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Evaluation of degradation potential of Free cell & Immobilized cell using Shake flask technique and lab scale Bioreactor technique for remediation of Nitroaromatics in aqueous phase

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ABSTRACT

In this study, Free cell & Immobilized cell using Shake flask technique and lab scale Bioreactor technique was evaluated for the remediation of TNT in aqueous phase. In shake flask study, all samples with Free and Immobilized cell culture were kept in a shaker for 24 hours at 120 rpm at room temperature whereas the immobilized cell bioreactor was set at airflow 1 ml/l, agitation 50-60 rpm for 8 hours at 25 °C. The quantitative analysis by HPLC in shake flask study revealed 82.37% and 98.12% of TNT degradation by Free cell culture and immobilized cell culture respectively after 24 hours and 81.19% of TNT degradation was found in Bioreactor. The results depicted that Immobilized cell culture had better performance than free cell culture and immobilized cell bioreactor proved more effective than shake flask culture technique.

Key words*: Shake flask technique, Immobilization, TNT, Bioreactor, HPLC.*

Introduction

In the current scenario, presence of wide range of organic chemicals in environment has become a global concern throughout the world. Organic chemicals released from water/solid wastes is one of the dangerous contaminants causing threat to the environment and becoming very difficult to control and mitigate. 2,4,6- Trinitrotoluene (TNT) is a nitroaromatic explosive that enters into environment from solid/liquid waste resulting from processing of munitions at large scale.

During the manufacturing of TNT, large volume of water is required for purification (Levsen *et al.,* 1993) and this aqueous waste water (red water), contains up to 30 nitroaromatics besides TNT (Urbanski, 1985). In addition, during loading, packing or assembling munitions, a high concentration of other nitroaromatic explosive is released to the water (NDCEE, 1995). The toxic, carcinogenic, mutagenic and chemical persistency properties of TNT may cause major environmental hazards. Thus, the degradation of TNT is still a challenge for environmental research (Spain and Hughes, 2000).

Several chemical methods have been widely used to treat TNT contained wastewater or soils including advanced oxidation, incineration (Marcio *et al.,* 2009), adsorption (An FQ *et al.,* 2009). These methods have their own limitations. Among other methods, bioremediation method is a cost effective, feasible and environmentally safe practice (Hawari *et al.,* 2000). Thus, microbial bioremediation is an effective method due to its diverse metabolic pathways and versatility of the microorganism.

The use of Free cells for degradation of various toxic compounds for industrial applications has a number of disadvantages in comparison to immobilized cells. Different methods of cells immobilization can be used as covalent binding, adhesion, adsorption, aggregation, entrapment using diverse support matrices (Su *et al.,* 2006), such as sodium alginate (Bazot and Lebeau, 2009; Ya˜nez-Ocampo *et al.,* 2009), polyvinyl alcohol (PVA) (Cunninghama *et al.,* 2004), activated carbon, zeolite (Liang *et al.,* 2009), and vermiculite (Su *et al.*, 2006). Among these, Entrapment within insoluble calcium alginate has been found more effective due to its simple non-toxic, low cost, easily available and effective particle size.

The objective of present study was to evaluate and compare the degradation of TNT by free cells and immobilized cells of *Pseudomonas* sp. in sodium alginate beads using Shake flask and bioreactor.

Materials and Methods

Chemicals

2, 4, 6- Trinitrotoluene (TNT) was used in pure form. All the chemicals and reagents were used of analytical grade.

Microorganisms and culture media

Pseudomonas sp. was isolated from TNT contaminated (400 ppm) soil of *Vinca rosea* plant. Primary cultures were prepared using pour plate method in Nutrient agar media and secondary culture and Agar slants were also prepared. The bacterium was morphologically characterized as *Pseudomonas* sp. by staining techniques.

Immobilization of Bacterial Cells in Sodium alginate beads

The sodium alginate (SA) entrapment of the cells was performed by adopting the protocol from Institute of Microbial Technology, Chandigarh. SA (2%, wt/vol) was dissolved in distilled water (60-70 ºC) and autoclaved at 121 ºC for 15 min. The distinct colonies of microbial cells were taken from streaked plates or agar slants and mixed into 0.1% saline solutions till solution became turbid. The cell suspension was mixed into SA solution in 1:1 ratio by stirring on a magnetic stirrer. This SA cell mixture was extruded drop by drop in sterile $CaCl_2$ (1%, wt/vol.) solution and kept immersed in calcium chloride solution for 24 hours to get harden. Immobilized sodium alginate beads of approximately 3 mm diameter were obtained and beads were washed several times with distilled deionized water and pat dried with filter paper.

Evaluation of degradation potential of free and immobilized microbes using Shake flask study of TNT solution range

A range of TNT solutions of 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm concentration was prepared in 100 ml reagent bottles and added constituents of trypticase soy broth into it. In Shake flask study using Free microbial cells, 10-12 loopful scoops of microbial cells were inoculated to each 100 ml reagent bottles whereas in Immobilized study, 30 beads per sample were used. All these samples were kept in a shaker for 24 hours at 120 rpm at room temperature.

Lab scale Bioreactor study on evaluation of degradation potential of immobilized microbes of TNT solution range

In the present study, a type of Immobilized Cell Bioreactor (Fluidized Bed Bioreactor) made of borosilicate glass and stainless steel was used. The reactor was 25 cm in height and 16 cm in diameter. It had removable head space lid made up of stainless steel and vessel made up of borosilicate. It had mechanical agitation system consisting of a shaft with 2 impellers and 3 baffles. Air sparger had sintered disc at the base and the sampling port 30 cm from bottom. The reactor was fed with 3.5 litre 20 ppm TNT with constituents of trypticase soy broth (casein peptone – 17 g/l, soya peptone – 3 g/l, sodium Chloride – 5 g/l, di- Potassium Phosphate – 2.5 g/l and dextrose – 2.5 g/l) and 50 g sodium alginate beads. The experiment was set under controlled conditions of temperature – 25 °C, airflow – 1 ml per litre and agitation – 50-60 rpm for 8 hours.

Analysis of TNT Concentration

TNT degradation potential of Free microbial cells and Immobilized microbial cells was evaluated using high-performance liquid chromatography (HPLC). In shake flask study, 5 ml supernatant was taken after 24 hours, from each 100 ml reagent bottle of various concentrations and added 10 ml acetonitrile in ambered colored vials. In lab scale bioreactor study, 5 ml sample was taken after every 2 hours during 8 hours study. The samples were sonicated for one hour at 18 °C and kept undisturbed for 15-20

minutes. The sample of 3 ml supernatant was mixed with 3 ml HPLC water and filtered by using 0.45 micrometer Teflon filter.

Analytical separation was carried out using Flexar HPLC of Perkin Elmer Inc. C18 column, (3 ìm, 150 x 4.6 mm) was used as stationary phase and the mobile phase was methanol: water (50:50 v/v) mixture at a flow rate of 1 ml/min. Detection was performed by Photo diode array detector.

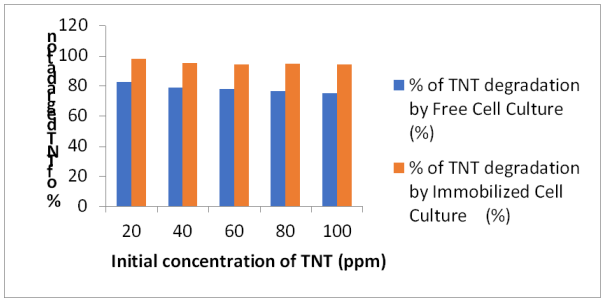

Fig. 1. Percentage of TNT degradation by Free and Immobilized cell culture

Results and Discussion

The present study involved the evaluation of degradation potential of Free cells, immobilized cells in shake flask and lab scale bioreactor on degradation of TNT solution range.

The quantitative analysis by HPLC, revealed the percentage of TNT degradation in case of shake flask study using free cells was 82.37%, 79.00%, 77.89%, 76.21% and 75.36% after 24 hours with respect to 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm as shown in Table 1 and Fig. 1. Whereas, in case of immobilized cell, the percentage of TNT degradation 98.12%, 95.23%, 94.12%, 94.79% and 94.07% was observed after 24 hours with respect to 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm as shown in Table 2 and Fig. 1.

TNT degradation by Free cell culture showed the significant degradation of TNT, but immobilized cells showed better performance as compared to free cells. The order of magnitude difference of TNT degradation was due to the physical entrapment of the cells in alginate gel beads.

In immobilized cell culture, the bacterial cells are two times more viable in comparison to free cells in TNT degradation. In case of immobilized cell culture, the immobilized material 'alginate' has increased the longer operating life times by providing enhanced stability to the cells. This binding agent is reusable and also simplifies the process of separation and recovery of the immobilized cells (Rahman *et al.*, 2006). The method 'entrapment' is an irreversible immobilization, and captured the cells by covalent or non-covalent bonds with in gel/matrix. The alginate -gelatin- calcium hybrid matrix provides the more mechanical stability to the cells and prevent the loss or leakage of viable cells but allowing the mass transfer of nutrients and metabolites (Datta

Table 1. TNT degradation after 24 hrs. by free cell culture of *Pseudomonas* sp.

Sr. No.	Initial conc. of TNT in samples (ppm)	R.T.(retention time in minutes)	Peak Area (µVsec)	Raw amount of TNT after 24 hrs (ppm)	% of TNT degradation after 24 hrs (%)
1	20	4.297	121312.54	3.526	82.370
2	40	4.144	288934.40	8.398	79.005
3	60	4.130	456625.07	13.272	77.890
4	80	4.228	654798.70	19.032	76.210
5	100	4.141	847742.74	24.64	75.360

Table 2. TNT degradation by Immobilized Cell Culture of *Pseudomonas* sp. after 24 hrs.

Sr. No.	Initial conc. of TNT in samples (ppm)	R.T(Retention time in minutes)	Peak Area (µVsec)	Raw amount after 24 hrs (ppm)	% of TNT degradation after 24 hrs (%)
1	20	4.105	14605.315	9.376	98.120
2	40	4.120	63456.96	1.906	95.235
3	60	4.124	133893.92	3.524	94.793
4	80	4.130	161784.94	4.165	94.126
5	100	4.238	230227.96	5.927	94.073

Table 3. Percentage of TNT degradation by immobilized cell culture in bioreactor

Sr. No.	Sample taken after every 2 hrs	20 ppm TNT (% degraded)	40 ppm TNT (% degraded)	60 ppm TNT (% degraded)
1	2	51.45	33.56	28.93
2	4	65.35	48.74	43.32
3	6	77.78	54.55	53.56
4	8	81.19	63.75	55.32

et al., 2013). Thus, it restricts the cellular mobility with in a defined space, thereby retaining catalytic activity; the particle structure allows contact between the substrate and microbes, greater cellular content in the support, enhanced cellular viability led to greater tolerance to high concentration of pollutants (Bhushan *et al.,* 2015).

In lab scale bioreactor study, experiments were performed for 20 ppm, 40 ppm and 60 ppm TNT concentrations for 8-hour duration. At the end of 8 hours, highest % of TNT degradation was observed for 20 ppm and lowest for 60 ppm as shown in Table 3. It showed as the concentration of the TNT increases, the percentage of TNT degradation decreases. It may be due to the lesser concentration of TNT made more favorable condition for bacteria and performed more efficiently than in higher concentration.

Buffiere *et al.*, 1995 has reported several factors which contribute to the treatment efficiency in a fluidized bed reactor. The first is the maximum contact between the liquid and the support medium. The second one is that the diffusional resistance of the liquid film is minimal due to the particle movement and fluid velocity. At the third place, the control and optimization of the biological film thickness is a major factor and lastly, it can be operated with a wide range of organic concentration and the degradation rates are proportional to this concentration.

The degradation of TNT in bioreactor showed less retention time in comparison to immobilized flask culture study. This can be achieved because of high biomass concentration and sufficient aeration to keep the gas, liquid and solid particles thoroughly mixed.

Conclusion

It was observed from the present study that immobilization technology was more effective technique. Immobilized Shake flask study was observed more effective than Free cell shake flask study. Moreover, bioreactor with immobilized cell culture showed more potential to degrade TNT in comparison to Shake flask study with immobilized and Free cell culture.

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