Screening and Characterization of Nitroglycerin Degrading Microorganisms

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Abstract

Biodegradation process is a novel and economically feasible one for the degradation of many toxic compounds present in the environment in a sustainable manner. Nitroglycerin degrading organisms were isolated from soil samples using enrichment technique. The nitroglycerin biodegradation assay was carried out using all the bacterial species capable of growing in minimal medium containing nitroglycerin. Among the 5 isolates, three isolates were found to be potent in nitroglycerin degradation. It was selected based on their efficacy in substrate utilization and spectrophotometric analysis. The crude enzyme was extracted from the selected isolates by cell lysis method. Nitroglycerin biodegradation assay was also carried out using the enzyme extracts and subjected to UV spectrophotometric analysis. The selected isolates were tentatively identified as Arthrobacter sp., Agrobacterium sp. and Pseudomonas sp., as per the standard methods. Based on the results obtained above, one potent isolate N5 (Pseudomonas sp.) was selected and utilized for chemical characterization studies. Chemical characterization of the degraded samples was done by FTIR analysis, which showed potential degradation of nitroglycerin carried out by Pseudomonas sp.

Keywords: Biodegradation; Biotransformation; Recalcitrant; Nitroglycerin, glycerol trinitrate; Flavoprotein nitroester reductase.

Introduction

Nitroglycerin (NG) or Glycerol trinitrate (GTN) is an aliphatic nitrate ester containing compound that is important for manufacturing of explosives and rocket propellants and as a pharmaceutical vasodilator. It is commonly found in the waste streams and soils of munitions and fire cracker manufacturing facilities and pharmaceutical plants (Husserl et al., 2010). Concerns about toxicity and explosion hazards have led to increased efforts to develop safe and cost effective methods for treating GTN laden waste streams. A number of early studies on environmentally fate of NG revealed toxicity to algae, invertebrates and vertebrates and further suggested that NG was recalcitrant to degradation (Kaldneris et al., 2011). NG affects the cardiovascular system, blood and nervous system of experimental animals and was suffered by hypotension, tremors, ataxia, lethargy etc. Acute exposure to NG can cause headache, nausea, vomiting, occasionally diarrhoea, sweating and light headedness. High exposure can cause abdominal cramps, vomiting, depression or mania, mental confusion, convulsions, paresis or paralysis, apasia, impaired vision, breathing difficulties, methaemoglobinemia and blue skin (Cyanosis), bradycardia, circulatory collapse or death (Mirecki et al., 2006). Chronic exposure to NG can lead to the development of tolerance, and sudden withdrawal from exposure can result in angina like chest pains which may be accompanied by malaise, weakness, vomiting, dizziness, headache or impaired vision. Sudden death may also result. Chronic exposure may also result in severe headache, hallucinations and skin rashes. Allergic contact dermatitis may occur secondary to topical exposure to NG (Yinon, 1990; EPA, 2007; Rittman and McCarty, 2001).

Historically, the destruction of energetic materials and explosive mixtures has been accomplished through open – air burning, detonation or incineration techniques. Physicochemical methods of GTN destruction involve adsorption on activated carbon followed by reduction with inorganic chemicals (e.g. Na₂SO₃) or by alkaline hydrolysis yield glycerol, nitrite or nitrate. However, these techniques suffer from high operational cost, the presence of excess reactants that remain dissolved in the effluent, and the necessity for secondary treatment to remove nitrogenous products. As more stringent environmental regulations are enacted at the state and federal levels, these techniques are no longer considered viable. Preference would therefore be given to environmentally friendly biological treatment methods, provided that a robust GTN biotransformation technology that ensures complete transformation i.e. completed denitrification without accumulation of glycerol dinitrates (GDN) or glycerol mononitrate (GMN) and economic practicability could be developed. Completed denitrification is preferred since GDNs and GMNs are more soluble than GTN (Marshall and White, 2001).

Few reports on the metabolism of NG have been demonstrated in bacterial species (Binks et al., 1996; Blehert et al., 1996; Spain et al., 2000). Husserl et al., (2010) reported on the ability of pure bacterial cultures to utilize GTN as sole Nitrogen source with the help of the enzyme called Flavoprotein nitroester reductase purified from two Pseudomonas species. Since the flavoprotein nitroester reductase enzyme production is often associated with the capability of microorganism to utilize nitroglycerin as substrate (Fox and Karpalus, 1994; French et al., 1994; Zenno et al., 1994; Binks et al., 1996; Blehert et al., 1997; Williams and Bruce, 2002).

NG was metabolized to DNG & MNG intermediates by various bacterial cultures (Goronzy et al., 1994; Husserl et al., 2010), and in some cases glycerol nitrates could be removed from the culture medium. Recently,
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The biodegradation of *Bacillus thuringiensis*, *Bacillus cereus* and *Enterobacter agglomerans* has been reported and a hydrolytic reaction mechanism was suggested based on studies of dialysed cell extracts (Meng et. al., 1995). The denitrification of NG by pure cultures of *Agrobacterium radiobacter* has been also reported and in vivo nuclear magnetic resonance measurements showed that both isomers of DNG accumulated with 1, 3 DNG preferred by a roughly 8:1 ratio (corresponding to selectivity for denitration at the C- 2 position (White et. al.,1996; Marshall et. al., 2004). The main objective of the present study is to reveal the biodegradation of nitroglycerin by the soil microorganisms.

**Materials and Methods**

**Isolation of Nitroglycerin degrading Microorganisms**

Soil sample collected was used as a source for isolating nitroglycerin degrading bacteria. An enrichment culture technique was used to isolate nitroglycerin degrading microorganisms. Nitroglycerin used in the present study was obtained from fire factory in Sivakasi. Erlenmeyer flask (250ml), containing 50ml of minimal medium was sterilized. 0.1% (w/v) nitroglycerin was added to the flask. One gram of the soil sample was added to the above contents and incubated on the shaker at 150rpm at 30°C. Repeated subculturing was carried out by adding 2ml of the enriched culture from the above flask to the fresh medium. This was done in step wise manner after every one week of incubation and the concentration of the nitroglycerin was increased from 0.1% to 0.5%. After subsequent enrichment the sample was inoculated on the minimal agar plate with nitroglycerin. The process was repeated twice to obtain pure culture. The pure cultures were then transferred to freshly prepared nutrient agar slants and stored at 4°C for further studies. Five different isolates were obtained by purification based on colonial morphology.

**Nitroglycerin Biodegradation Assay**

The enrichment media supplemented with 0.1% of nitroglycerin sample were used for the degradation studies. The nitroglycerin sample was prepared by filter sterilized in 0.2 micron membrane filter, in order to make them free from contamination. The inoculum used for the biodegradation studies were prepared by inoculating a loopful of isolated culture in 50ml of enrichment media, incubated in shaker at 37°C for 24 hrs. After incubation 10ml of inoculum was centrifuged and the resultant supernatant was discarded. The cells were washed in 50mM Phosphate buffer pH (7.0) and absorbance was read at 660nm to obtain an O.D. value of 0.2. Then 1ml culture suspension of five different isolates were inoculated in 5 separate conical flasks containing 50ml of enrichment medium supplemented with 0.1% nitroglycerin sample. The flasks were placed in rotatory shaker at 37°C and kept for 5 days of incubation.

**UV Spectrophotometric Analysis**

After incubation, the degraded samples using 5 isolates were subjected to UV spectrophotometric analysis. The absorption shown by the treated samples were compared with the peaks obtained for control broth containing nitroglycerin.

**Enumeration of substrate utilization**

Serial dilutions of overnight culture of 5 different isolates were made and the diluted samples were spread plated on the solidified minimal media coated with nitroglycerin as a sole carbon and energy source. The plates were incubated at 37°C for 48 – 72 hrs. The colony forming units were counted and calculated as the number of utilizing bacteria. All tests were carried out in duplicates.

**Selection of Bacterial Isolates**

The 3 isolates were selected from the UV spectrophotometric analysis and colony forming units obtained in substrate utilization.

**Nitroglycerin Biodegradation Assay using Selected Bacterial Isolates**

The nitroglycerin biodegradation assay for the selected bacterial isolates was carried as per the procedure referred before.

**Enzyme Extraction**

The enzyme flavoprotein nitroester reductase responsible for the complete denitrification of NG can be extracted by cell lysis method. The incubated samples obtained from nitroglycerin biodegradation studies were taken, centrifuged and the resultant supernatant was used for crude enzyme extraction. The separation of crude enzyme extract was done by precipitating the protein by adding 10% TCA solution to the supernatant solution obtained from the degradation studies.

**Nitroglycerin Biodegradation Assay using Enzyme extract**

The nitroglycerin biodegradation assay for the selected bacterial isolates by using their respective crude enzyme extracts was carried as per the procedure mentioned above. After incubation, the resultant samples were subjected to UV spectrophotometric analysis for determining the degradation ability shown by the enzyme extracts.

**Nitroglycerin Biodegradation Assay using Selected Bacterial Isolates**

The nitroglycerin biodegradation assay for the selected bacterial isolate (N5) was carried out again for chemical characterization as per the procedure referred before.

**Characterization of Bacterial Isolate**

The selected bacterial isolates were identified based on microscopic and biochemical characteristics and screened as per the guidelines of Bergey's Manual of Bacteriology (Holt et. al., 1994; Cappucino and Natelie Sherman., 1999).

**Chemical Characterization**

After incubation, control sample and the degraded sample were analyzed by Fourier Transform Infra-Red Spectroscopic (FTIR) analysis. FT-IR - 8400S, SHIMADZU model was used for the analysis and the spectrum was taken in the mid IR region of 400 – 4000cm⁻¹.

**Results**

The preliminary screening of nitroglycerin degrading bacterial strains was isolated by their growth in the presence of nitroglycerin as a criterion. By using enrichment culture technique, 5 native isolates from the soil sample were found to possess the ability to grow in nitroglycerin containing medium and named as N1 to N5 (Figure.1). The nitroglycerin biodegradation assay was carried out by using the five different isolates obtained in enrichment procedure. After incubation, the resultant samples were subjected to UV spectrophotometric analysis. After incubation, the samples were used for determining the degradation ability of the nitroglycerin by 5 isolates using UV spectrophotometer.
The maximum absorption peak for the degraded samples by the bacterial cultures was compared with the peak obtained for control (Figure 4; Table 1). Enumeration procedure was done to find out the ability of the bacterial species capable of utilizing nitroglycerin. In this, no of colony forming units were counted for each bacterial species growing on minimal medium containing nitroglycerin (Table 2). Selection of strains was mainly based on the results obtained from UV spectrophotometric analysis and enumeration procedure. Three isolates were found to show maximal growth in utilizing nitroglycerin as substrates. The crude enzyme was extracted from the selected isolates by cell lysis method.

The nitroglycerin biodegradation assay was carried out using the crude enzyme extracts from the selected isolates as per the procedure referred before. After incubation they were subjected to UV spectrophotometric analysis for determining the degradation ability shown by the enzyme extracts (Figure 2; Table 3). According to Bergey’s Manual of Bacteriology, the efficient native isolate was tentatively identified as Arthrobacter sp., Agrobacterium sp., and Pseudomonas sp. (Figure 3; Table 4). The nitroglycerin biodegradation assay was carried out using the one selected isolate based on earlier results. The samples were used for FTIR analysis. Degradation analysis of Nitroglycerin samples was done using the one selected bacterial isolate. The analysis was carried out in a control and test flask (Figure 4 & 5).
Discussion

Many biodegradation systems are often set up with little knowledge about the microbial communities, which are being used. In order to better understand what is happening in an operational biodegradation system an understanding of the microbial community is needed. A comprehensive study using liquid system was chosen, since liquid systems are relatively inexpensive, easy to set up and control parameter, which can be monitored and manipulated, suited for large bioremediation.

Soil samples contain a considerable number of bacteria which are capable of using nitroglycerin with complex structure even as sole carbon source. It was confirmed by defined growth on mineralizing bacteria (Accashian et. al., 1998; 2000). In the present study the isolation of nitroglycerin degrading native bacterial isolates from soil was done on minimal medium enriched with nitroglycerin from soil. Similar studies were carried out on the isolation of nitroglycerin degrading bacteria from the soil sample (Blehert et. al., 1996; White and Snape, 1993).

Of the different isolates, 3 isolates were selected based on substrate utilization and spectrophotometric analysis. The efficient native isolate was tentatively identified as *Arthrobacter sp.*, *Agrobacterium sp.*, and *Pseudomonas sp.* Husserl et. al., (2010) have also been reported that *Pseudomonas sp.*, *Agrobacterium sp.*, & *Arthrobacter sp.*, are involved in the utilization of nitroglycerin degrading system respectively. However, they were only identified to genus level and thus comparison between degradative ability of specific species is difficult.

The crude enzyme extracts were isolated by cell lysis method and the efficacy of crude enzyme extract for the biodegradation of nitroglycerin by the isolate has been done by UV-Spectrophotometric analysis. The results showed that crude enzyme extracts for selected isolates are also found to be much more effective in degradation of nitroglycerin. The results are in conformity with early findings (Blehert et. al., 1996; Williams and Bruce, 2002; Oh et. al., 2004).

Regarding the FTIR analysis the degradation of nitroglycerin by *Pseudomonas sp.* has been studied by using peaks obtained for functional groups in control and treated sample. FTIR spectrum of control sample showed peaks at 3900 cm<sup>-1</sup>, 3854 cm<sup>-1</sup>, 3797 cm<sup>-1</sup>, 3349 cm<sup>-1</sup> indicating aromatic groups; 2962 cm<sup>-1</sup>, 2926 cm<sup>-1</sup>, 2871 cm<sup>-1</sup>, indicating C-H aliphatic peaks; 2285 cm<sup>-1</sup>, 2182 cm<sup>-1</sup>, 2032 cm<sup>-1</sup>, 1901 cm<sup>-1</sup> indicating triple bonded groups; 1464 cm<sup>-1</sup>, 1378 cm<sup>-1</sup> indicating C-H aliphatic peaks; 2285 cm<sup>-1</sup>, 2182 cm<sup>-1</sup>, 2032 cm<sup>-1</sup>, 1901 cm<sup>-1</sup> indicating aromatic groups; 2962 cm<sup>-1</sup>, 2926 cm<sup>-1</sup>, 2871 cm<sup>-1</sup>, indicating C-H aliphatic peaks (Fig.4). The spectrum analysis of treated sample showed peaks at 2962 cm<sup>-1</sup>, 2926 cm<sup>-1</sup>, 2871 cm<sup>-1</sup>, indicating C-H aliphatic peaks (Fig.2). Comparing the degraded samples with the control, the aromatic peaks were not observed. In addition to that, the aliphatic stretch peaks at 6, 7 and 8 positions in the IR spectrum were noticed at 3000nm in the control and the same aliphatic stretch in the treated sample was noticed between 2500 and 1500nm (Acasshian et. al., 1998; 2000).

Conclusion

Future research in this area will range from clean-up directives for explosives manufacturing and munitions development to sustaining military readiness by appropriately managing training and testing ranges in an environmentally responsible manner. Assessing the potential for explosives contamination and the potential for exposure of environmental and human receptors resulting from various military activities will be necessary. Research will be needed to refine environmental and human health risk assessment methods and develop tools for effective management of necessary military training operations to minimize adverse environmental and human health effects.
Acknowledgements

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References


Table 4. Identification of the Selected Isolates
NA – No reports available

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