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Effect of rhamnolipid biosurfactant on the degradation of pentaerythritoltetranitrate (PETN)

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This study investigated the bioremediation of explosive pentaerythritoltetranitrate (PETN) in presence of rhamnolipid (RL) biosurfactant by aerobic process. Microbial inoculate were obtained from a textile wastewater treatment plant activated sludge. Addition of rhamnolipid surfactant (60 mg/l) increased the removal efficiency of PETN from 33% to 76% and COD removal from 20% to 58%. Application of rhamnolipid led to mineralization of PETN. Degradation of PETN is expressed to be of first-order and the kinetic reaction parameters are calculated based on different initial concentrations of PETN. The first-order rate constant of the rhamnolipid amended experiments were at least 3.6 orders of magnitude higher for PETN than those found for not amended rhamnolipid experiments. Inoculated bacteria have capability to use of PETN as source of nitrogen and energy. It seems that the addition of rhamnolipid showed great potential for treatment of explosives by textile activated sludge.

Key words: PETN, COD, rhamnolipid, textile activated sludge, mineralization.

INTRODUCTION

A massive production and use of different synthetic chemicals in the form of pesticides, herbicides, plasticizers, explosives, dyes, drugs, and industrial products in our life, continually contaminate soil, water, and air which have shown direct or indirect adverse effect on our and animal health[1-3]. Toxicity, mutagenicity or carcinogenicity of these chemicals to humans and animals has been reported. Explosives, anthropogenic nitro-organic compounds, are main group of pollutants for the environment because of their extensive use, toxicity, recalcitrance and bioaccumulation. [3]. These organic nitrate contaminants are very recalcitrant and not typically found in the environment, thus classifying these compounds as xenobiotic [4]. Among the nitro energetic compounds, pentaerythritoltetranitrate (PETN) is extensively used as a powerful explosive [5]. In addition to observable physical hazards related to explosives, toxicity to biological systems is as well of concern. The vasodilatory effects of the nitrate esters GTN and PETN, and their capability to cause methaemoglobinaemia, which affects the ability of red blood cells in transportation of oxygen, is one example of toxic effects of nitrate esters[4]. With respect to hazards related to nitrate esters, remediation of this compounds by effective and environment friendly approach must be concern[6, 7]. Although researches about PETN degradation were conducted previously, however, compared with other explosives, the fate and transport of PETN in the environment has received little research attention.

Studies on degradation of PETN has been done previously. For example degradation of PETN by granular iron was performed by Zhung[8]. More ever biodegradation of pentaerythritoltetranitrate (PETN) by anaerobic consortia was evaluated by Zhung et al [5]. Also Binks et al. [9] isolated an

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*Enterobacter cloacae*PB2strain from a soil enrichment under aerobic and nitrogen-limiting conditions and concluded that PB2 utilized 2 mol of nitrogen per mol of PETN, producing metabolites of pentaerythritoltrinitrate (PETriN) and pentaerythritoldinitrate (PEDN). PEDN was then oxidized to dinitratedialdehyde. Nitrate esters and other explosive compounds have also been shown susceptible to biodegradation, with adapted indigenous microbial populations which have been exposed to contaminants for a long period of time presenting the greatest possibilities[10]. Compared with other methods, biological methods for remediation of explosive contaminated sites can be biosurfactant) the cell surface hydrophobicity (CSH) improved due to the removal of lipopolysaccharide (LPS) from cell surface of Gram-negative bacteria and the successive exposure of hydrophobic phospholipid tails or by the adsorption of rhamnolipids modifying the cell surface properties of Gram-positive bacteria[14]. In a study conducted by Whang et al. [11] it was specified that rhamnolipid (RL) have capability in lowering surface tension, increasing diesel solubility, and therefore enhancing biodegradation of diesel in diesel/water systems. Also Zhou and Zhu [15] show that the rhamnolipid is capable to shorten the extended lag phase of bacteria for PAH biodegradation. Moreover, they revealed that the biochemical pathways of the biodegradation of PAHs depend, generally, on aerobic condition with the rhamnolipid. It was assumed that the activated sludge wastewater contain nutrients and /or bacteria that could improve the biodegradation process[16]. So in this study activated sludge from a textile wastewater treatment plant was added as organic amendment and as seeding agent. Presently, available information concerning the effects of rhamnolipid addition on improved biodegradation of PETN is sparse. In this study, rhamnolipids were selected to assessment the effect of biosurfactant on biodegradation of PETN. Also kinetics studies on reductive degradation of PETN in contaminated soil were investigated. The results obtained from this study were expected to deliver some additional information for developing approach to improve the remediation efficiency for PETN contaminated soils.

MATERIAL AND METHODS

Biosurfactant

The biosurfactant used in this study was a rhamnolipid ($C_{32}H_{58}O_{13}$) obtained from the Genetic Engineering and Biotechnology Institute (Iran). It is an extra-cellular natural substance produced during precisely controlled fermentation processes using more cost-effective and significantly reduce toxicity of the soil [4]. However relatively low solubility of PETN [8]could be limiting factor for its biodegradation. Thus addition of external agent such as surfactants to contaminated soil, at concentrations above their critical micelle concentration (CMC) values, can be a feasible approach to enhancing the solubility and therefore, increasing their biodegradation[11, 12]. The application of biosurfactants has increasingly augmented during the past decade as potential candidates because of their biodegradability, lower toxicity and greater diversity than the available synthetic surfactants [13].In the case of rhamnolipids (as common certain bacterial strains [3]. Its molecular weight was 650 g/mol.

Soil preparation

Eight soil-pan (1to 8) experiments were conducted. The clean natural soil was derived from a garden in university of Isfahan. The physical and textural characteristics of these soils are given in Table 1. Soil samples were sieved through a 2-mm sieve to remove coarse fragments. Contaminated soil was then prepared by dissolving an appropriate quantity of PETN in water/acetonitrile solution and a known weight of soil was added with continuous mixing. The resultant mixture was placed in a ventilation hood to allow the complete evaporation of the solvent. The contaminated soil was stored at room temperature for 7 days.

The experimental soil had an effective size of 1.00 mm with a uniformity coefficient of 2.23. The initial soil porosity was calculated at 36% on the basis of measured values for soil bulk density (1.63 mg/cm3).

Each pan experiment was prepared by placing 3000 g of contaminated soil in a square plastic pan (30 cm ×20 cm ×20cm in height). This mass of soil occupied the pans approximately two-thirds full. The bottoms of these pans were perforated to allow drainage of fluids during and after flooding phases, via 2-mm-diameter holes spaced 2 cm apart in a square grid as described by Boopaty[17]. Each of the plastic pan was placed inside a slightly larger plastic pan (33 cm × 22 cm × 22 cm) to provide secondary containment of deionized water. Since addition of amendment can improve soil management properties[17], wood chips was added to the contaminated soil. The potential benefits of the wood chips included improved drainage, moisture retention and aeration. In this treatment, contaminated soil represented nearly 95% of the total soil mixture, while the wood chips amendment comprised the remaining 5% (dry-weight basis).

Pans 1 and 5 served as control but were operated on a periodic cycle consisting of flooding for 2 d

2, 3, 4, 6, 7 and 8 were designed to be biologically active treatments. Activated sludge from a textile wastewater treatment plant was added to pans 2, 3 and 4, 6, 7 and 8 except that rhamnolipid at CMC of 60 mg/l was added to the 6, 7 and 8 pans. Pans 2 and 6, 3 and 7, 4 and 8 have a PETN concentration of 50,100 and 200 mg/kg respectively. Kinetic studies for each set of experiments were conducted. But results obtained from pans 4 and 8 were reported for biodegradation study (highest concentration). The length of soil aeration depended on the general with deionized water, followed by draining and aeration for several days. Pans

drying conditions in all of the aerated pans. All of specified biological Pans were operated on a periodic cycle consisting of flooding with deionized water for 2 d, followed by draining and aeration for several days. These pans were drained simultaneously by lifting the smaller plastic pans vertically above the larger plastic pans, putting support slats across the top edges of the larger plastic pans, and then remained on the support as described by Boopaty[17].

Table 1. Properties of soil samples used in this study

Parameter	Value (%)
Clay	16
Sand	34
Silt	46
Total carbon	4

Beginning of soil aeration indicated by draining of the pans. Tillage was done when the soils in the aerated pans were dry enough to break apart. The period of aeration depended on soil drying specifications but generally happened in the range of 7 to 11 days.

Chemicals

All chemicals used were of analytical grade; pentaeritritholtetranitrate (PETN) was from Zarrinshahr Chemical Industries (Esfahan, Iran) and was purified by recrystallizing. All other chemicals were obtained from Sigma–Aldrich and Merck.

Sampling and chemical analyses

Samples of soil and liquid filtrates were taken periodically during the experiment for analyses of PETN. Sampling were done once every two weeks .The three grab samples was collect from the top 3 cm of soil in the pans during each sampling event. And liquid filtrates was collected after drainage events at the bottom of larger pans for all of biological pans.

The PETN in soil samples were extracted in accordance with the US EPA Method 8330[18]. Soil samples were air-dried; then 5 g (mixture of three grab samples) of soil was transferred to a clean glass vial and extracted with 20 ml of acetonitrile. The mixture was then centrifuged for 5 min at 3,000 rpm, followed by filtration with 0.22 μm Pall membrane. The prepared sample was analyzed for PETN removal with high-performance liquid chromatography (HPLC). The HPLC system used was from Waters (Milford, MA, USA), consisted of a Model 600E pump, fitted with a Rheodyne 7725i injector valve, a Model 486 UV programmable multi wavelength detector, a data module, a Model 600E system controller, Detector and a Nova-pak C18 guard column. The analytical column was an ODS2-Optimal column (25cm×4.6mm id, 5μm) from Capital HPLC (West Lothian, UK). A water-acetonitrile mixture (20:80, v/v) was used as the mobile phase at a flow rate of 1.0 mL/min. The injection volume was 20μL and the absorbance was measured at a wavelength of 210 nm. The measurement of chemical oxygen demand (COD) was carried out according to standard methods[17].

Analysis of biodegradation products by LC–MS

PETN biodegradation products were analyzed by LC–MS using a Shimadzu LCMS- 2010 EV (Japan) equipped with two pumps (LC-10 ADvp), controller (SCL- 10Avp), autoinjector (SIL- 10ADvp) and a UV2000 UV/VIS detector. The analytes were separated on 250 mm 4.6 mm 5 μm C18 Hypersil GOLD column (Thermo, Waltham, MA) by acetonitrile–water gradient elution (90:10, v/v), at a flow rate of 0.2 mL/min.

Soil bacteria density

Cell growth in pans was determined using the standard total plate count method [16]. At first, 1mL of soil sample was mixed with 9mL sterile phosphate buffer and stirred for 2min to detach the bacteria from soil matrix. Then, serial dilutions of the treated samples were performed in the range of 10^{-4}–10^{-8} and 0.1mL of each diluted sample was spread onto nutrient agar plates (as duplicate). The colonies on each plate were counted after 48 h of incubation at 30°C and the average of the two measurements was reported as bacterial growth in colony forming units per milliliter of soil (CFU/mL-soil).

RESULT AND DISCUSSION

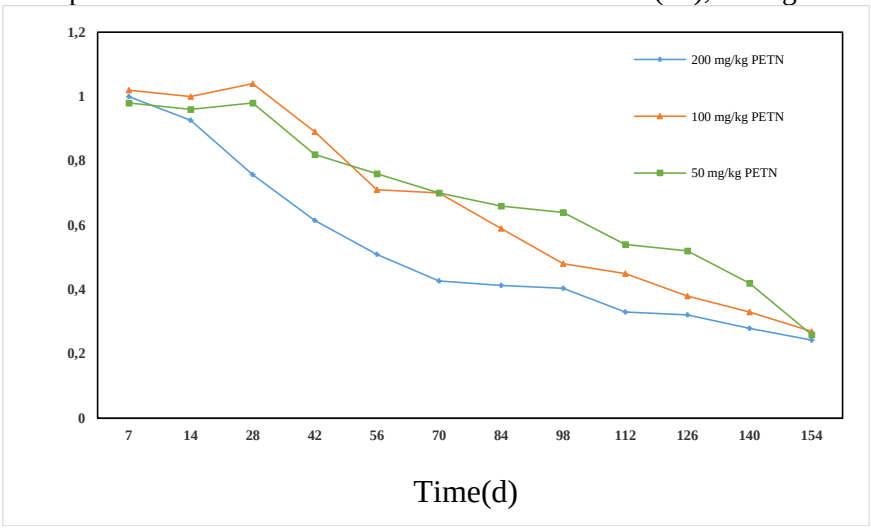
Kinetic studies

In order to determine the kinetics of the explosive degradation, different concentration of PETN were used. Fig. 1a,bshows biodegradation of PETN as a function of time. Analysis of the data showed that, PETN biodegradation followed a general exponential equation representative of first-order kinetics (Table 2). Rate constant k [d^{-1}] is calculated from the slope of the line for ln [C_0/C] vs. reaction time.

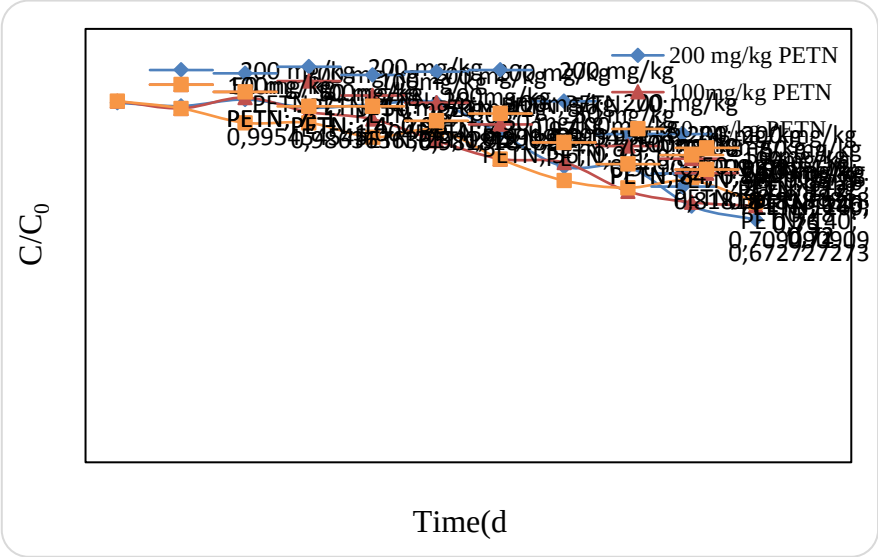
$$dC/dt = -kt \quad (1)$$

$$C = C_0 \exp(-kt) \quad (2)$$

Where C_0 = initial PETN concentration (mg/kg); k is rate constant (d^{-1});t is degradation time (d).



(a)



(b)

Fig.1. Disappearance of PETN with time by addition of rhamnolipid (a) and without addition of rhamnolipid (b)

In general, removal rate of PETN was higher in experiments that rhamnolipid was added. As Table 2 shows the first-order rate constants of the rhamnolipid amended experiments were at least 3.6 orders of magnitude higher for PETN than those found for the non-amended rhamnolipid experiments. This can be due to addition of rhamnolipid which result in increasing the solubility of explosives and then enhancing the explosive biodegradation. Surfactants can increase the surface area of hydrophobic materials, so increasing their water solubility and subsequently increase the biodegradation of complex hydrocarbons[19].

Table.2 Reductive degradation of PETN with different initial concentrations. Experimental data was fit to the first-order kinetic equation.

PETN Concentration(mg/kg)		With rhamnolipid			Without rhamnbolipid		
		50	100	200	50	100	200
Equation	R^2	0.94	0.97	0.96	0.92	0.94	0.98
$-Ln(C/C_0)=Kt$	$Kd[^{-1}]$	0.0074	0.0091	0.0093	0.0021	0.0026	0.0026

Effect of rhamnolipid surfactant on the degradation of PETN

Tests of PETN biodegradation with and without amendment of rhamnolipid (pan8 and 4 respectively), were shown in Fig 2. Data showed that PETN removal in pan 4 during the first 7 weeks of experiment was insignificant. In the absence of rhamnolipid, approximately 1% of the initial PETN removed by 84 days (7 weeks). After 154 d, the removal rate of PETN was slightly improved and reached 33%. As shown in Fig.3 degradation of PETN began with a lag phase of 84 days. At first we assumed that low bacterial population may be cause of the low PETN removal. But significant removal of PETN in pan 8 at the same period of time reject our assumption. Although at the beginning of the study density of bacteria was low, but low solubility of PETN was the main cause of its negligible removal The low solubility of PETN(<40mg/L)[10], limit its ability to be transported into microbial cells and thus be biodegraded. However addition of external agent that increase the solubility of PETN, could enhanced its biodegradability. As can be seen in Fig.3 at the end of 154 days PETN removal rate in pan 8 was 76%. Compare to pan 4 which no rhamnolipid was added, PETN removal in pan 8 was 2.38 times higher than that of the pan 4. This can be due to addition of rhamnolipid. Addition of rhamnolipid led to enhanced mobilization and increases the bioavailability of PETN. This is in agreement with the result obtained by Moldes*et al*[20] who showed that in the presence of biosurfactants, biodegradation efficiency of octane in soil was much higher than that in their absence. The authors proposed that mobilization of octane molecules and consequent intensification in their bioavailability was the main reason of the observed differences.

Although inoculated bacteria have been encountered previously with aromatic compound in wastewater, but additional time to acclimate to new environment is needed. As fig 3 shown in pan 4 which no rhamnolipid was added, about 84 days was need for acclimation of bacteria. This is in agreement with the result of Avramova *et al.* [13] who reported that it was possible that 12 days incubation was enough for adapting of resting cells to the presence of hydrophobic substrate phenanthrene. But in pan 8 it was observed that much lower time was needed for acclimation of degrading bacteria. In other word rhamnolipid reduced the required time for initiation of degradation. In the study performed by the Zhou and Zhu [15] it was found that rhamnolipid is capable to shorten the extended lag phase of bacteria for PAH biodegradation. Also higher removal rate of PETN in the experiment that amended by rhamnolipid (pan8) can be related to the fact that rhamnolipid decreased toxicity of PETN for degrading bacteria. In the study performed by Das et al. [21] it was found that rhamnolipid decreased the toxicity of PAHs and therefore facilitating their consumption by the microorganism (Figure 2).

Microbial growth

Bacterial plate counts were done several times during the experiment on soil samples taken from each of the pans. The bacterial population densities in the soil contained rhamnolipid (Pan 8) were consistently higher than those in the pan 4 and control pans (Table.3). Much higher densities were present in pan 8 compared to pan 4 and control during the experimental period, specifying that rhamnolipid improves bacterial activity in explosive contaminated soil.With high lycontaminated soil, furthermore addition of organic material successfully dilutes the concentration of explosives and may prevent toxicity to the microbial[22]. As mentioned earlier rhamnolipid can have protective potential against the toxicity of PETN for bacteria. The result obtained by the Boopathy*et al.* [17] show that in cultures that enriched with molasses, higher bacterial growth was observed. They conclude that molasses is the suitable source for bacteria compare to the other source. Although bacterial population in pan 8 was higher at first but it was decreased gradually. The drop in bacterial density very likely indicates that the rhamnolipid had been used up or environment condition has undergone change.

Table 3. Bacterial plate counts (colony forming units/g of soil)

Pan	Day 30	Day 60	Day 90	Day 120	Day 150
control	8×10^4	17×10^4	12×10^4	15×10^4	21×10^4
4	5×10^5	7×10^5	6×10^5	1.7×10^6	7×10^6
8	2.8×10^6	3.3×10^6	3.5×10^6	3×10^6	1.9×10^6

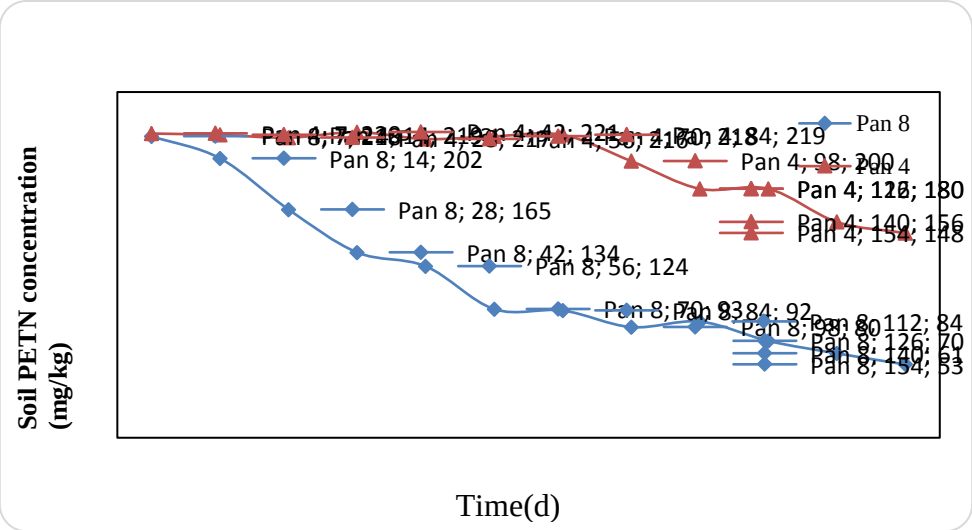


Fig.2. Effect of rhamnolipid on bioremediation of PETN. Pan 4 amended with sludge. Pan 8 amended with rhamnolipid plus sludge

Removals of COD

The COD is a gross parameter of the concentration of organic materials in a solution, so any reduction in COD level was reflected the mineralization [19]. COD was measured according to standard methods[17]. In this study effect of rhamnolipid at CMC of 60 mg/l on mineralization of PETN was evaluated. Sampling of filtrate was done and analyzed for COD removal. Fig.3 shows the effect of rhamnolipid on removal of COD. As Fig.3 indicated COD removal in pan 8 which amended by rhamnolipid was 58 % while only 20 % of COD was removed in 154 days in pan 4. Compare to pan 4 which no rhamnolipid was added, COD removal in pan 8 was 2.9 -fold higher than that of the pan 4. It seems that applications of rhamnolipid increase the solubility of PETN, and thus, facilitate its biodegradation. Sponza and Gök[23] found that addition of rhamnolipid surfactant (15 mg l^{-1}) to a petrochemical wastewater increased the removal efficiencies of PAHs and soluble COD from 72% and 90% to 80% and 99%, respectively. Considering that reduction in COD level can serve as an indicator for mineralization[19] higher removal of COD in pan 8 show that rhamnolipid resulted in higher level of PETmineralization.

Metabolic pathway

Since microorganisms have the capability of producing many different types of enzymes, different metabolic pathways and mechanisms for explosives biodegradation have been suggested in recent years. For instance Zhuang *et al*[5] proposed three potential metabolic processes for PETN degradation: (1) PETN served as a primary substrate for bacterial growth by using the carbon and nitrogen atoms in the PETN structure. (2) PETN served as an electron acceptor. (3) PETN degraded via co-metabolic processes which reduced by a non-specific enzyme or co-factor from metabolism of primary substrates. In the present work, LC–MS analysis of PETN intermediate showed that pentaerythritoldinitrate, 3-hydroxy-2, 2 bis [(nitrooxy) methyl] propanal, 2, 2-bis [(nitrooxy) methyl] propanedial and pentaerythritol were observed during PETN metabolism. This is in consistent with the result obtained by others[8, 9] who reported that transformation of PETN by PETN reductase yield intermediates such as identified in our study. Based on the identified intermediate productsit is proposed that PETN degradation in the aerobic condition follows a successive reductive degradation pathway with the release of NO_2^- in each denitrification step. Since there is no addition of nitrogen source, our study conducted under nitrogen limited condition. Growth of degrading bacteria and successive degradation of PETN indicated that these degrading bacteria have ability to use of PETN as source of nitrogen. Result of other researchers [9, 24] confirmed our result.

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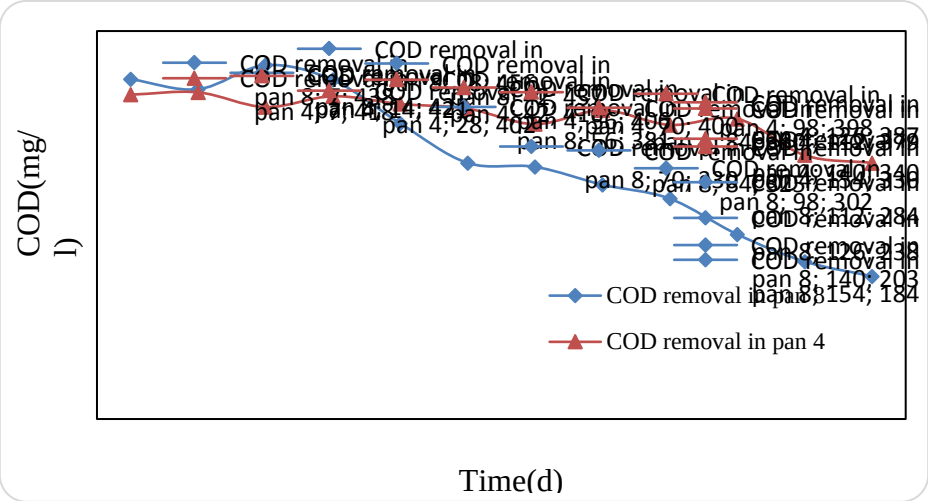

Fig.3. Effect of rhamnolipid on COD removal. Pan 8 contained rhamnolipid and pan 4 contained no rhamnolipid.

CONCLUSION

This study showed the removal of PETN from the soil in aerobic conditions. Application of rhamnolipid biosurfactant was effective in biodegradation of PETN and its mineralization and caused higher removal rate in shorter time. Also rhamnolipid was useful in lowering of acclimation time for degrading bacteria. Activated sludge obtained from textile wastewater treatment plant was a good source of nutrient for degrading bacteria. This study show that application of this method is promising option for bioremediation of explosives contaminated area.

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