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Implications of Benzoate Induced Alterations in Cell Morphology and Physiology in *Pseudomonas aeruginosa* TMR2.13 for Potential Application in Bioremediation and Monitoring Approaches

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Abstract

The aromatic hydrocarbon sodium benzoate induced significant alterations in cell morphology with parallel changes in physiological processes like denitrification and pigment formation during growth of a bacterial strain TMR2.13 in denitrification medium. The isolate obtained from a low nutrient level ecosystem (coastal sand dunes) was identified as *Pseudomonas aeruginosa*. The culture was capable of growing in up to 3% benzoate as the sole carbon source. The culture, a strong denitrifier, showed changes in nitrite levels in response to benzoate. Presence of benzoate induced a 50% reduction in intermediate nitrite accumulation under well aerated conditions. High benzoate levels caused a delay in growth rate, nitrate reduction activity and prominent decrease in transitional nitrite levels from 1528 µM (without benzoate) to 508 µM (with 1% benzoate). Further, the intensity of pigment production was also enhanced in presence of sodium benzoate indicated by a significant increase in absorbance at 380 nm (pyoverdin peak) and 680 nm (pyocyanin peak). The impact of benzoate was greater on pyoverdin production than on pyocyanin. The alterations depicted by TMR2.13 at high benzoate concentrations in nitrite and pigment levels can be projected with potential application in bioremediation and monitoring studies.

Keywords: Denitrifying bacteria; Denitrification; Aromatic hydrocarbon; Pyoverdin; Pyocyanin; Bioremediation

Abbreviations: DM: Denitrification Medium; SEM: Scanning Electron Microscopy

Introduction

Hydrocarbon contamination particularly that derived from petroleum products and other anthropogenic activities is a major cause of concern in the marine environment and has therefore garnered interest in bioremediation studies [1-4]. Environments rich in hydrocarbons particularly coastal and estuarine ecosystems generally have a high nitrate load [5]. If not contained, elevated nitrate levels trigger the proliferation of algal blooms causing fish mortality, water anoxia and the formation of dead zones [6,7]. Removal of nitrate and nitrite from these systems is primarily done by denitrification [8,9]. Denitrification is the dissimilatory reduction of nitrogen oxides to nitrogen gas mediated by microorganisms and occurs ubiquitously [5,9]. The distinctive and flexible mechanism of respiration, using nitrate as the terminal electron acceptor in lieu of oxygen can be exploited efficiently in the degradation of hydrocarbons under mixed aerobic / microaerophilic conditions [10]. Biodegradation of hydrocarbons, especially the aromatics by indigenous strains of hydrocarbon-degrading denitrifying bacteria, therefore represents an efficient approach towards pollution remediation [5,11-13].

The abundant organic matter in coastal and estuarine systems generates favourable conditions for the heterotrophic denitrifiers to proliferate [14]. However, in such ecosystems, the persistent accumulation of aromatic hydrocarbons will inevitably render an effect on the physiological activities of the autochthonous microbial communities [12], such as, membrane transport [15], denitrification [16] and pigment production [17]. Such physiological changes can consequently influence the overall productivity of the marine ecosystem [18]. Therefore, in order to generate an effective bioremediation design employing indigenous denitrifiers, it is important to understand the effect of such hydrocarbons on their physiological processes, in particular, their denitrification efficiency.

In the present investigation, a denitrifying bacterial strain TMR2.13 was isolated from a coastal sand dune ecosystem. Sodium benzoate was used as a model compound representing aromatic compounds to simulate a pollutant. We describe the changes induced by the aromatic hydrocarbon on its cell morphology and physiological processes such as denitrification and pigment production. Relevant emphasis was drawn towards bioremediation and monitoring studies.

Materials and Methods

Growth media

The culture used in the present study was isolated from low nutrient sand dune samples collected at Miramar beach, Goa located on the West Coast of India [19]. The isolate was grown and maintained on denitrification medium (DM) [20] containing (per litre) 4.72 g sodium succinate, 1.5 g KH_2PO_4, 0.42 g Na_2HPO_4, 0.6 g NH_4Cl, 5 g tryptone, 1 g $MgSO_4.7H_2O$, 1 g KNO_3 and 2 ml trace metal solution. The medium was supplemented with sodium benzoate whenever required.

Growth and cell morphology by scanning electron microscopy (SEM)

Cells were grown in DM and DM supplemented with 0.5% filter sterilized sodium benzoate. The flasks were incubated at 28˚C at 100 rpm. Growth was monitored every 2 h as increase in turbidity measured

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using Shimadzu UV-Visible spectrophotometer (UV-2450). After 24 h, culture broth was centrifuged and the washed cell pellet was used for SEM studies. Cells were fixed overnight in 2% glutaraldehyde prepared in 50 mM potassium phosphate buffer. The fixed cells were washed with phosphate buffer and dehydrated using an increasing gradient of acetone in distilled water (30, 50, 70, 90%) for 10 min each and finally in 100% acetone for 30 min and air dried. The specimens were then sputter coated with platinum using an auto fine coater (JEOL JFC 1600) and visualized using SEM (JEOL JSM-6360).

Nitrate reduction in DM and DM supplemented with benzoate

Cells previously grown in DM (70 ml in 150 ml capacity Erlenmeyer flask) were washed with de-ionized water and inoculated (5%) in 2 sets of DM (70 ml) in Erlenmeyer flasks (150 ml capacity). One set was without sodium benzoate and the other was supplemented with 0.1% filter sterilized sodium benzoate. The flasks were maintained at different aeration conditions of static, 50 rpm, 100 rpm and 150 rpm and incubated at 28˚C. Nitrate and nitrite concentrations were monitored every 2 h until the nitrite in the medium was completely reduced.

Nitrate estimation: Nitrate was estimated spectrophotometrically (Shimadzu UV-Visible spectrophotometer UV-2450) as described by Nagaraja et al. [21]. To 1 ml of cell-free supernatant, 5 ml of 14.4 M H_2SO_4 and 3 ml of a mixture containing dopamine (3-hydroxy tyramine) and methyl-2-benzothiazolinone hydrazone hydrochloride were added. After an incubation period of 15 min, the absorbance was measured at 530 nm. Nitrate concentration was estimated using KNO_3 as the standard (0-500 µM).

Nitrite estimation: Nitrite was measured using 1 ml each of sulphanilic acid and α-naphthylamine [22]. A reaction time of 15 min was allowed after the addition of each reagent to 1 ml of cell-free supernatant. Absorbance at 540 nm was noted. Nitrite concentration was calculated using $NaNO_2$ as the standard (0-200 µM).

Effect of different concentrations of benzoate on nitrite levels

Cells grown earlier in DM were washed with de-ionized water. 5% of this was inoculated in Erlenmeyer flasks containing DM broth supplemented with increasing concentrations of filter sterilized sodium benzoate [0, 0.1, 0.2, 0.5, 0.7 and 1% (w/v)]. The flasks were incubated at 100 rpm at 28˚C. Nitrate and nitrite levels were monitored every 2 h by the methods mentioned earlier.

Effect of benzoate on pigment profiles

Cells were inoculated in DM and DM supplemented with 0.1% and 0.5% filter sterilized sodium benzoate and incubated under static conditions at 28˚C. After regular time intervals, 5 ml of the culture broth was centrifuged. The cell-free supernatant was extracted twice with equal volumes of distilled chloroform in a separating funnel. Scans of the upper green aqueous layer and the lower blue organic layer were taken from 190-800 nm using UV-Visible spectrophotometer (Shimadzu UV-2450). Fluorescence was detected using a spectrofluorimeter (Shimadzu RF-5301 PC).

All experiments were carried out in triplicates and the graphs were plotted with error bars depicting standard deviation.

Results and Discussion

Bacterial isolate TMR2.13 was identified as *Pseudomonas aeruginosa* (GenBank accession number: HM030825) based on cultural, biochemical and 16S rRNA gene sequence analyses [19]. *Pseudomonas aeruginosa* is a well documented denitrifying bacterium [23,24] and is known to play a typically active and functional role as a denitrifier [25]. Strain TMR2.13 also exhibited an efficient denitrification of nitrate (up to 31 mM) in DM. *P. aeruginosa* is known to mineralize a wide variety of organic compounds including aliphatic [24], aromatic [26] and polycyclic aromatic hydrocarbons [27]. Isolate TMR2.13 when inoculated in a mineral salts medium [28] with sodium benzoate as the sole carbon source, showed luxuriant growth between 0.1-1.5% and tolerated up to 3% benzoate. The culture exhibited the ortho mode of ring cleavage on testing with Rothera's test [29] and the specific activity of catechol 1, 2-dioxygenase was 3.65 enzyme units [30].

Effect of benzoate on cell morphology

Isolate TMR2.13 showed good growth in DM and DM with 0.5% benzoate (Figure 1). The presence of the aromatic compound did not significantly alter the growth profile of the culture. However, it increased the viability of the cells by providing an extra carbon source. Interestingly, the presence of sodium benzoate in the medium caused a change in cell morphology and reduction in cell length as observed in scanning electron micrographs (Figure 2). In contrast to the normal cells obtained in DM (Figure 2a), the cells appeared shorter and swollen in the presence of benzoate (Figure 2b). Cells exposed to hydrocarbons, particularly the aromatic group, are reported to undergo changes in structure and permeability of the cell membrane [15]. This helps them overcome the toxicity or adapt to the altered carbon source. These effects are invariably reflected on the cell morphology in terms of size reduction, destructive openings, wrinkling and other deformations [17,31].

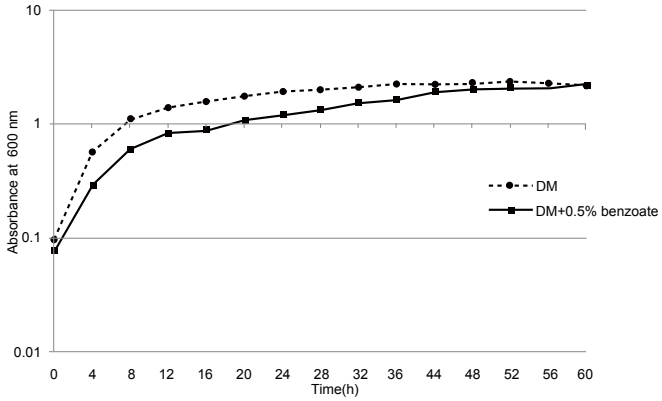

Figure 1: Growth profile of strain TMR2.13 in DM and DM supplemented with 0.5% sodium benzoate.

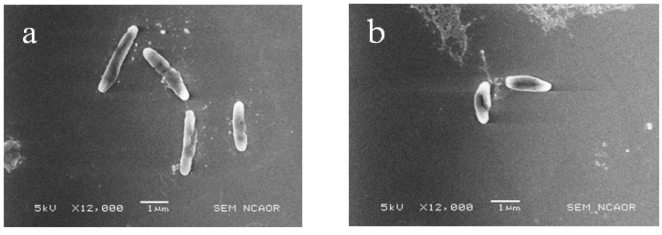

Figure 2: Scanning electron micrographs of cells grown in a) DM b) DM supplemented with sodium benzoate.

Constant exposure of bacterial cells to hydrocarbons also influences important susceptible physiological processes [15] such as denitrification [16] and pigment production [17].

Effect of benzoate on denitrification

During denitrification most of the nitrate is usually converted to nitrogen gas with nitrite as an intermediate [32]. The rate of conversion, however, depends on the oxygen levels [24] and the type of carbon sources available [33].

In our study, we observed a significant difference in the rate of nitrate reduction during growth of strain TMR2.13 in DM incubated under static / shake (50 rpm, 100 rpm and 150 rpm) conditions. Under static conditions the reduction of nitrate was initiated after 2 h with maximum nitrite levels (3578 µM) detected at 8 h and completely reduced at the end of 10 h. At 100 rpm, nitrite was detected within 2 h of inoculation of the culture with maximum nitrite levels (1528 µM) detected by 4 h and further completely reduced within 6 h. However, at 150 rpm, there was a tremendous increase in nitrite between 2-6 h with maximum nitrite (5383 µM) formed by 6 h followed by a sharp decline in its concentration after 6 h. In all cases, both nitrate and nitrite were completely reduced before the cells entered the stationary phase.

Denitrification is essentially an anaerobic process occurring under microaerophilic to anaerobic conditions. However, several studies have shown that both oxygen and nitrate can function concurrently during respiration irrespective of the availability of oxygen [20,22]. Such a phenomenon is termed as aerobic denitrification and is more prevalent in Gram negative bacteria such as *P. aeruginosa* [34]. Our results reflect that although static conditions were conducive for denitrification in strain TMR2.13, aeration created favourable conditions for growth of the aerobic culture. The prolific accumulation of intermediate nitrite at 150 rpm can be considered to be a manifestation of the insufficiency of carbon that arises due to the profuse growth under well aerated conditions [35]. Inadequate aeration under static conditions slowed down the growth of the aerobic strain thereby delaying the reduction reactions. Notably, aeration at 100 rpm simulated conditions which were ideal for growth and denitrification to occur concomitantly.

Significantly, on addition of the aromatic hydrocarbon sodium benzoate, although there was no major effect on the growth profile of the culture, a prominent drop in intermediate nitrite accumulation was observed (Figure 3). Under static conditions, when 0.1% benzoate was added to DM, it resulted in a drop in maximum nitrite level accumulation (2606 µM) (Figure 3a). Interestingly, at 150 rpm, the concentration of intermediate nitrite (2264 µM at 4 h) was 50% less than that formed in the absence of sodium benzoate (Figure 3b). Furthermore, as the concentration of the aromatic hydrocarbon was increased (0-1%); growth in DM depicted a significant fall in intermediate nitrite levels especially in the presence of 1% benzoate (Figure 3c). Her and Huang [35] also reported a similar decrease in intermediate nitrite levels in presence of aromatic compounds.

Brezonik [36] explained that benzoate during denitrification gets converted to nitrosobenzoate followed by the release of nitrogen gas as a consequence of tautomerism. This phenomenon induces a decrease in intermediate nitrite accumulation in the presence of the aromatic hydrocarbon. Accordingly, as the benzoate concentration is increased in the medium the nitrite formed binds to the excess benzoate available, thereby preventing its accumulation in the medium. It is important to note that nitrite is known to be toxic to aerobic bacteria. It inhibits various physiological activities such as proton-dependent active transport, functional activity of crucial metabolic enzymes and oxygen uptake and oxidative phosphorylation ensuing interferences to energy conservation [37,38]. Furthermore, nitrite accumulation is also detrimental to aquatic life disrupting their respiratory physiological processes including blood oxygen transport [39]. The observations made during this study implicate that the presence of aromatic compounds decreases the amount of intermediate nitrite levels accumulating during denitrification. Such a mechanism could find relevance in alleviating nitrite toxicity in natural ecosystems contaminated by aromatic hydrocarbons as well as excessive nitrates.

The results also depict that in the presence of high benzoate concentrations, there is a prominent delay in the overall reduction of nitrate and nitrite. In presence of 0.5% and 1% benzoate, nitrite was maximum at 4 h (1380 µM) and 10 h (508 µM), and gradually reduced by 8 h and 12 h, respectively (Figure 3c). Gilbert et al. [16] reported that hydrocarbons either stimulate or inhibit denitrification depending on their concentration and correlated it to several factors like availability of organic compounds and nitrate. We envisage that this stimulation or inhibition of denitrification is more to do with the oxygen requirement

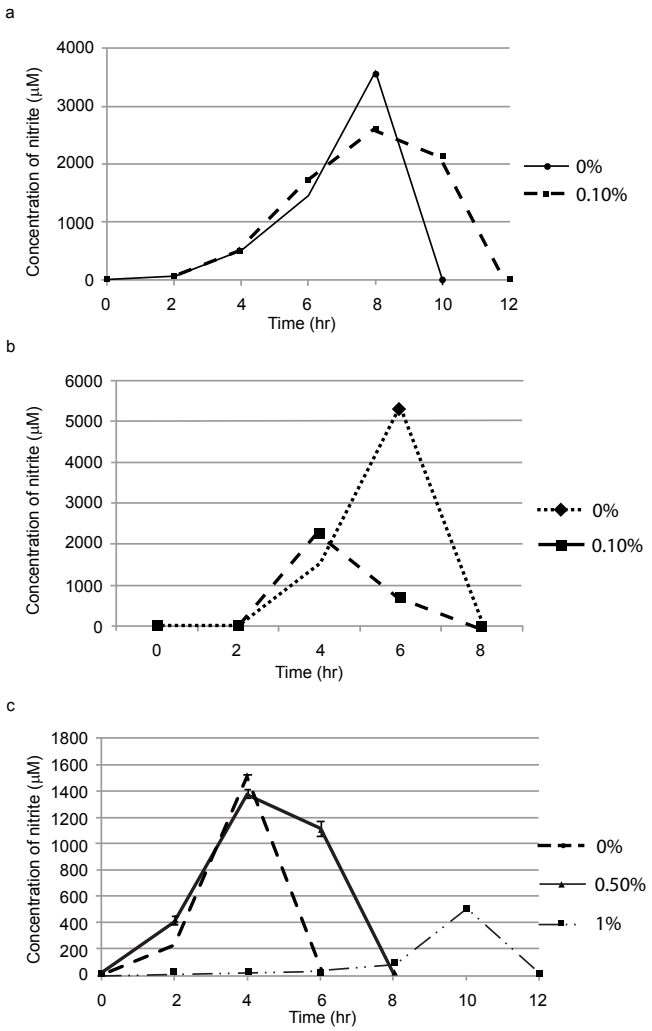

Figure 3: Nitrite levels detected during growth of TMR2.13 in DM: a) under static conditions in the absence and presence of 0.1% sodium benzoate; b) at 150 rpm in the absence and presence of 0.1% sodium benzoate; c) in presence of increasing concentrations of sodium benzoate.

during degradation of the hydrocarbons especially in the case of aromatic compounds [40]. TMR2.13 degrades sodium benzoate via the aerobic ortho mode of ring cleavage (β-ketoadipate pathway) mediated by catechol 1, 2-dioxygenase [41] as confirmed by the purple ring obtained during Rothera's test [29]. The culture therefore needs oxygen for this enzymic reaction [41] resulting in oxygen depletion during growth in DM, which favours the denitrification pathway. However, higher concentrations of benzoate put an excessive demand on the oxygen requisite of the aerobic culture. This limits its growth and subsequently delays its denitrification activity thereby inducing an overall effect on the physiological processes of the organism [2].

Effect of benzoate on pigment production

Growth of TMR2.13 in succinate containing denitrification medium under static conditions shows the production of extracellular yellow-green and blue-green pigments (Figure 4a). UV-Vis spectrophotometric analysis showed a strong absorbance at 380 nm and 680 nm indicating the presence of pyoverdin (yellow-green, fluorescent) and pyocyanin (blue-green, non-fluorescent) pigments, respectively. The presence of pyoverdin was further confirmed using spectrofluorimetric analysis which depicted an emission at 472 nm when excited at 398 nm [42]. Pyoverdin, enhanced by succinate [43], was produced within 8 h of growth, that is, during the late-log phase of the culture [44]. On the other hand, pyocyanin was detected only at the end of 24 h when the cells were in the stationary phase [45,46]. Production of pyoverdin and pyocyanin correlated with the growth pattern and were easily separated by extraction with chloroform (Figure 4b) giving characteristic profiles. Further, production of both pyoverdin and pyocyanin increased on prolonged incubation (Figure 5).

Earlier reports suggest that this simultaneous outburst in the two pigments during the stationary phase is on account of the reduction in oxygen under iron limitation [47,48]. The production of pyoverdin and pyocyanin is also influenced by various carbon and nitrogen sources [23]. Our results showed enhanced pigment production on addition of sodium benzoate (Figure 4a), with a significant increase in absorbance at 380 and 680 nm in the presence of 0.5% sodium benzoate on prolonged incubation (Figure 5). At this stage, the cells having utilized the easier metabolizing substrates viz. sodium succinate and tryptone from the medium now turn to sodium benzoate for a carbon source. Utilization of such aromatic compounds results in a greater demand for oxygen [41] resulting in a stress on the organisms thus imposing enhanced production of pigments. These microbial pigments influence the metabolism of the bacteria that produce them boosting their competitiveness and survival [46,49]. Dao et al. [48] suggested them to be stress related and influenced by various stress agents like metals and oxygen radicals. Several reports have also indicated an interrelation between hydrocarbons particularly the aromatics and these pigments [17,49].

Interestingly, the impact of benzoate was greater on pyoverdin production in comparison to pyocyanin (Figure 5). This was supported by the increase in fluorescence in proportion to the concentration of the aromatic compound (Figure 6). Pyoverdin is a water-soluble aromatic chromophore derivative and acts as a siderophore in low-iron media [50]. Benzoate augments pyoverdin production since benzoate grown cells have a specific iron requirement. Iron acts as a cofactor for the enzyme catechol 1, 2-dioxygenase involved in the degradation of the aromatic compound. Benzoate grown cells therefore readily become iron deficient [19,51]. Pyoverdin being a siderophore supports iron chelation and renders it available to the cells. Pyocyanin on the other hand, is a chloroform-soluble phenazine pigment and is known to be stimulated under low aeration conditions [47] like those developed during denitrification.

Research devoted to the influence of stress agents like aromatic compounds on the critical aspects of microbial physiology, although limited, is ecologically valuable especially for toxicity studies. In natural ecosystems exposed to an influx of hydrocarbons, such regulation in physiological activities would affect the overall community structure and consequently the productivity of the system. The ability of the denitrifying bacterial strain TMR2.13 to degrade aromatic hydrocarbons with simultaneous reduction in nitrite levels is relevant and important in natural ecosystems. The bioremediation of hydrocarbons could also reduce nitrite toxicity bringing about a more effective and consolidated environment remediation design. The increase in pigmentation in response to high benzoate concentrations

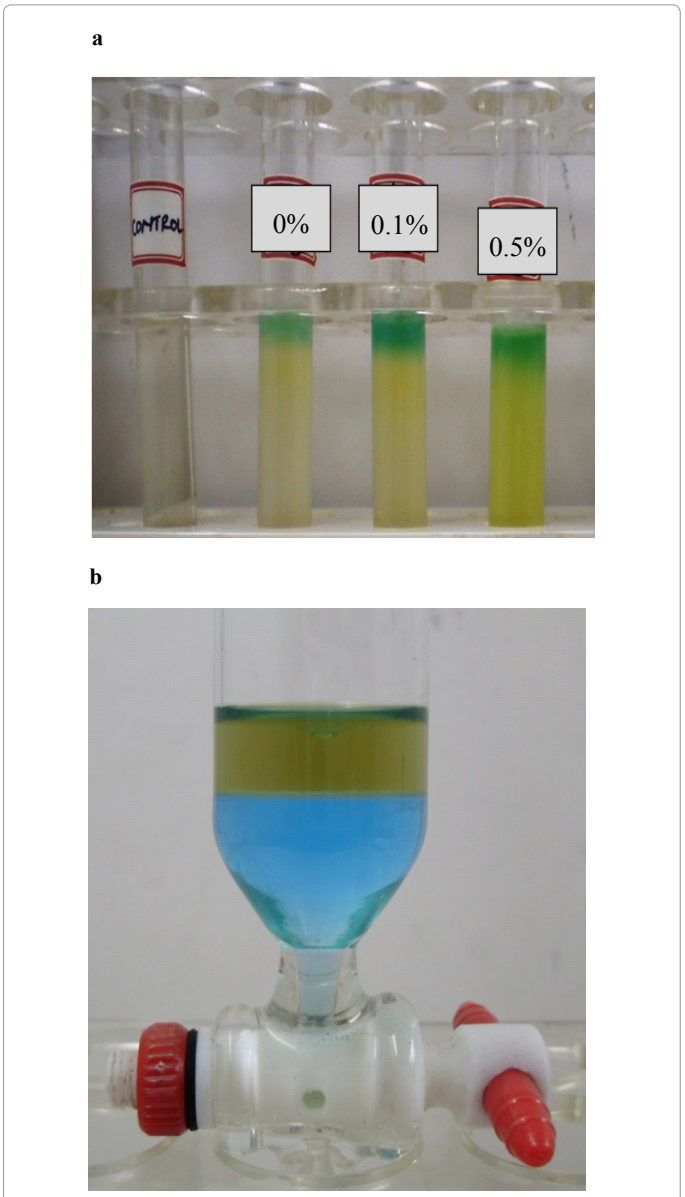

Figure 4: Pigment formation and extraction: a) Production of pigments pyoverdin and pyocyanin during growth of TMR2.13 in DM under static conditions in the presence of different benzoate concentrations; b) Separation of pigments by extraction with distilled chloroform in a separating funnel

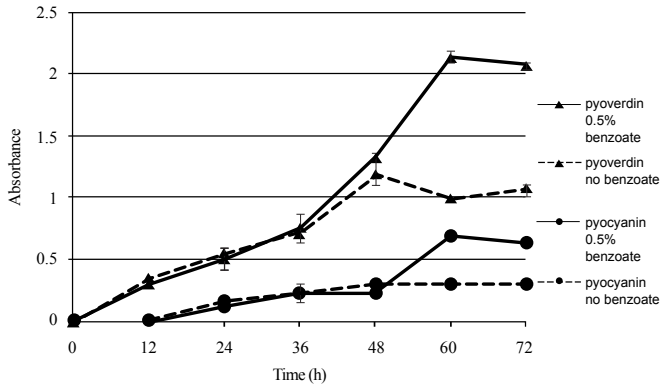

Figure 5: Increase in pyoverdin and pyocyanin in presence of 0.5% sodium benzoate on prolonged incubation during growth of TMR2.13 in DM.

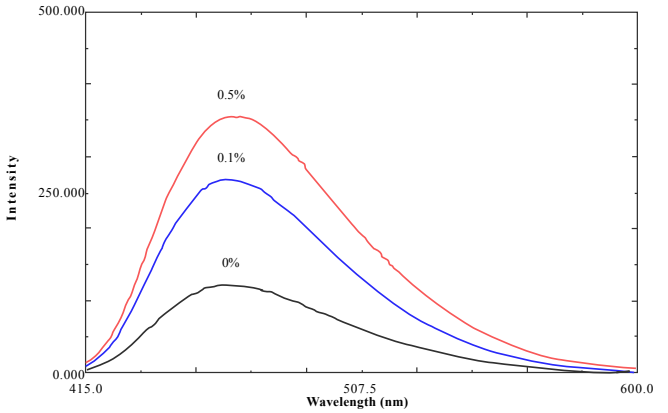

Figure 6: Emission spectrum of pyoverdin (Excitation wavelength: 398 nm) with increase in sodium benzoate concentrations: Pigment extracts were diluted 100-fold and the fluorescence was detected using a spectrofluorimeter (Shimadzu RF-5301 PC).

would prove to be a useful indicator of hydrocarbon pollution and needs to be developed for monitoring studies. Further investigations are required on the effect of such xenobiotics on the physiological aspects of indigenous microbial communities in natural systems. Such studies will provide comprehensive insights on the ecological impact and pave new avenues for bioremediation approaches.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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