

BIODEGRADATION OF TRINITROTOLUENE BY IMMOBILIZED *BACILLUS* SP. YRE1

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Abstract

The present study focus on the biodegradation of trinitrotoluene (TNT) by *Bacillus* sp. YRE1 isolated from red effluent in free state and also cells immobilized on charcoal and polystyrene. TNT degradation was monitored weekly for 168 hours, at 262 nm. Immobilized *Bacillus* sp. YRE1 was checked for its ability to degrade TNT by exposing it to different temperatures. It was found that both charcoal and polystyrene immobilized bacteria degraded TNT more efficiently at 37°C. Maximum percentage reduction in case of charcoal immobilized *Bacillus* sp. YRE1 at 37°C was calculated as 73.35%. Whereas, polystyrene immobilized bacteria showed 70.58% reduction. *Bacillus* sp. YRE1 immobilized on charcoal, showed maximum degradation at pH 7 with 93.81% reduction in TNT. Similarly, pH 5 was found to be optimum for the degradation of TNT by polystyrene immobilized bacteria, with percentage reduction as 94%. Charcoal immobilized cells showed increased transformation with 96% reduction in the presence of Tween 20, whereas, polystyrene immobilized cultures showed 87.77% reduction in TNT.

Introduction

Contamination of soil and water resources by explosives is mainly the result of ineffective (if any) treatment of wastewater generated during explosives manufacturing and inappropriate waste-disposal practices (Pennington *et al.*, 2001). The biodegradation of 2,4,6- trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), the most widely used nitro-organic explosives detected in soil and groundwater (Hawari *et al.*, 2000), has received a great deal of attention. Several bioremediation methods for explosives contaminated soils, such as soil-slurry reactors, composting and land farming, have been developed (Clark & Boopathy, 2007) and have shown that microbial activity significantly influences the fate of explosives.

2,4,6-Trinitrotoluene contamination is a major environmental concern due to its toxicity and mutagenicity. TNT is not only a source of environmental contamination, but it also remains potentially explosive for years after it is produced (Yinon, 1990; Collie *et al.*, 1995). Some of the nitro aromatics and nitramines that have been found in the vicinity of munitions plants are known to be mutagenic, carcinogenic or otherwise toxic to aquatic and terrestrial organisms (McCormick *et al.*, 1976; Kaplan & Kaplan, 1982). The presence of the highly toxic and persistent explosive compound 2,4,6-trinitrotoluene (TNT) in groundwater, soils and sediments has caused extensive environmental degradation and pollution worldwide (Rieger & Knackmuss, 1995; Sheremata *et al.*, 1999; Halasz *et al.*, 2002; Lewis *et al.*, 2004).

Bioremediation is a natural process that uses microorganisms and plants to transform hazardous materials into more benign substances (Halasz *et al.*, 2002). 2, 4, 6-

Trinitrotoluene (TNT) and other explosive compounds are major soil contaminants at many former military installations. These compounds are very recalcitrant and research has been done on their biodegradation and transformation (Boopathy *et al.*, 1993; Gorontzy *et al.*, 1994; Kaplan & Kaplan, 1982; Khan *et al.*, 1997; Pasti-Grigsby *et al.*, 1996). The biodegradation pathways of TNT as single compounds have been thoroughly described in the literature (Bernstein *et al.*, 2008; Balakrishnan *et al.*, 2003; Esteve-Nuñez *et al.*, 2001; Fournier *et al.*, 2004; Yin *et al.*, 2005). The initial products formed from TNT biotransformation are reduced amino derivatives, such as 4-amino-2,6-dinitrotoluene (4-Am-2,6-DNT) and 2-amino-4,6-dinitrotoluene (2-Am-4,6-DNT) (Hawari *et al.*, 2000). Both aerobic and anaerobic bacteria consortia and isolates are believed to utilize reductases that are responsible for TNT degradation (Bradley *et al.*, 1994). For the past two decades, a search for a suitable microbe that could mineralize TNT has been going on. TNT is known to be retained for longer time in soil and water, and hence it poses an environmental hazard.

Immobilized cells have been used extensively in the production of useful chemicals, the degradation of wastewaters (Chibata *et al.*, 1986; Bisping & Rehm, 1988), and the bioremediation of numerous toxic chemicals including phenol (Bisping & Rehm, 1988), 4-chlorobenzoate (Sahasrabudhe *et al.*, 1988), 4-chlorophenol (Balfanz & Rehm, 1991), pyridine (Lee and Long, 1974).

The present study was designed to isolate the microorganisms from red effluent which have the ability to degrade TNT, to compare the degradation of TNT by bacterial cells in free state and immobilized on different inert materials, to study the effect of pH, temperature and surfactants on biodegradation of TNT by immobilized cells.

Material and Methods

Microorganism: In the present study the bacterial strain, *Bacillus* sp. YRE1 was used to check its potential for degradation of 2,4,6-trinitrotoluene in free and immobilized state. This strain was previously isolated by Yasin *et al.*, (2007) from red effluent, collected from outside the ammunition factory, Pakistan. The morphological and biochemical identification (Holt, 1993) and the entire experimental work was carried out at Microbiology Research Laboratory, Quaid-i-Azam University, Islamabad.

Analytical method: Concentration of trinitrotoluene was calculated by the absorbance at 262nm. An Agilent UV-Visible Recording Spectrophotometer UV-8453 was used. Concentration of 2,4,6-trinitrotoluene was calculated from the absorbance maximum at 262nm. Samples were taken at different time intervals on daily basis. Bacterial degradation was determined by measuring the absorbance of samples at 262nm (Yasin *et al.*, 2007).

Whole cell immobilization of bacillus sp. YRE1 using charcoal granules: *Bacillus* sp. YRE1 culture was refreshed by culturing on nutrient agar plates. About 5ml nutrient broth was taken in test tube, autoclaved at 121°C and 15 psi for 15 minutes, inoculated with a loop of *Bacillus* sp. YRE1 and incubated at 37°C for 24 hours in an orbital shaker at 120 rpm. About 95 ml of nutrient broth was prepared in a flask, sterilized and inoculated by 5ml of the inoculum and incubated at 37°C for 24 hours.

For the immobilization of *Bacillus* sp. YRE1 on charcoal granules (2 nm), the granules were sterilized and added to 100 ml of medium inoculated with *Bacillus* sp. YRE1. This mixture was incubated at 37°C for 36 hours for cells immobilization. After 36 hours, media was discarded and charcoal granules having immobilized cells were

washed two times by sterilized distilled water. These charcoal granules were then added into 250 ml flask containing 150 ml mineral salt medium and 50 ppm 2,4,6-trinitrotoluene having pH 7.4 under aseptic condition. The medium was then incubated in orbital shaker (150 rpm) at 37°C and the samples were taken after every 24 hours upto 168 hours on daily basis. Samples were analyzed spectrophotometrically for 2,4,6-trinitrotoluene degradation at 262nm.

Optimization of various parameters for biodegradation of TNT by whole cells immobilized on charcoal granules

Effect of temperature on biodegradation of TNT (2,4,6-Trinitrotoluene): The experiments for shake flask transformation of 2,4,6-Trinitrotoluene were performed at different temperatures (30, 37 and 50°C) for *Bacillus* sp. YRE1 with 50 ppm TNT concentration. Samples were collected at 0, 24 to 168 hours. Samples were analyzed spectrophotometrically for 2,4,6-trinitrotoluene disappearance at 262 nm.

Effect of pH on biodegradation of 2, 4, 6-Trinitrotoluene: A set of experiment was conducted to demonstrate the effect of pH on the transformation of TNT. Culture of *Bacillus* sp. YRE1 was incubated with 50ppm TNT concentration in mineral salt broth at different pH values (7, 7.4, 8 and 8.5). The flasks were incubated at 37°C (optimizing temperature) in a shaker at 150 rpm and samples were collected periodically at 0, 24 to 168 hours. Samples were analyzed spectrophotometrically for 2,4,6-Trinitrotoluene disappearance at 262nm.

Effect of surfactant on biotransformation of 2,4,6-Trinitrotoluene: An experiment for shake flask transformation of 2,4,6-Trinitrotoluene was performed with Tween 80 (3%) and Tween 20 (2%) at 50 ppm 2,4,6-TNT concentration to study the effect of surfactants. Samples were collected at 0, 24- 168 hours. Samples were analyzed spectrophotometrically for 2,4,6-trinitrotoluene disappearances at 262nm.

Whole cells immobilization of *Bacillus* sp. YRE1 using polystyrene film: *Bacillus* sp. YRE1 culture was refreshed by culturing on nutrient agar plates. About 5ml medium (nutrient broth) was taken in test tube and autoclaved at 121°C and 15 psi for 15 minutes and inoculated with a loop of *Bacillus* sp. YRE1 and incubated at 30°C for 24 hours in orbital shaker at 120 rpm. Nutrient broth (95 ml) was sterilised and inoculated by 5ml of the inoculum and incubated at 30 °C for 24 hours.

About 0.8 g of packing material (polystyrene) was dissolved in 20 ml chloroform for thin film formation. After film formation, it was cut into pieces and gently washed by spirit for sterilization and then washed with sterilized distilled water. Sterilized pieces of film were put into the 100 ml culture and incubated at 30°C in orbital shaker at 150 rpm for 36 hour for cells immobilization. After 36 hours, medium was discarded and were washed the film pieces of polystyrene having immobilized cells two times by sterilized distilled water. These washed film pieces were then added into 250 ml flask containing 150ml mineral salt medium and 50ppm 2,4,6-trinitrotoluene having pH 7.4 in aseptic condition .The medium was then incubated in orbital shaker150 rpm at 30°C and the samples were taken after 24 up to 168 hours on daily basis. Samples were analyzed spectrophotometrically for TNT degradation at 262nm.

Optimization of various parameters for biodegradation of TNT by whole cell immobilization using polystyrene film

Effect of temperature on biodegradation of TNT (2,4,6-Trinitrotoluene): The experiment for shake flask transformation of 2,4,6-trinitrotoluene was performed at different temperatures (30, 37 and 50°C) for *Bacillus* sp. YRE1 with 50 ppm 2,4,6-TNT concentration. Samples were collected at 0, 24, 48, 72, 96, 120, 144 and 168 hours. Samples were analyzed spectrophotometrically for 2,4,6-trinitrotoluene disappearance at 262 nm.

Effect of pH on biodegradation of trinitrotoluene: A set of experiments was conducted to demonstrate the effect of pH on the transformation of 2,4,6-trinitrotoluene. Culture of *Bacillus* sp. YRE1 was incubated with 50 ppm 2,4,6-Trinitrotoluene TNT concentration in mineral salt broth at different pH values (7.0, 7.4, 8.0 and 8.5). The flasks were incubated at 37°C in a shaker at 150 rpm and samples were collected periodically at 0, 24 to 168 hour. Samples were analyzed spectrophotometrically for TNT disappearance at 262 nm.

Effect of surfactants on biodegradation of 2,4,6-Trinitrotoluene: An experiment for shake flask transformation of 2,4,6-trinitrotoluene was performed with surfactants; Tween 80 (3%) and Tween 20 (2%) with 50 ppm 2,4,6-trinitrotoluene. were used to study the effect of surfactants. Samples were collected at 0, 24 to 168 hours. Samples were analyzed spectrophotometrically for 2,4,6-trinitrotoluene disappearances at 262 nm.

Results

Bacillus sp., YRE1, isolated from red effluent, was selected to check their ability to transform nitroaromatic compounds. The present research was conducted to exploit the potential of this bacterial strain for the transformation of 2,4,6-trinitrotoluene in free and immobilized state. *Bacillus* sp. YRE1 was isolated from effluents collected from area contaminated with TNT outside the ammunition factory *Bacillus* sp. YRE1 was identified previously in Microbiology Research Laboratory Quaid-i-Azam University, Islamabad. The strain was found to be capable for degradation of 2, 4, 6-TNT.

Optimization of various parameters for biodegradation of TNT by whole *Bacillus* sp. YRE1 immobilized on charcoal granules

Effect of temperature on biodegradation of TNT: *Bacillus* sp. YRE1, immobilized on charcoal, was used to check degradation of TNT at different temperatures (30, 37, and 50°C) in mineral salt medium. Maximum degradation or transformation was observed at 37°C. Absorbance recorded at 37°C was 0.69 after 168 hours (Fig. 1). Maximum percentage reduction at 37°C was 73.35%. At 30°C and 50°C reduction was not very considerable.

Effect of pH on biodegradation of TNT: The *Bacillus* sp. YRE1 immobilized on charcoal was subjected to trinitrotoluene transformation at 50 ppm TNT concentration at different pH values (7.0, 8.0 and 8.5) in mineral salt medium. This strain showed maximum degradation at pH 7.0 giving absorbance value of 0.12 after 168 hours in comparison with degradation at pH 8.0 and pH 8.5 (OD as 0.60 and 0.41 respectively, at 262 nm), after 168 hours (Fig. 2). Percentage reduction recorded at pH 7.0 was 93.81%. The percentage reduction at pH 8.0 and pH 8.5 were 2 % and 74% respectively.

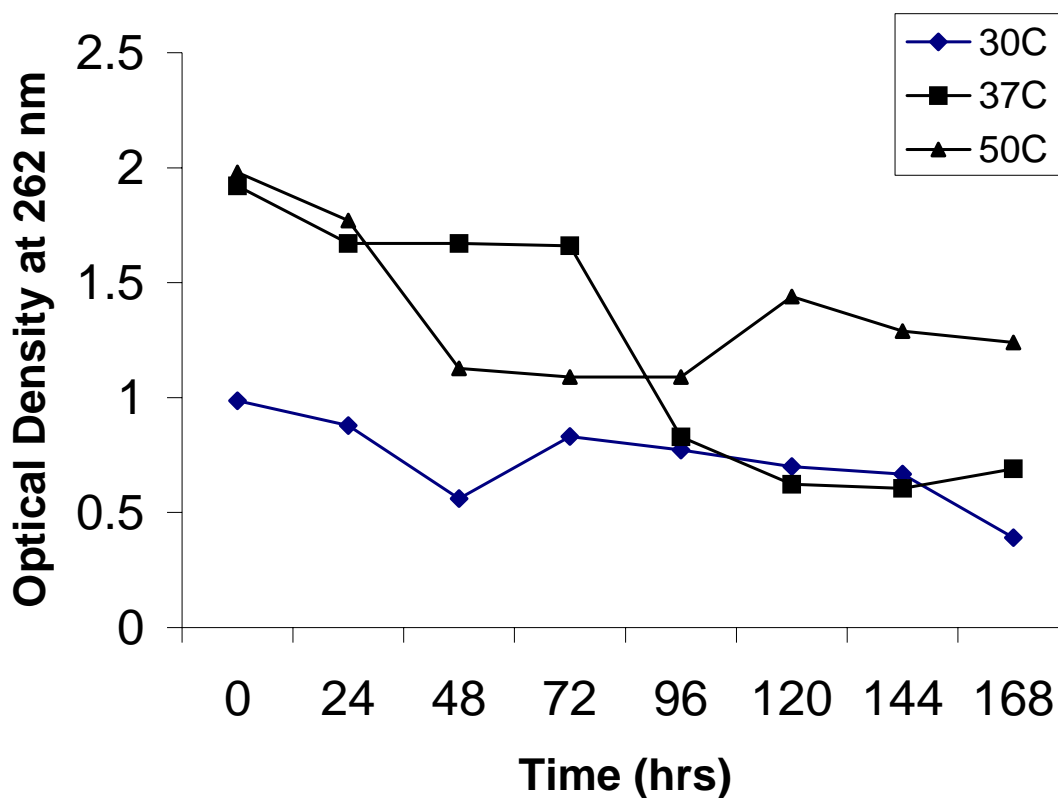


Fig. 1. Effect of temperature on biodegradation of TNT at 50 ppm concentration by charcoal immobilized *Bacillus* sp., YRE1.

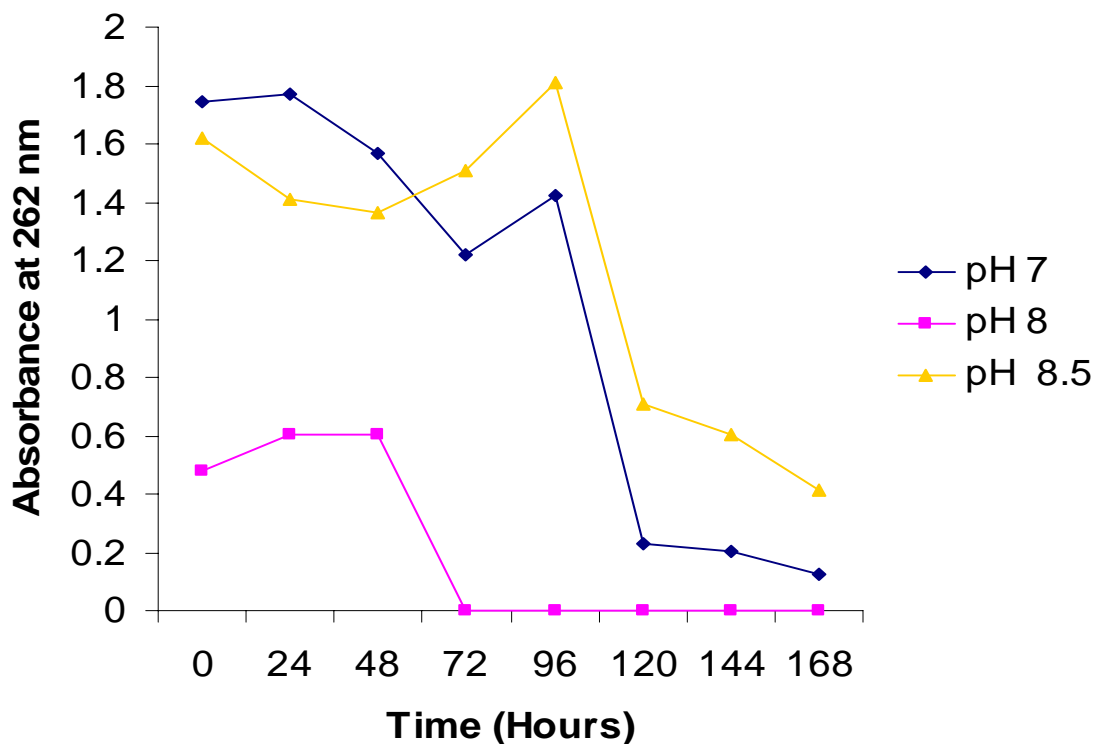


Fig. 2. Effect of pH on biodegradation 2,4,6-Trinitrotoluene at 50ppm concentration by charcoal immobilized *Bacillus* sp., YRE1.

Effect of surfactants on biodegradation of TNT: To study the effect of surfactants, TNT was subjected to transformation in the presence of Tween 80 and Tween 20. The immobilized cultures in the presence of Tween 20 showed more biodegradation with percentage reduction of 96% in comparison with cultures containing Tween 80, showing 82.11% percentage reduction. Absorbance value for Tween 80 was 0.27 and Tween 20 was 0.10, respectively, after 168 hour (Fig. 3). The medium supplemented with Tween 20 showed more reduction in TNT as compared to Tween 80.

Optimization of various parameters for biodegradation of TNT by *Bacillus* sp. YRE1 immobilized on polystyrene film

Effect of temperature on biodegradation of TNT: Trinitrotoluene was subjected to degradation by *Bacillus* sp. YRE1 at different temperatures (30, 37, and 50°C) in mineral salt medium. Maximum degradation or transformation was observed at 37°C. Absorbance value recorded at 37°C was 0.10 after 168 hours. Absorbance values observed at 30°C and 50°C were 0.50 and 0.71, respectively, after 168 hour (Fig.4). Percentage reduction at 37°C was 70.58% whereas, at 30°C and 50°C reduction recorded was 46.80% and 5.5%, respectively, after 168 hours.

Effect of pH on biodegradation of TNT: The selected strain of *Bacillus* sp. YRE1 was subjected to trinitrotoluene degradation at 50 ppm TNT concentration at different pH values (4, 5 and 6) in mineral salt medium. This strain showed maximum degradation at pH 5 giving absorbance value observed as 0.04 after 168 hours in comparison with degradation at pH 4.0 and pH 6 giving absorbance values of 3.1 and 0, respectively, after 168 hours (Fig. 5). Percentage reduction recorded at pH 5.0 was 94%. The percentage reduction at pH 4 and pH 6 were 6% and 0%, respectively, after 168 hours.

Effect of surfactants on biodegradation of TNT: To study the effect of surfactants, TNT was subjected to degradation with Tween 80 and Tween 20. Result indicated that the cultures with Tween 20 showed more degradation with percentage reduction of 87.77% in comparison with cultures with Tween 80, showing, 71.06% percentage reduction. Absorbance values for Tween 80 reduced from 0.38 to 0.11 and Tween 20 were from 1.2 to 0.15 (Fig. 6). The medium supplemented with Tween 20 showed more reduction as compared to Tween 80.

Discussion

Microbial transformation of TNT by aerobic, anaerobic or combined pathways had been well demonstrated (Funk *et al.*, 1993; Bruns-Nagel *et al.*, 1994; Esteve-Nuñez & Ramos, 2001). Some of these microorganisms reported to degrade or transform TNT under aerobic conditions included *Bacillus* sp. Nitrite release from TNT and transformation to 2-amino-4-nitrotoluene (Kalafut *et al.*, 1998).

It has been well documented that the high productivity obtained in immobilized cells is partially due to high-cell density as well as immobilization-induced cellular or genetic modifications. It is noted that there is a high density of cells in the immobilized system, which contributed to the higher fermentation rate. Therefore a higher concentration of substrate is converted to final products in the immobilized cell fermentation (Zhu & Yang, 2003). The cometabolic transformation of 2,4,6-trinitrotoluene (TNT) by an immobilized *Phanerochaete chrysosporium* culture was investigated, 100% TNT biotransformation was achieved at 1,100 mg L⁻¹ d⁻¹ glycerol feeding rate (Rho *et al.*, 2001). In the present work an attempt has been made to increase the efficiency of TNT degradation by a *Bacillus* sp. YRE1 immobilized on inert material like charcoal and polystyrene.

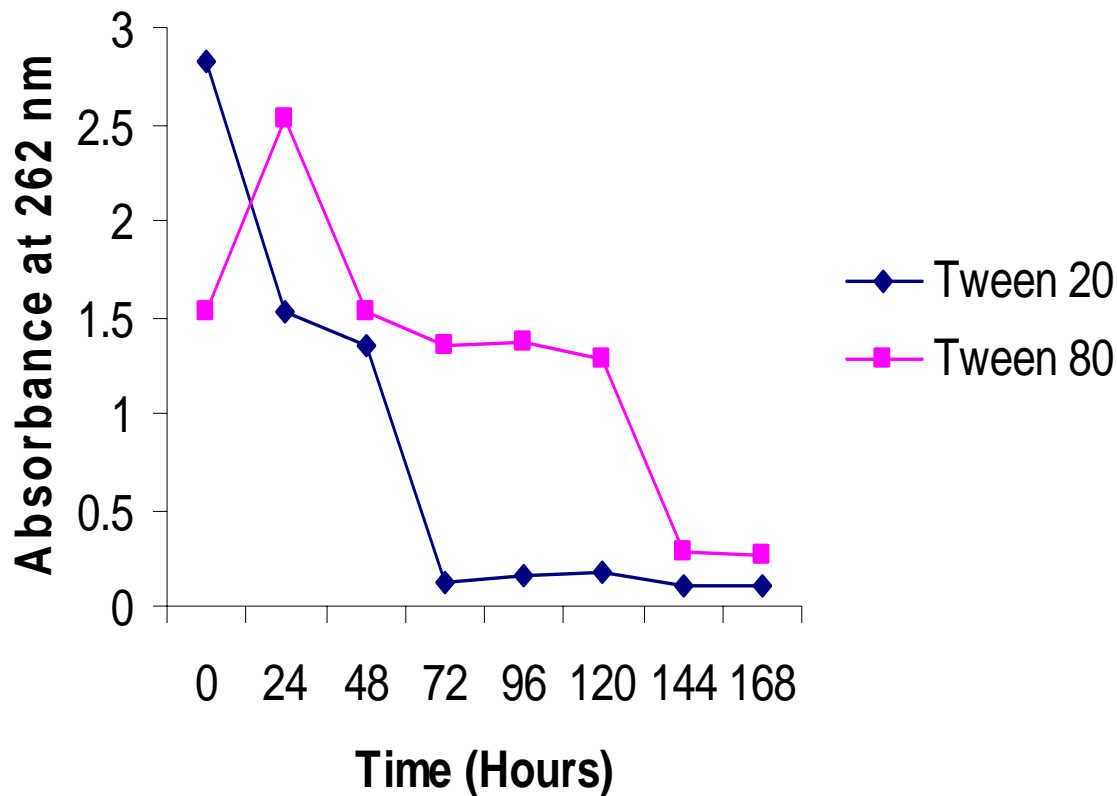


Fig. 3. Effect of surfactants on biodegradation of TNT at 50ppm concentration by charcoal immobilized *Bacillus* sp., YRE1.

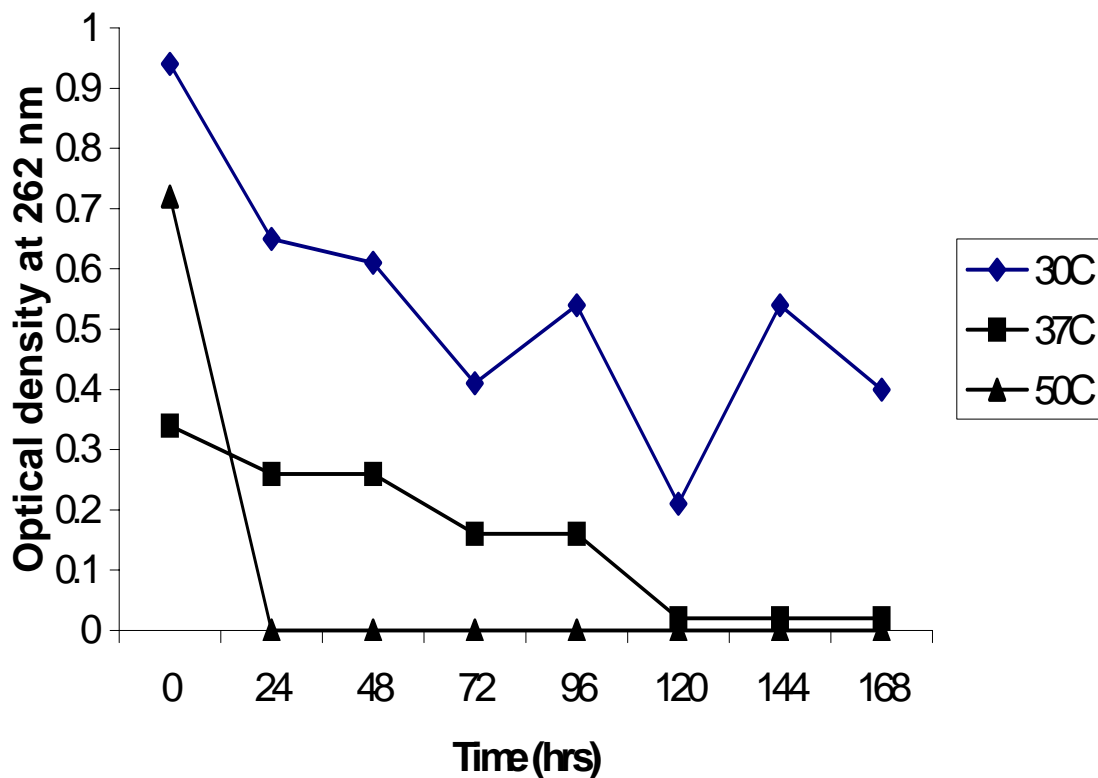


Fig. 4. Effect of temperature on biodegradation of TNT at 50ppm concentration by polystyrene immobilized *Bacillus* sp., YRE1.

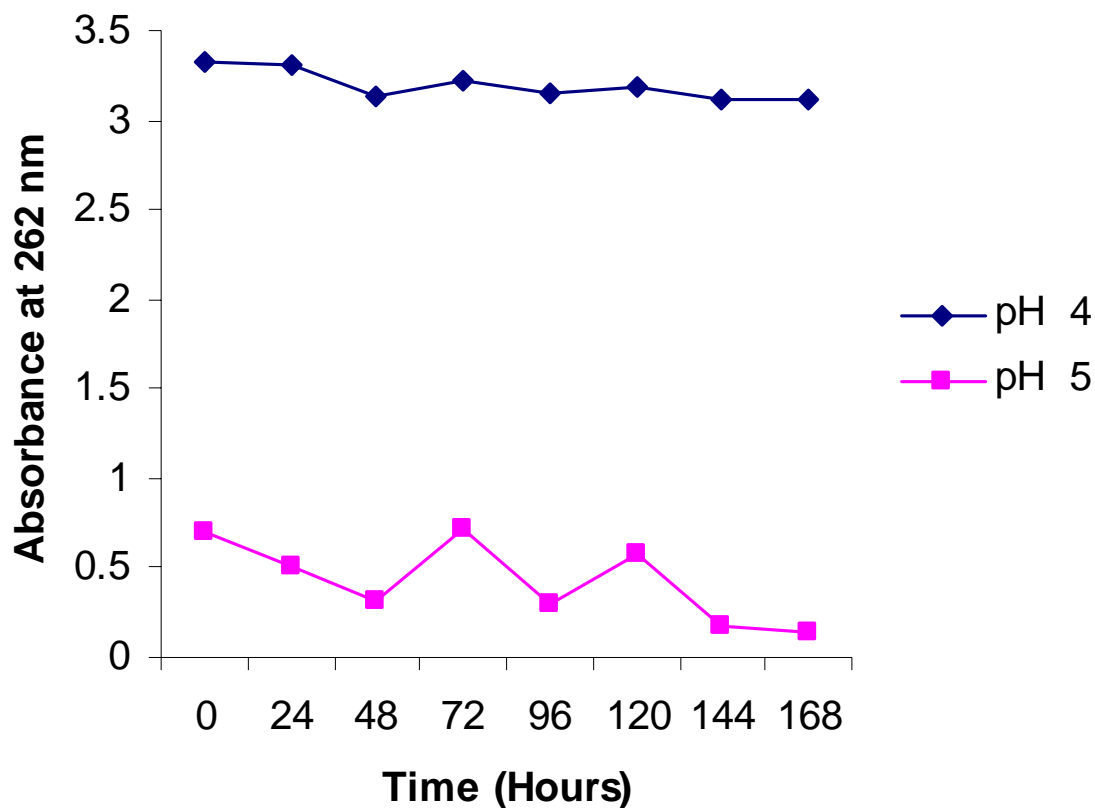


Fig. 5. Effect of pH on biodegradation 2,4,6-Trinitrotoluene at 50ppm concentration by polystyrene immobilized *Bacillus* sp., YRE1.

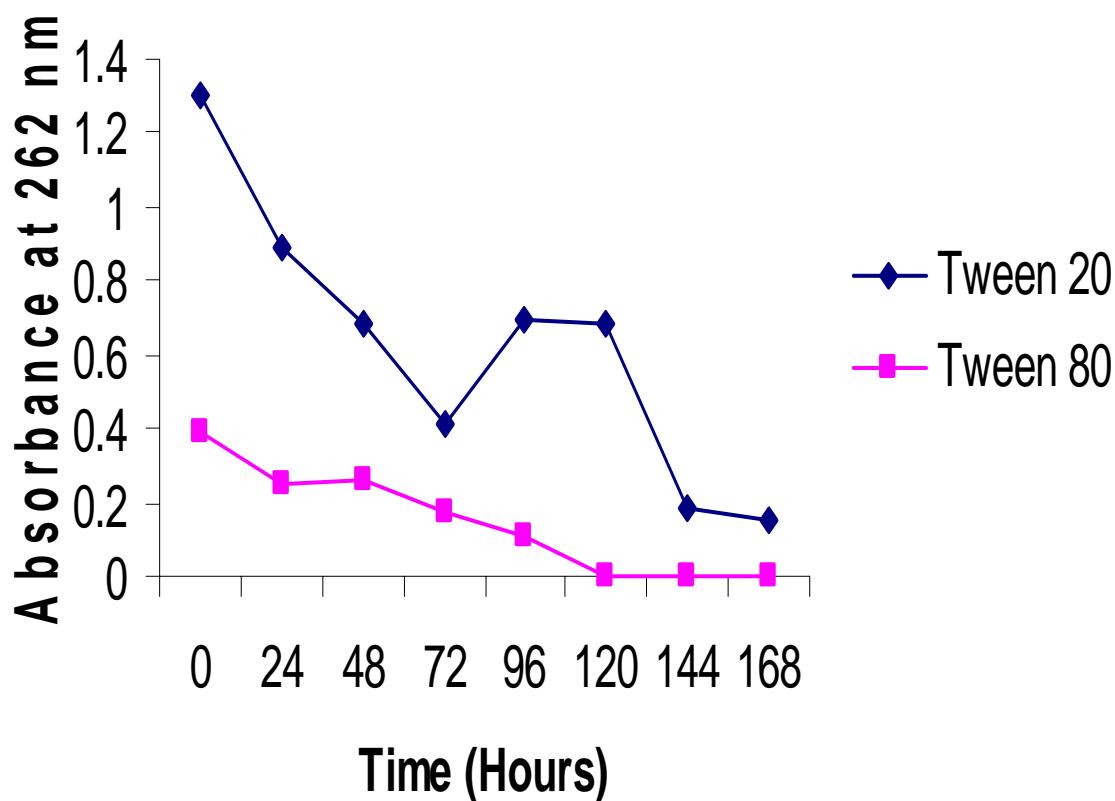


Fig. 6. Effect of surfactants on biodegradation of TNT at 50ppm concentration by polystyrene immobilized *Bacillus* sp., YRE1.

In the present study, the selected charcoal immobilized strain of *Bacillus* sp. YRE1 was subjected to degradation of TNT at 50 ppm concentration at different pH values (7.0, 8.0 and 8.5) in mineral salt media and polystyrene immobilized bacteria at different pH values (4.0, 5.0 and 6.0). The results showed that TNT transformation by the isolated strain was pH dependent. Both charcoal and polystyrene immobilized *Bacillus* sp. YRE1 showed best TNT reduction at pH 7.0 and pH 5.0, respectively, whereas Sublette *et al.*, (1992) observed cultures incubated at different pH values (6.0, 7.0, 7.5, 8.0 and 9.0). They observed that 2,4-DNT (TNT product) transformation by the isolated strains was pH dependent. Degradation was monitored for 168 hours at different pH values. Both *Pseudomonas* and *Bacillus* strains showed best 2,4-DNT reductions at 7.5 pH value. Oh *et al.*, (2003) reported that the optimal pH range for TNT transformation was between 7.0 and 8.0. pH has a significant influence on the transformation of the TNT-hydride complexes. While transformation of TNT to DNT occurs at similar rates in treatments with initial pH values of 6 and 7, this reaction is inhibited at pH values below 4 (Finogenova *et al.*, 2005).

In the present study, trinitrotoluene was subjected to transformation by *Bacillus* sp., YRE1 immobilized on charcoal and polystyrene at different temperatures (30, 37, and 50°C) in mineral salt medium. Optimum temperature for TNT degradation by charcoal and polystyrene immobilized bacteria *Bacillus* sp. YRE1 was 37°C. At low temperature (30°C) TNT removal was slower than at 37°C with maximum TNT degradation. At 50°C TNT removal decrease significantly. Similar results were obtained by Kalafut *et al.*, (1998) which stated that optimal temperature for TNT reduction by *Bacillus* sp., and *Staphylococcus* sp., was 37°C. Boopathy *et al.*, (1993) also observed that the incubated culture at 20°C, 25°C, and 30°C showed better performance than other cultures

In our study, the effect of surfactants (Tween 20 and Tween 80) on TNT degradation was observed with culture containing charcoal and polystyrene immobilized *Bacillus* sp. YRE1. The result showed better TNT degradation in medium containing charcoal immobilized bacteria supplemented with Tween 20 with percentage reduction as 96%, whereas, the culture containing Tween 80 showed 82% reduction. Similarly, medium containing polystyrene immobilized bacteria supplemented with Tween 20 showed more TNT reduction up to 87.09% as compared to the culture with Tween 80 that showed 71.7% reduction. The effect of a nonionic surfactant (Tween 80) on 2,4,6-trinitrotoluene (TNT) mineralization by the white-rot fungus *Phanerochaete chrysosporium* strain BKM-F-1767, was investigated in a liquid culture at 20, 50, and 100 mg TNT·L⁻¹. The presence of 1% (w/v) Tween 80, at 20 mg·L⁻¹ TNT, added to a 4-d-old culture, allowed the highest TNT mineralization level, that is 29.3% after 24 days (Hodgson *et al.*, 2000).

Immobilization of pure cultures has a significant role to play in industrial processes but their significance in degradations has not been fully exploited. An immobilized consortium or single bacteria can be viable strategy for degradation of TNT found in red and yellow water of TNT- producing units.

Conclusions

Bacillus sp. YRE1 effectively grows and degrades TNT optimally at 37°C. Maximum percentage reduction at 37°C was 73.35% by charcoal immobilized *Bacillus* sp., YRE1 and 70.58% by polystyrene immobilized bacteria. Optimum pH for TNT degradation was found to be 7.0 in case of bacteria immobilized in charcoal and 5.0 in case of polystyrene immobilized bacteria. The maximum percentage reduction of TNT in the presence of Tween 20 was found to be 96% in case of charcoal immobilized *Bacillus* sp., YRE1.

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