

Anaerobic Biodegradation of Three Aromatic Sulfur Compounds by *Desulfovibrio Psychrotolerans* JS1^T, in Soil and Sludge Microcosms



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Abstract

The sulfate reducing bacterium, *Desulfovibrio psychrotolerans*, JS1^T could biodegrade three aromatic Sulphur compounds namely P-Toluene Sulfonic acid (PTSA), Sulfanilic acid (SFA) and Thiophene - 2 -acetic acid (TPA) when provided as sole source of carbon under strict anaerobic conditions. The strain could grow in 25 mM and tolerate up to 50 mM of all the three test compounds supplemented as sole carbon source with optimum growth at 3 or 4 mM. Though none of the tested compounds were completely metabolized within the experimental period of three months, PTSA was degraded up to 82%, SFA up to 65.5% and TPA was degraded up to 72% in liquid culture. The soil and sludge microcosm studies revealed that strain JCM14597^T could degrade the test compounds more efficiently as pure culture when compared to that with its consortium, with role of native microorganisms being insignificant. The biodegradation of PTSA & TPA was significantly reduced in sludge microcosm than in soil while SFA degradation was similar in both soil and sludge microcosms. The present work in laboratory scale is a preliminary study conducted at ambient conditions of naturally occurring soils and sludge and thus indicates the potential of *D. psychrotolerans*, strain JCM14597^T for biodegradation of contaminated soils and sludge. To the best of our knowledge, this is the first report of a sulfate reducing bacterium capable of degrading three aromatic Sulphur compounds under anaerobic conditions and the isolate will be potentially useful in bioremediation of contaminated soils.

Keywords: Anaerobic biodegradation; Aromatic sulphur compounds; Microcosm studies; Sulfate reducing bacteria

Abbreviations: SRB: Sulfate Reducing Bacteria; PTSA: Para-Toluene Sulfonic Acid; SFA: Sulfanilic Acid; TPA: Thiophene-2-acetic acid

Introduction

Aromatic compounds form the second largest group of organic compounds in nature after carbohydrates [1]. Hazardous aromatic compounds get into the environment in the form of diverse detergents, with oil spills, sewage from petroleum refineries and chemical plants, and with municipal waste waters. Many environments are anoxic or rapidly become anoxic due to contamination with carbon rich compounds like wastes from industrial effluents, gasoline, crude oil etc [2]. Removal of such recalcitrant compounds becomes important as these potentially hazardous molecules may enter into drinking water supplies.

As a major part of natural environment has little or no access to atmospheric oxygen, anaerobic microbes hold a major role in the processing of the nutrient cycles in nature and also in waste treatment plants where the aerobic processes may not completely remove aromatic compounds, turning researchers' interest to the study of the anaerobic metabolism of these compounds [3]. In this regard, Sulfate Reducing Bacteria (SRB) have been extensively recognized and studied due to their ubiquitous distribution and capability in anaerobic biodegradation and biotransformation of a

number of environmental pollutants under sulfate reducing conditions [4].

The aim of the present investigation was to study the ability of a sulfate reducing bacterium reported from our lab, *Desulfovibrio psychrotolerans*, strain JS1^T [5] to degrade three aromatic sulfur compounds namely Para-Toluene Sulfonic Acid (PTSA), sulfanilic acid (SFA) and thiophene-2-acetic acid (TPA) under strict anaerobic conditions. These pollutants enter into environment through effluents from textile, dye and chemical industries and petroleum products. The aerobic degradation pathway of toluene was demonstrated in *Pseudomonas testosterone* [6]. A 40% degradation of sulfanilic acid under aerobic conditions by fungal strains *Phanerocheate chrysosporium* [7] and *Aspergillus niger* RH19 [8] were demonstrated. *Sphingomonas subartica* strain was reported to utilize sulfanilic acid as sole carbon, nitrogen and sulfur source indicating its degradation by the strain [9]. Aerobic microbiological conversion of thiophenes has been studied extensively [10,11]. However, very little information is available concerning the anaerobic conversion of these aromatic sulfur compounds.

As the true fulfillment of any laboratory studies on pollutant biodegradation is accomplished only when they are applied on field for bioremediation of contaminated sites, microcosm studies on the degradation of the test compounds were conducted in sludge and soil microcosms to understand the effects of physico-chemical properties of sludge and soil and other biological parameters on the survival and growth of strain JS1^T and subsequently on the degradation of test compounds before switching on to on-site studies. The present study demonstrating the degradation of aromatic sulfur compounds by *D. psychrotolerans*, JS1^T in liquid culture and in microcosms is the first such study of degradation, by any pure culture of SRB.

Materials and Methods

Growth medium for degradation studies

The pure culture of *D. psychrotolerans* strain JS1^T was grown in Postgate's B medium (PBM) [12] consisting of (g L⁻¹) KH₂PO₄·7H₂O, 0.5; NH₄Cl, 1.0; Na₂SO₄, 4.5; CaCl₂·2H₂O, 0.06, MgSO₄·7H₂O, 2.0, Yeast extract, 1.0, FeSO₄·7H₂O, 0.004, Sodium citrate, 0.3, Sodium Lactate, 3.5, Sodium ascorbate solution (1M), 1 mL and Na₂S₉H₂O solution (1M), 1mL. A one percent (v/v) inoculum of pure culture of strain JS1^T washed twice in sterile saline, centrifuged and the culture pellet was inoculated into Postgate's B medium with the aromatic sulfur test compounds namely, Para-Toluene Sulfonic Acid [PTSA], Sulfanilic acid [SFA] and Thiophene 2-Acetic acid [TPA] supplemented as either sole source of Carbon (3mM) replacing lactic acid or electron acceptor (0.5mM) replacing FeSO₄ respectively in 7.5 mL screw capped tubes incubated at 30±2 °C. The culture was frequently sub cultured in the same medium and used for various biodegradation studies mentioned in the present report. Turbidometry was used to monitor the growth of *D. psychrotolerans*, strain JS1^T. Increase in optical density (OD) (turbidity) was measured for every 24 h starting from time 0 h to 96 h. Optical density of the bacterial suspension was directly measured in a Systronics make (model 112) colorimeter at 540 nm (filter 7) against un-inoculated medium as blank.

Maximum biodegradability and biosorption test

Different concentrations of the test compounds (i.e. 0, 1, 2, 3, 4, 5, 10, 25 and 50 mM) were supplemented as sole carbon source replacing lactic acid and growth as increase in optical density was measured colorimetrically at 24 h interval starting from time 0 h (i.e.0, 24,48,72, 96 and 120 h). For reading the degradation of the test compounds, 2 mL of the liquid culture was taken in 2.0 mL Eppendorf tube, centrifuged at 5008 g for 15 minutes, 0.5 mL of the culture supernatant was diluted 10 times with deionized water. The diluted sample was measured for absorbance at the λ maxima of 229nm for PTSA, 280nm for SFA and TPA on a Spectron Genesys 2 Spectrophotometer. Cells were simultaneously observed under phase contrast light microscope (Olympus BH-2) for viability from each test sample.

Test to rule-out biosorption of test compounds by JS1^T

In order to see whether the aromatic sulfur compounds PTSA, SFA and TPA under test were being adsorbed or truly utilized

by the strain JS1^T, live and dead (heat killed) culture/biomass of strain JS1^T were separately inoculated into Postgate's B medium supplemented with 3mM of the test compound as sole carbon source and incubated at 30±2 °C. Growth in terms of increase in OD was measured colorimetrically and concentrations of each of the test compounds were estimated by U.V. absorption at their respective absorption maxima of the test compounds.

HPLC analysis for biodegradation of test compounds

The HPLC analysis of aromatic sulfur test compounds, PTSA, SFA and TPA in culture supernatants was performed at room temperature using a Shimadzu SPD-10AVP isocratic system. Luna 5 μ C18 (2) 100A column (250 x 4.6 mm) was used for the detection of metabolites in a UV-VIS detector. SFA and TSA were detected in a solvent system containing methanol: Potassium Phosphate buffer 0.05M at pH 6.5 (40:60) at 1.0 mL.min⁻¹ flow rate with the detection done at 280nm. The PTSA was detected in a solvent system containing methanol water (40:60) at 1.0 mL.min⁻¹ flow rate with the detection done at 229nm. The retention times (TR in minutes) of SFA, PTSA and TPA were 2.6, 3.2 and 3.4 respectively in their specific solvent systems and flow rates as mentioned above. The degradation/disappearance of three aromatic sulfur compounds PTSA, SFA and TPA by the strain JS1^T was determined though HPLC at regular intervals from 0 h to 15 days of incubation. Three months (90 days) old inoculated sample was also analyzed for each test compound.

Soil and Sludge microcosm studies for biodegradation

The microcosm studies were carried out in soil collected from JNTU (Jawaharlal Nehru Technological University) campus and anaerobic sludge collected from the sedimentation tank of JETL (Jeedimetla Effluent Treatment Limited), Hyderabad. The sludge used in this experiment had a pH of 7.58, EC (Electrical conductivity) of 16.42 μ S, alkalinity of 440 mg L⁻¹, COD of 4200 mg L⁻¹ (for 10 times diluted sample), TDS (Total Dissolved Solids) of 4308 mg L⁻¹. These parameters were analyzed according to the methods suggested in the standard methods for determination of water and wastewater. The soil used had a pH of 7.2. The carbon, nitrogen, hydrogen and sulfur content in the soil were analyzed by Elementar make CHNS analyzer comprising Vario Micro software. The soil contained 1.8% organic carbon, 0.2% hydrogen, 0.4% nitrogen and 0.02% sulfur.

The strain JS1^T grown in PBM supplemented with 3 mM of the test compound as sole source of carbon was centrifuged and the cell pellet was suspended in the PBM without any carbon source. This was used as inoculum in the soil and sludge microcosms spiked with the test compound. Microcosms were carried out in test tubes (25 X 150 mm) with 20g soil and/or 20 ml sludge spiked with one aromatic sulfur test compound in each at 3 mM concentration supplemented with 2 ml PBM without any carbon source in two sets of tubes. One set was autoclaved which indicated the degradation of test compound by the inoculated strain JS1^T alone and another set was left unautoclaved to assess the degradation of the test compound by the native microorganisms

if any. Within the autoclaved and unautoclaved sets of tubes, one set was inoculated with 2 ml of the strain JS1^T and another was left uninoculated. The culture was thoroughly mixed with the soil and sludge, then closed with rubber seal followed by flushed with Argon gas to maintain anaerobic conditions and incubated at 28 ± 2 °C for 20 days. Soil and sludge samples (1g and/or 1mL) were drawn and analyzed for concentrations of each test compound at 5 days intervals.

Results

Growth of JS1^T on aromatic sulfur compounds

For extraction of PTSA, SFA and TPA from soil and for sludge, 1g of soil or sludge was suspended in 10ml of deionized water and vortexed thoroughly followed by centrifugation at 7845 g for 10 min. The supernatant was extracted and diluted 10 times (10 µL supernatant in 90 µL deionized water) and was analyzed by HPLC as mentioned in the previous segment. All experiments carried out in triplicate. Numerical data are presented as mean ± SD.

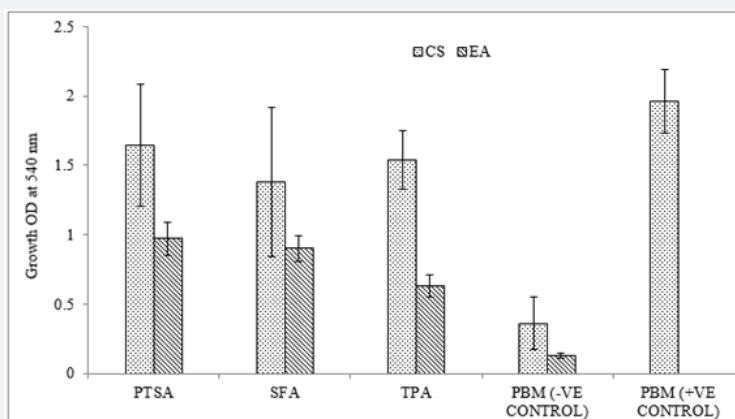


Figure 1: Growth of *Desulfovibrio psychrotolerans* strain JS1^T on the three aromatic sulfur compounds supplemented as either sole carbon source or electron acceptor source with reference to the positive and negative controls.

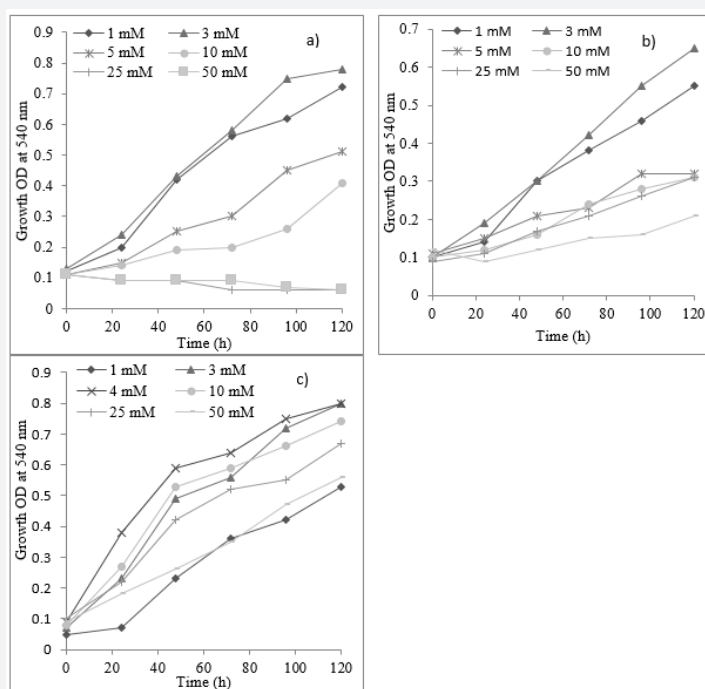


Figure 2: Effect of various concentrations of aromatic sulfur test compounds on the growth of *D. psychrotolerans* strain JS1^T (a) para-toluene sulfonic acid, (b) sulfanilic acid and (c) thiophene-2-acetic acid.

Strain JS1^T showed a gradual increase in growth within the tested period of 10 days in all the three aromatic sulfur test compounds. Growth in terms of OD at 540 nm was maximum (1.98) in

positive control where PBM (with 3 mM lactate as carbon source and 0.5 mM FeSO₄ as electron acceptor) was used and was minimum (0.1 & 0.3) where PBM without any carbon or electron ac-

ceptor source was used respectively. Growth of JS1^T was comparatively more in medium with the test compound supplemented as Carbon Source (CS) than as Electron Acceptor (EA). The decreasing order of growth in terms of OD at 540 nm after 10 days of incubation of JS1^T in the test compounds was PTSA-CS (1.64) > TPA-CS (1.54) > SFA-CS (1.38) > PTSA-EA (0.97) > SFA-EA (0.90) > TPA-EA (0.63) (Figure 1).

The effect of various concentrations from 0 mM to 50 mM of each of the three aromatic sulfur test compounds, i.e., PTSA, SFA and TPA when given as sole carbon source on the growth of strain JS1^T within a time course of 5 days showed that the strain JS1^T grew optimally in all three test compounds at 3 or 4 mM concentration. Growth was feeble at and above 25 mM concentration of PTSA, while the strain could tolerate up to 50 mM concentration of SFA and TPA (Figure 2).

Degradation of Aromatic sulfur compounds by live and dead biomass of strain JS1^T

In the experiment conducted with live and dead biomass of JS1^T to understand the loss of each test compound in the course of degradation, due to passive adsorption, if any, it was observed that the compound adsorbed was ignorable in all the three cases. There was no decrease in concentration of the test compounds inoculated with dead biomass of JS1^T until 5 days of observation. Among the three test compounds, 42% of PTSA (Figure 3-a), 47% of SFA (Figure 3-b) and 49% of TPA (Figure 3-c) were degraded after 5 days of incubation by the strain. None of the tested compounds were completely degraded by strain JS1^T within 5 days of incubation. The compounds PTSA and SFA were gradually degraded up to day 5 while the degradation of TPA was rapid up to day 2 after inoculation and then the degradation slowed down gradually till the end of day 5 (Figure 3).

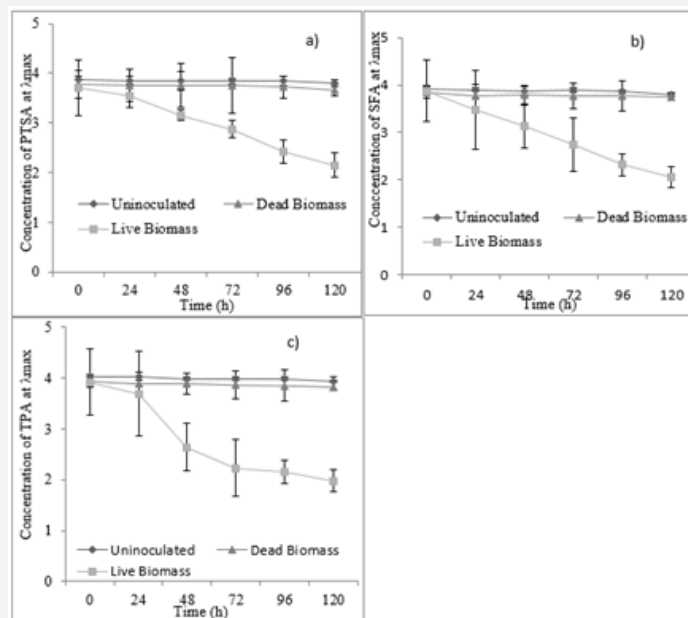


Figure 3: Degradation of aromatic sulfur compounds by the live and dead (heat killed) biomass of *Desulfovibrio psychrotolerans* strain JS1^T (a) para-toluene sulfonic acid, (b) sulfanilic acid and (c) thiophene-2-acetic acid.

HPLC determination of degradation of aromatic sulfur compounds

The HPLC analysis of degradation of the aromatic sulfur test compounds by the strain JS1^T showed a decrease of PTSA by 13%, 34% and 60% after 2, 4 and 15 days of incubation respectively and a maximum decrease of 83% after 90 days of incubation. Neither additional peaks nor peak shift was observed up to 15 minutes of run of the test compound in HPLC (Figure 4-a). A decrease in concentration of SFA by 10%, 20% and 33% after 2, 4 and 15 days of incubation respectively and a maximum decrease of 75% were observed after 90 days of incubation. The decrease in the SFA peak (T_R 2.6) was associated with an increase in an unknown peak (T_R 3.0). But the increase in the unknown peak was not proportional to the decreasing SFA peak (Figure 4-b). A decrease in concentration of TPA was noted by 11%, 23% and 47% after 2, 4

and 15 days of incubation respectively and a maximum decrease of 68% was observed after 90 days of incubation. The decrease in the TPA peak (T_R 3.4) was associated with an increase in an unknown peak (T_R 2.6). But this was not proportional to the decrease in TPA peak (Figure 4-c). None of the test compounds were degraded completely within the tested period of three months.

Degradation of the selected aromatic sulfur compounds by *Desulfovibrio psychrotolerans*, JS1^T

The degradation of aromatic sulfur compounds in sludge microcosms due to the individual and combined activities of *D. psychrotolerans*, JS1^T and the native microorganisms (if any, present in the sludge sample) analyzed through HPLC observed after 10 days of incubation revealed a degradation of PTSA up to 38% by strain JS1^T alone (Figure 5-1a) and up to 47% by the combined

activity of JS1^T and native microbes of the sludge (Figure 5-1b). SFA was degraded up to 64% by strain JS1^T alone (Figure 5-2a) and 58% by the combined activity of JS1^T and native microbes of the sludge (Figure 5-2b). TPA was degraded up to 31% by strain JS1^T alone (Figure 5-3a) and 48% degradation was observed by

the combined activity of JS1^T and native microbes of the sludge (Figure 5-3b). However, no significant decrease in concentrations of PTSA, SFA and TPA was observed in uninoculated sludge samples either autoclaved or unautoclaved (Figure 5).

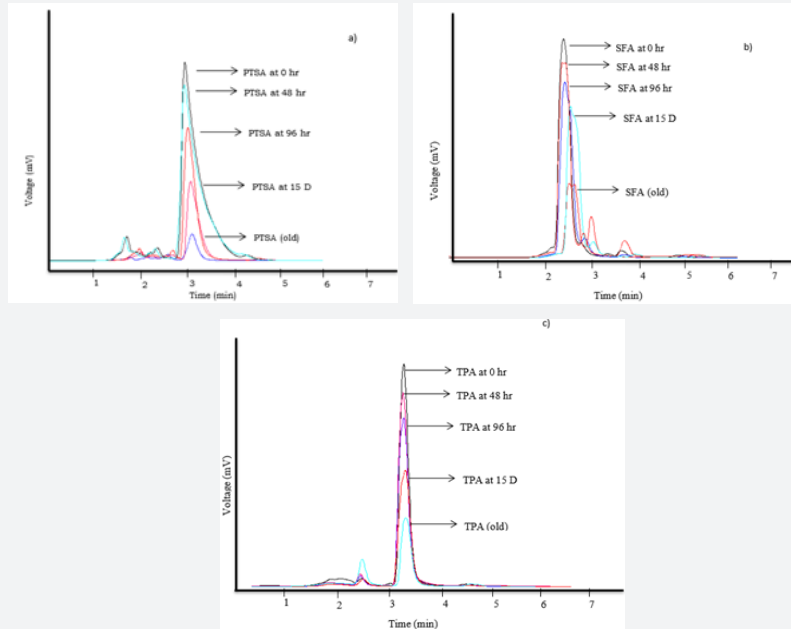


Figure 4: Overlay of HPLC chromatograms showing the degradation of three aromatic sulfur compounds by *D. psychrotolerans* strain JS1^T a) para-toluene sulfonic acid, (b) sulfanilic acid and (c) thiophene-2-acetic acid.

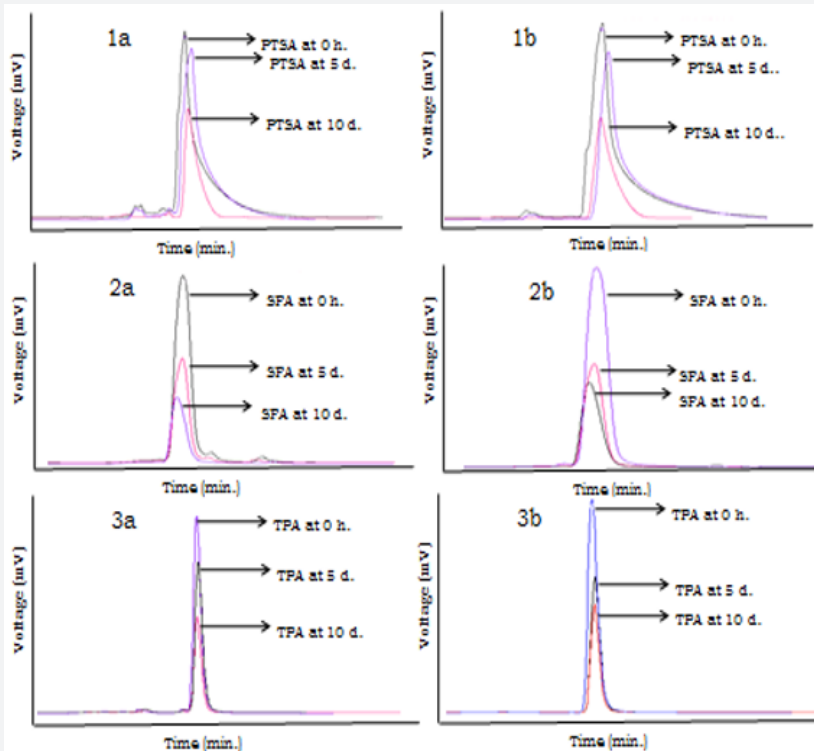


Figure 5: Overlay of HPLC chromatograms showing the degradation of the three aromatic sulfur test compounds by *Desulfovibrio psychrotolerans* strain JS1^T in sludge microcosms.

Similarly, the degradation of aromatic sulfur compounds in soil microcosms revealed a PTSA degradation of up to 66% by strain JS1^T alone (Figure 6-1a) and 63% degradation by the consortium of JS1^T and native microbes of the soil (Figure 6-1b). SFA was degraded up to 65% by strain JS1^T alone (Figure 6-2a) and 40% degradation was observed by the consortium of JS1^T and na-

tive microbes of the soil (Figure 6-2b). TPA was degraded up to 72% by strain JS1^T alone (Figure 6-3a) while only 30% degradation was observed by the consortium of JS1^T and native microbes of the soil (Figure 6-3b). However, no significant decrease in concentrations of PTSA, SFA and TPA was observed in uninoculated soil samples either autoclaved or unautoclaved (Figure 6).

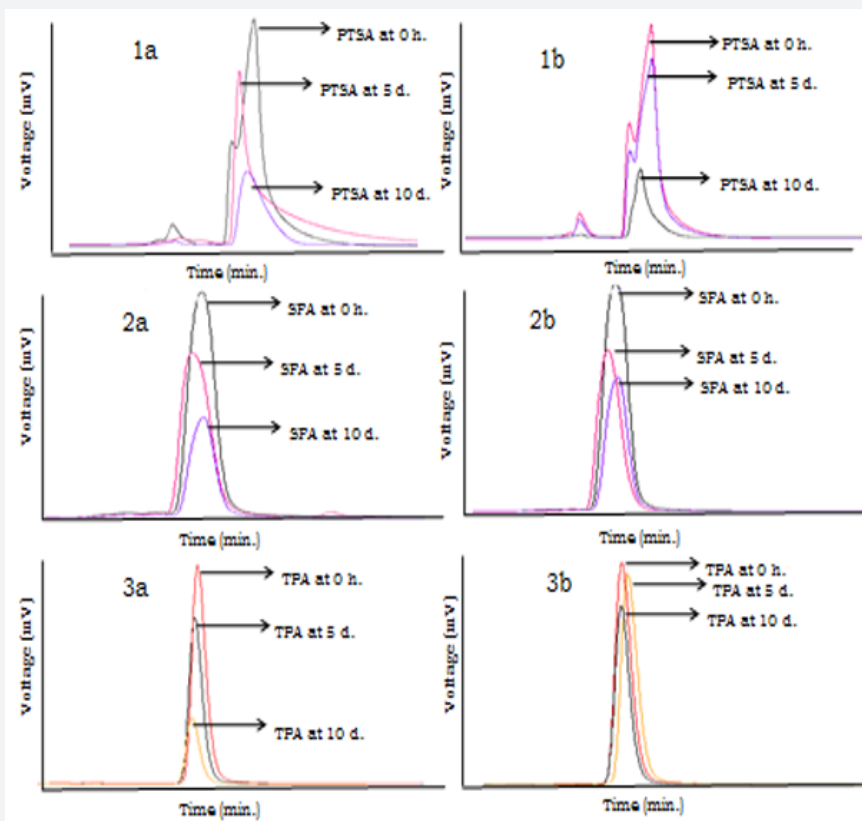


Figure 6: Overlay of HPLC chromatograms showing the degradation of the three aromatic sulfur test compounds by *Desulfovibrio psychrotolerans* strain JS1^T in soil microcosms.

Discussion

Sulfate reducing bacteria are phylogenetically and physiologically diverse group of bacteria, characterized by their versatile metabolic capabilities to use various electron acceptors and donors [13,14]. They are generally considered as the terminal oxidizers in the natural recycling of organic compounds to CO₂ in anoxic environments. Due to these exceptional capabilities, SRB are studied and applied in various bio-degradative tasks under anoxic regions. In the present study, three test compounds were selected for biodegradation studies, namely Para-Toluene Sulfonic Acid (PTSA), Sulfanilic acid (SFA) and Thiophene-2-acetic acid (TPA). These pollutants enter into environment through effluents from textile, dye and chemical industries and petroleum products. These compounds were used as sole sulfur source or as sole carbon/electron donor source in many previous reports [15-17]. In the present study, the biodegradability of these compounds by strain JS1^T was tested by supplementing them as either sole carbon source and/or sole electron acceptor. Shcherbakova, et al. [18] reported that a sulfate reducing strain *Desulfovibrio* sp. SR1

utilized PTSA as an electron acceptor. PTSA and SFA have reducible sulfur moiety in the chemical structure, and hence can also serve as electron acceptor in addition to being utilized as carbon source. TPA on the other hand was reported to undergo degradation in soil under oxygen limiting conditions when no other external electron acceptor was added and dibenzothiophene served as an electron acceptor in anaerobic respiration in a report by Annweiler, et al. [19]. Hence the degradation of TPA was also tested in the absence of any electron acceptor. *Desulfovibrio psychrotolerans*, JS1^T was selected as the test organism for studying the degradation of aromatic sulfur compounds, as this was the only bacterium among 5 cultures tested, that could grow in a medium supplemented with the test compounds.

Our efforts to enrich SRB degrading these compounds from 20 different environmental samples also were not successful (data not shown). The *D. psychrotolerans*, JS1^T could grow in medium with the aromatic sulfur compounds supplemented as either sole carbon source or as sole electron acceptor. But as *D. psychrotolerans*, JS1^T could grow well in medium with the aromatic sulfur

compounds supplemented as sole carbon source than as sole electron acceptor source, the test compounds were supplemented as sole carbon source in further degradation studies. This result also supports the earlier report that aromatic compounds are metabolized by SRB much efficiently when they are supplemented as sole carbon source [20, 21].

Hulshoff Pol [22] has reported that many SRB are quite resistant to overloads of certain organic compounds and to toxic upsets from aromatic compounds like alkanes, ethylbenzene, toluene, chloroform and other long chain fatty acids and can dominate in growth by competing with other anaerobic bacteria. A similar observation was made with respect to the tolerance of the test compounds by strain JS1^T. The *D. psychrotolerans*, JS1^T was not sensitive to even very high concentrations of the test compounds. PTSA was tolerated up to a concentration of 25 mM while SFA and TPA were tolerated even up to the highest concentration of 50 mM tested. However, optimum growth was observed when the aromatic sulfur compounds were supplemented as sole carbon sources within a concentration of 3 and 4 mM. The pattern of degradation of the test compounds by *D. psychrotolerans*, JS1^T was similar to that of its growth on these compounds. A few SRB have been reported to adsorb certain metals and pollutants especially in activated sludge [23, 24]. Hence, it was essential to understand whether disappearance of the compound observed only in the presence of *D. psychrotolerans*, JS1^T was due to the biodegradation or simple passive adsorption. It was concluded that passive adsorption did not play a significant role since there was no significant loss (<5% loss) in the concentration of the compounds when inoculated with dead biomass (heat killed), while there was rapid decrease in their concentration when inoculated with live biomass within 5 days of incubation.

Though there are no reports of degradation of aromatic sulfur compounds by any SRB, *Desulfobacula toluolica* and *Desulfobacula phenolica* [20,25], *Desulfosarcina cetonica* have been demonstrated to degrade toluene completely to CO₂. The mixed cultures of SRB and other methanogenic bacteria were reported to degrade toluene. Degradation of PTSA under anaerobic conditions by mixed cultures was reported by Shcherbakova, et al. [18]. Also, anaerobic degradation of dibenzothiophene [17,26] and anaerobic desulfurization of benzothiophene and dibenzothiophene have been reported in mixed cultures of SRB earlier, no reports exist on the anaerobic degradation of either PTSA, SFA or TPA by pure cultures of SRB. Hence, the present study on degradation of aromatic sulfur compounds by *D. psychrotolerans*, JS1^T is the first such study of degradation by any pure culture of SRB. However, none of the aromatic sulfur compounds were completely degraded within the experimental period of three months (PTSA was degraded by 82%, SFA by 65.5% and TPA by 72%).

In general, laboratory studies are not accurate predictors of field degradation rates [27]. Bioremediation via environmental introduction of degradative microorganisms requires that microbes survive in substantial numbers and effect an increase in the rate

and extent of pollutant removal in these natural habitats [28,29]. Measuring biodegradative activity and efficiency of the selected microbes in natural habitats is difficult due to limited accessibility of samples as well as sorption and abiotic transformation of contaminants [30]. Due to these reasons, microcosm studies are generally used to understand these important parameters before switching on to on-site studies.

Microcosm studies are also known as the bio feasibility studies. Microcosms are artificial, simplified ecosystems that are used to simulate and predict the behavior of natural ecosystems under controlled conditions [31,32]. These studies are useful in understanding the effect caused due to a pollutant or to determine the role of microbes in eliminating or reducing the effect of these pollutants in natural systems. The microcosm studies of degradation of pollutants by SRB conducted till now have mainly concentrated on degradation by mixed populations. An example is the degradation of toluene by SRB in oil contaminated soils [33,34].

Conclusion

In the present study, the degradation of aromatic sulfur compounds and their subsequent degradation by pure culture of *D. psychrotolerans*, JS1^T was carried out in both soil and sludge microcosms that mimic the naturally existing environments of contaminants. *D. psychrotolerans* JS1^T was employed independently (in sterile soil and/or sludge) and also in consortium with the native microbiota (unsterile soil and/or sludge) inhabiting the soil and sludge samples. The viability of spiked pure culture of strain JS1^T in soil and sludge microcosm could be checked by streaking the spiked samples on to PBM at regular time intervals during the experiment and observing for the pure colonies of the strain JS1^T.

The role of native microbiota alone in the degradation of the test compounds was insignificant as observed in the unsterile and uninoculated microcosms of soil and sludge spiked with the aromatic sulfur compounds. PTSA was degraded by strain JS1^T much efficiently in soil microcosm (66%) than in sludge microcosm (38%). Degradation of PTSA by indigenous soil and sludge microbiota in consortium with JS1^T was not very significant, but a slight increase of degradation to 47% (from 38%) in sludge and a slight decrease of degradation to 63% (from 66%) in soil were observed. The degradation of SFA by pure culture of JS1^T was almost similar in sludge and soil microcosms (64 and 65% respectively) but the degradation in consortium with soil and sludge microflora was lowered to 58% (from 64%) in sludge and 40% (from 65%) in soil microcosms respectively. The degradation of TPA was the least in sludge microcosm (31%) while the highest in soil microcosm (72%). There was a significant decrease in degradation to 30% (from 72%) due to contribution of soil consortia and an increase to 48% (from 30%) due to contribution of sludge consortia. The present work in lab scale is a preliminary study conducted at ambient conditions (pH, temperature, nutritional conditions etc.) of naturally occurring soils and sludge and thus indicates the potential of *D. psychrotolerans*, JS1^T for bioremediation of contaminated soils and sludge.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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