprillum fulvum* group and its photosynthetic pigments. Arch. Microbiol. 51:258-266.

Pfennig, N. and Wagener, S. (1986). An improved method of preparing wet mounts for photomicrographs of microorganisms. J. Microbiol. Meth. 4: 303-306.

Pfennig, N. (1989). Ecology of phototrophic purple and green sulphur bacteria. In "Autotrophic Bacteria", (Schlegel, H. G., and Bowien, B.) 97–116.

Perei, K., Rakhely, G.,Kiss, I.,Polyak, B.and Kovacs, K.L.(2001)Biodegradation of sulfanilic acid by Pseudomonas paucimobilis.ApplMicrobiol Biotechnol 55:101-107.

Prasertsan, P., Jaturapornpipat, M. and Sirpatana, C. (1997). Utilization and treatment of tuna condensate by photosynthetic bacteria. Pure Appl. Chem. 69:2438- 2445.

Rabus, R. and F. Widdel. (1995). Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163:96-103.

Rabus, R., Nordhaus, R., Ludwig, W. and Widdel F (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Applied and Environmental Microbiology 59: 1444-1451.

Rahul, V.K., Akhil, N.K., Mayur, B. K. and Sanjay, P. G. (2011). Phytoremediation potential of Portulaca grandiflora Hook. (Moss-Rose) in degrading a sulfonated diazo reactive dye Navy Blue HE2R (Reactive Blue 172). Bioresource technology. 102:6774-7.

Ramana, Ch. V., Sasikala, Ch., Arunasri, K., Anil Kumar, P., Srinivas, T. N. R., Shivaji, S., Gupta, P., Suling, J. and Imhoff, J. F. (2006). *Rubrivivax benzoatilyticus* sp. nov., an aromatic, hydrocarbon-degrading purple betaproteobacterium International Journal of Systematic and Evolutionary Microbiology 56: 2157–2164.

Ranjith N. K., Ramana, Ch. V. Sasikala, Ch. (2007a). Catabolism of L-phenylalanine and L-tyrosine by Rhodobacter sphaeroides OU5 occurs through 3,4-dihydroxyphenylalanine Research in Microbiology 158 :506-511.

Ranjith, N.K., Sasikala, Ch. and Venkata Ramana, Ch. (2007b). Rhodethrin: a novel indole terpenoid ether produced by *Rhodobacter sphaeroides* has cytotoxic and phytohormonal activities Biotechnol Lett 29:1399–1402.

Ranjith N. K., Ramana, Ch. V. and Sasikala, Ch. (2010). L-Tryptophan catabolism by *Rubrivivax benzoatilyticus* JA2 occurs through indole 3-pyruvic acid pathway Biodegradation. 21:825–832.

Ranjith N. K., Ramana, Ch. V. Sasikala, Ch. (2011). Rubrivivaxin, a new cytotoxic and cyclooxygenase-I inhibitory metabolite from *Rubrivivax benzoatilyticus* JA2 World J Microbiol Biotechnol 27:11–16.

Riediker, S., Ruckstuhl, S., Suter, M.J.F., Cook, A.M. and Giger, W. (2000). p-Toluenesulfonate in Landfill Leachates: Leachability from Foundry Sands and Aerobic Biodegradation. Environmental science and technology 34: 2156-2161.

Ripin, M.J., Noon, K.F. and Cook, T.M. (1971). Bacterial Metabolism of Arylsulfonates.

Benzene Sulfonate as Growth Substrate for Pseudomonas testosteroni H-81Appl Microbiol. 21:495–499.

Romain Duc, Tomas, V., Petr, S. and Schwitzguébel, J.P. (1999). Accumulation and Transformation of Sulfonated Aromatic Compounds by Rhubarb Cells (*Rheum palmatum*) International Journal of Phytoremediation. 1:255-271.

Ronald, H.O., Jerome, J. K. and Kaphammer, B. (1994). A Novel Toluene-3-Monooxygenase Pathway Cloned from *Pseudomonas pickettii* PKO1. Journal Of Bacteriology.176:3749-3756.

Ruff, J., Hitzler, T., Rein, U., Ritter, A. and Cook, A.M. (1999). Bioavailability of water-polluting sulfoaromatic compounds. Appl. Microbial. Biotechnol. 52: 446-450.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.

Santos, S.R. and Ochman, H. (2004). Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. Environ. Microbiol. 6:754–759.

Sasikala, Ch.,. Ramana Ch. V. and Raghuveer Rao, P. (1994). Photometabolism of heterocyclic aromatic compounds by *Rhodopseudomonas palustris* OU 11 Appl. Environ. Microbiol., 60:2187-2190.

Sasser, M. (1990). Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids. Newark, DE: MIDI Inc.

Schmalenberger, A.A., Michael, A. and Kertesz, M.A. (2007). Desulfurization of aromatic sulfonates by rhizosphere bacteria: high diversity of the asfA gene. Environ Microbiol 9**:**535-45.

Schocher, R. J., Seyfried, B. Vazquez, F. and Zeyer. J. (1991). Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. 157:7- 12.

Schwitzguébel, J.P., Aubert, S., Grosse, W. and Laturnus, F. (2002). Sulphonated aromatic pollutants. Limits of microbial degradability and potential of phytoremediation. Environ Sci Pollut Res Int. 9:62-72.

Schulze, K. (1996). Der westeuropa¨ische Tensidmarkt 1994/1995. Tenside Surfactants Deterg. 33:94–95.

Schulz, S., W. Dong, U. Groth, and A. M. Cook., (2000). Enantiomeric degradation of 2-(4-sulfophenyl)butyrate via 4-sulfocatechol in Delftia acidovoransDelftia acidovorans SPB1. Appl. Environ. Microbiol. 66:1905-1910.

Schwitzguébel, J.P., Braillard, S., Valérie, P. and Sylvie, A. (2008). Sulfur assimilation and abiotic stress in plants.16: 335-354.

Serrano, W., Amann, R. and Fischer, U. (2009). A new moderately thermophilic and high sulphide tolerant biotype of Marichromatium gracile, isolated from tidal sediments of the German Wadden Sea: Marichromatium gracile biotype thermosulfidiphilum. Syst. Appl. Microbiol. 32:1–7.

Serrano, W., Amann, R., Rosello-Mora, R. and Fischer, U. (2010). Evaluation of the use of multilocus sequence analysis (MLSA) to resolve taxonomic conflicts within the genus Marichromatium. Syst. Appl. Microbiol. 33, 116–121.

Shcherbakova, V.A, Chuvilskaia, N.A., Golovchenko, N.P., Suzina, N.E., Lysenko, A.M., Laurinavichus, K.S. and Akimenko, V.K. (2003). Analysis of the anaerobic microbial community capable of degrading p-toluene sulphonate. Mikrobiologiia. 72:752-8.

Shiaris, MP. (1989.) Seasonal biotransformation of naphthalene, phenanthrene and benzo[a]pyrene in surficial estuarine sediments. App Environ Microbiol 55:1391–1399.

Shinoda, Y., Sakai, Y., Uenishi, H., Uchihashi, Y., Hiraishi, A., Yukawa, H., Hiroya., Y. and Nobuo, K. (2004). Aerobic and Anaerobic Toluene Degradation by a Newly Isolated Denitrifying Bacterium, Thauera sp. Strain DNT-1 Appl Environ Microbiol. 70: 1385–1392.

Shivali, K., Sasikala, Ch. and. Ramana, Ch.V (2012). MLSA barcoding of *Marichromatium* spp. and reclassification of *Marichromatium fluminis* (Sucharita et al., 2010) as *Phaeochromatium fluminis* gen. nov. comb. nov. Systematic and Applied Microbiology 35:221– 225.

Shivali, K., Venkata Ramana, V., Ramaprasad, E.V.V., Sasikala, Ch. and Ramana, Ch.V. (2011). *Marichromatium litoris* sp. nov. and *Marichromatium chrysaorae* sp. nov. isolated from beach sand and from a jelly fish (*Chrysaora colorata*) Systematic and Applied Microbiology 34:600– 605.

Singh, S., Singh, P., S. K. Anjali Pandey,A and Leela, I. (2008). Mineralization of 2-aminobenzenesulfonate by a bacterial Consortium World J Microbiol Biotechnol 24:841–847.

Singh, P., Mishra, L.C. and Iyengar, L. (2004). Biodegradation of 4-aminobenzenesulfonate by a newly isolated bacterial Strain PNS-1. W. J. Microbiol. Biotechnol. 20: 845–849.

Singh, P., Birkeland, N.K., Leela, I. And Ramanathan, G. (2006a). Mineralization of 4-aminobenzenesulfonate (4-ABS) by *Agrobacterium* sp. strain PNS-1 Biodegradation 17:495–502.

Singh, P., Mishra, L,C., Pandey, A. and Iyengar, L., (2006b). Degradation of 4-aminobenzenesulfonate by alginate encapsulated cells of *Agrobacterium* sp. PNS-1. Bioresour Technol. 97:1655-9.

Soeder, C. J., Hegewald, E. and Kneifel, H. (1987). Green micro algae can use naphthalenesulfonic acids as sources of sulfur.Arch Microbiol. 148:260- 263.

Song, Z., Suzanne, R., Edwards, and Richard, G. B. (2005). Biodegradation of naphthalene-2-sulfonic acid present in tannery wastewater by bacterial isolates *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC Biodegradation 16: 237–252.

Stöffler B, Luft G.Chemosphere., (1999). Oxidative degradation of p-toluenesulfonic acid using hydrogen peroxide. 38:1035-47.

Stöffler, B. and Luft, G. (1999) ;. Oxidative degradation of p-toluenesulfonic acid using hydrogen peroxide. Chemosphere.38:1035-47.

Stolz, A. (1999). Degradation of substituted naphthalenesulfonic acids by *Sphingomonas xenophaga* BN6. J Ind Microbiol Biotechnol. (4-5):391-399.

Stolz A. (2001). Jul;Basic and applied aspects in the microbial degradation of azo dyes. Appl Microbiol Biotechnol. 56:69-80.

Strzeszewski, B. (1913). Beitr¨agezurKenntnisderSchwefelfloraundder Umgebung vonKrakau.Bull.Acad.Sci.Cracov.Ser.B 309–334.

Sucharita, K., Shiva Kumar, S., Sasikala, C., Panda, B.B., Takaichi, S., Ramana, C.V., (2010). Marichromatium fluminis sp. nov., a slightly alkaliphilic, phototrophic gamma proteobacterium isolated from river sediment. Int. J. Syst. Evol. Microbiol. 60, 1103–1107.

Sunayana, M. R., Sasikala, Ch.and Ramana, Ch. V. (2005a). Production of a novel indole ester from 2-aminobenzoate by Rhodobacter sphaeroides OU5 J Ind Microbiol Biotechnol 32: 41–45.

Sunayana, M.R., Sasikala, Ch. and Ramana, Ch.V. (2005b). Rhodestrin: a novel indole terpenoid phytohormone from Rhodobacter Sphaeroides Biotechnology Letters 27: 1897–1900.

Sun, X., Harder, J., Krook, M., Sjoberg, B.M. and Reichard, P. (1993). A possible glycine radical in anaerobic ribonucleotide reductase from Escherichia coli: Nucleotide sequence of the cloned nrdD gene (ribonudeoside-triphosphate reductase/adcal

enymogy/Fnr binding site/pyruvate formate-lyase/bacteriophage T4 sunY gene). Proc. Nati. Acad. Sci. Biochemistry. 90 :577-581.

Tabor, C.F. and Barber, L.B. (1996). Fate of linear alkylbenzene sulfonate in the Mississippi River. Environ. Sci. Technol. 30, 161- 171.

Takewaki, S., Okusumi, K., Manabe, I., Tanimura, M., Miyamura, K., Nakahara, K., Yazaki,Y., Ohkubo, A. and Nagai, R. (1994). Nucleotide sequence comparison of the mycobacterial dnaJ gene and PCR-restriction fragment length poly- morphism analysis for identification of M*ycobacterial* species. Int.J.Syt. Bacteriol. 44,159–166.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.

Tan, N.G.C., van, Leeuwen, A., van Voorthuizen, E.M., Slenders, P., Prenafeta-Boldu, F.X., Temmink, H., Lettinga, G., Field, J.A., 2005. Fate and biodegradability of sulfonated aromatic amines. Biodegradation. 16: 527-537.

Thiel, V., Tank, M., Neulinger, S. C., Gehrmann, L., Dorador, C. and Imhoff, J. F., (2010). Unique communities of anoxygenic phototrophic bacteria in saline lakes of Salar de Atacama (Chile): evidence for a new phylogenetic lineage of phototrophic Gammaproteobacteria from pufLM gene analyses. FEMS Microbiol. Ecol. 74:510-522.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G., (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Thurnheer, T., Kohler, T ., Cook, A. M. and Leisinger, T., (1986). Orthanilic acid and analogues as carbon sources for bacteria: growth physiology and enzymic desulphonation. Journal of General Microbiology 132:1215-1220.

Thurnheer, T., Cook, A.M. and Leisinger, T. (1988) Co-culture of bacteria to degrade seven sulfonated aromatic compounds: e¤ciency, rates and phenotypic variations. Appl. Microbiol. Biotechnol. 29:605-609.

Thurnheer, T., Zürrer, D., Höglinger, O., Leisinger, T. and Cook, A.M. (1990). Initial steps in the degradation of benzene sulfonic acid, 4-toluene sulfonic acids, and orthanilic acid in *Alcaligenes* sp. strain O-1.Biodegradation. 1:55-64.

Tourova T. P. and Antonov, A. S. (1987). Identification of microorganisms by rapid DNA-DNA hybridization. Meth. Microbiol. 19:333-355.

Tralau, T., Cook, A.M. and Ruff, J. (2001). Map of the IncP1beta plasmid pTSA encoding the widespread genes (tsa) for p-toluenesulfonate degradation in *Comamonas testosteroni* T-2. Appl Environ Microbiol. 67:1508-16.

Tralau, T., Yang, E.C., Tralau, C., Cook, A.M. and Küpper, F.C.(2011). Why two are not enough: degradation of p-toluenesulfonate by a bacterial community from a pristine site in Moorea, French Polynesia. FEMS Microbiol Lett. 316:123-9.

Trüper, H. G. and Pfennig, N., (1981). Isolation of members of the families Chromatiaceae and Chlorobiaceae. In "the Prokaryotes: a Handbook on Habitats, Isolation, and Identification of Bacteria", (Starr, M. P., Stolp, H., Tru¨per, H. G., Balows, A. and Schlegel., H. G. (eds)), p. 279-289. Berlin: Springer.

Uchihashi, K., Misawa, T., Takeo, M. and Negoro, S. (2003). Mutational analysis of the metabolism of 2,6-naphthalenedisulfonate by *Pigmentiphaga* sp. NDS-2. Journal of Bioscience and Bioengineering. 95:476-482.

Usha, P., Sasikala, Ch. and Ramana, Ch.V. (2007). Light-Dependent Assimilation of trans-Cinnamate by *Rhodobacter sphaeroides* OU5. Current Microbiology. 54:410–413.

Vairavamurthy, M.A., Zhou, W., Eglinton, T. and Manowitz, B. (1994). Sulfonates: a novel class of organic sulfur compounds in marine sediments. Geochimica and Cosmochimica Acta 58, 4681–4687.

Valérie, Schwitzguébel and Jean-Paul. (2009). Metabolism of sulphonated anthraquinones in rhubarb, maize and celery: the role of cytochromes P450 and peroxidases Source: Plant Cell Reports, 28: 1725-1735.

Valli, N.C., Vijayalakshmi, K., Muralidharan, D. and Suseela R.G. (2007). Mineralization of metanilic acid by Pseudomonas aeruginosa CLRI BL22 World Journal of Microbiology & Biotechnology - world j microbiol biotechnol. 23:1733-1738.

Verfurth, K., A. Pierik, C. Leutwein, S. Zorn, and J. Heider. (2004). Substrate specificities and electron paramagnetic resonance properties of benzylsuccinate synthases in anaerobic toluene and *m*-xylene metabolism. Arch. Microbiol. 181:155-162.

Vijay Shanker, Sunayana, M.R., Ranjith N. K., Sasikala, Ch. and Ramana, Ch. (2006). Light-Dependent Transformation of Aniline to Indole Esters by the Purple Bacterium *Rhodobacter sphaeroides* OU5 current microbiology. 52: 413–417.

Wang, L.T., Lee, F.L., Tai, C.J. and Kasai, H. (2007). Comparison of gyrB gene sequences, 16SrRNA gene sequences and DNA-DNA hybridization in the Bacillus subtilis group. Int.J.Syst.Evol.Microbiol.57:1846–1850.

Wanga,Y.Q., Zhanga, J.S., Zhoua, J.T. and Zhangb. Z.P. (2009) Biodegradation of 4-aminobenzenesulfonate by a novel *Pannonibacter* sp. W1 isolated from activated sludge Journal of Hazardous Materials. 169 :1163–1167.

Washer, E. and Edwards, A. (2007). Identification and Expression of Benzylsuccinate Synthase Genes in a Toluene-Degrading Methanogenic Consortium *Appl. Environ. Microbiol* 73:1367-69.

Weelink, S.A, Van Doesburg, W., Saia, F.T., Rijpstra, W.I., Röling, W.F., Smidt, H. and Stams, A.J.A. (2009). Strictly anaerobic betaproteobacterium *Georgfuchsia toluolica* gen. nov., sp. nov. degrades aromatic compounds with Fe(III), Mn(IV) or nitrate as an electron acceptor. FEMS Microbiol Ecol. 70:575-85.

White, R.H. (1986). Intermediates in the biosynthesis of coenzyme M (2-mercaptoethanesulfonic acid). Biochemistry. 25:5304- 5308.

Whited, G. M., and D. T. Gibson. 1991. Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to p-cresol in *Pseudomonas mendocina* KR1. J. Bacteriol. 173:3010-3016.

Winderl, C., Schaefer, S. and Lueders, T. (2007). Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (bssA) genes as a functional marker. Environ Microbiol. 9:1035-46.

Wittich, R. M., Rast, H. G. and Knackmuss, H.J. (1988). Degradation of naphthalene-2,6- and naphthalene-1,6-disulfonic acid by a *Moraxella* sp. Appl Environ Microbiol 54, 1842–1847.

Worsey, M. J. and. Williams. P. A. (1975). Metabolism of toluene and xylenes by *Pseudomonas putida* (arviUa) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. 124:7-13.

Wright, G. E. and Madigan, M. T. (1991). Photocatabolism of aromatic compounds by the phototrophic purple bacterium *Rhodomicrobium vannielii.* Appl. Environ. Microbiol. 57:2069- 2073.

Yamanaka, K., Moriyama, M., Minoshima, R. and Tsuyuki, Y. (1983).Isolation and Characterization of a methanol-utilizing phototropic bacterium, *Rhodopseudomonas acidophila* M402 and its growth on vanillin derivatives. Agric and Biol Chem 47:1257–1267.

Yutin, N., Suzuki, M. T., Teeling, H., Weber, M., Venter, J. C., Rusch, D. B. and Béjà, O. (2007). Assessing diversity and biogeography of aerobic anoxygenic phototrophic

bacteria in surface waters of the Atlantic and Pacific Oceans using the Global Ocean Sampling expedition metagenomes. Environ. Microbiol. 9: 1464-1475.

Zaar, A., Fuchs, G., Golecki, J.R. and Overmann, J. (2003). Anew purple sulfur bacterium isolated from a littoral microbial mat, *Thiorhodococcus drewsii*, sp. nov., Arch.Microbiol. 179: 174–183.

Zahra, Y., Besharati, H. and Fallah, A.R. (2012). Survey effect of salinity and different amounts of nitrogen on performance of isolated petroleum–degrading bacteria from bousher province soil 3International Journal of Agriculture: Research and Review.2: 879-885.

Zengler, K., Heider, J., Rossello-Mora, R. and Widdel, F. (1999). Phototrophic utilization of toluene under anoxic conditions by a new strain of *Blastochloris sulfoviridis*. Archives of Microbiology. 172: 204-12.

Zerbinati, O., Vincenti, M., Pittavino, S. and Gennaro, M.C. (1997). Fate of aromatic sulfonates in fluvial environment. Chemosphere 35: 2295-2305.

Zurrer, D., Cook, A. M. and Leisinger, T. (1987). Microbial desulfonation of substituted naphthalenesulfonic acids and benzenesulfonic acids. Appl. Environ. Microbiol.53:1459-1463.

Zylstra, G.J., Richard Mccombie,W., Gibson, D.T and Finette, B.A.(1988). Toluene Degradation by Pseudomonas putida Fl: Genetic Organization of the tod Operon. Appl. Environ. Microbiol.54:1498-1503.