



Degradation of *p*-nitrophenol by immobilized cells of *Bacillus* spp. isolated from soil

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ABSTRACT

Two species of *Bacillus*, *Bacillus pantothenicus* and *Bacillus aminovorans*, capable of degrading *p*-nitrophenol (PNP), were isolated from soil following selective enrichment. Washed cells of PNP-grown cultures were entrapped in alginate beads and assessed for their potential in degrading PNP. Degradation of PNP by immobilized cells in batch cultures was slow when compared with those in shake cultures. Cells of *B. pantothenicus* entrapped in 3% calcium alginate were more efficient in degrading PNP than those immobilized in 4 or 5% alginate beads. The rate of PNP degradation by immobilized cells was directly proportional to the stocking density of cells in the beads. The potential of PNP biodegradation in immobilized cells of *B. pantothenicus* lasted even after two years of storage of the beads in medium supplemented with PNP at refrigerated temperature of 4 °C. Our results clearly indicate that these soil isolates of *Bacillus* spp. have great potential in bioremediation of aqueous systems contaminated with PNP.

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1. Introduction

p-Nitrophenol (PNP) is an intermediate in the synthesis of organophosphorous insecticides, parathion and methyl parathion (Lichtenstein and Schulz, 1964). About 27% of its use in industry is for pesticide manufacture and 13% is for synthesis of dye components (Markle et al., 1980). PNP is found as a major metabolite of microbial degradation of these agrochemicals (Munnecke and Hsieh, 1974; Barik and Sethunathan, 1978; Nelson, 1982; Sharmila et al., 1989), and is thus an important environmental pollutant (Jones and Alexander, 1986), posing even odor problems to water bodies (Barik, 1984; Bruhn et al., 1987). PNP has therefore been listed as a priority pollutant by the US Environmental Protection Agency (Keith and Telliard, 1979). Available research suggests that several microorganisms, implicated in soil fertility, are in great risk due to the presence of PNP in soil ecosystem (Venkateswarlu, 1993; Megharaj et al., 1993a;

Umamaheswari and Venkateswarlu, 2003, 2004; Ramakrishnan et al., 2010, 2011).

The use of immobilized cells has many of the operational advantages over the use of immobilized enzymes together with a number of additional merits (D'Souza, 1989). The immobilization of whole cells tends to improve the stability of enzymes by retaining them in their natural surroundings during immobilization and subsequent operation (Venkatasubramanian and Vieth, 1979). Immobilized bacterial systems have been used for the degradation of benzene (Somerville et al., 1977), phenol (Dwyer et al., 1986) and aromatic rings of xenobiotics (Dhulster et al., 1984). It has already been established that several pure cultures of bacteria metabolize PNP with removal of nitro group as nitrite (Spain and Gibson, 1991; Hanne et al., 1993; Jain et al., 1994; Kadiyala and Spain, 1998; Pakala et al., 2007). However, there is only one report available in the literature on the use of immobilized bacteria (*Nocardioide* sp. NSP41) for simultaneous degradation of PNP and phenol (Cho et al., 2000). Our preliminary studies indicated that *Bacillus pantothenicus* and *Bacillus aminovorans*, isolated from a local agricultural soil following selective enrichment, degraded PNP with the concomitant removal of the nitro group as nitrite. Using PNP-grown cells immobilized in calcium alginate beads, the present study compared the two soil isolates of *Bacillus* spp. for their potential to degrade PNP.

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2. Materials and methods

2.1. Chemicals

p-Nitrophenol (PNP) and sodium alginate were purchased from Sigma Chemical Company, St. Louis, USA. Aqueous stock solution of PNP was prepared by dissolving in sterilized distilled water.

2.2. Bacterial strains

Two bacterial strains capable of degrading PNP were isolated from a soil following enrichment culture technique. These bacteria were identified as *B. pantothenicus* and *B. aminovorans*, on the basis of morphological, cultural and biochemical tests, and the identity was confirmed by the Institute of Microbial Technology, Chandigarh, India. Unless otherwise mentioned, these isolates were maintained by culturing routinely in the presence of 50 μ M PNP supplemented to mineral salts medium (MSM) with the composition (g/L): KH_2PO_4 , 4.8; K_2HPO_4 , 1.2; NH_4NO_3 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.04; and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; pH 7.0.

2.3. Immobilization studies

To study PNP biodegradation by immobilized cells of *Bacillus* spp., entrapment of cells within beads was carried out by slightly modifying the method of Megharaj et al. (1992). Cells of *Bacillus* spp., grown in MSM containing 180 μ M PNP and 0.01% yeast extract, were aseptically harvested by centrifugation at 6000 rpm for 20 min and suspended in 50 ml sterile distilled water. Sodium alginate was added to another aliquot of 50 ml sterilized distilled water and gently stirred for 1 h, boiled and cooled. Bacterial cell suspension was then added to sodium alginate solution and stirred for 30 min to ensure proper mixing. All these steps were done under aseptic conditions in a laminar flow hood. The mixture, with a final concentration of 3% (w/v) sodium alginate and bacterial cells was run down, drop-wise, through a burette into 0.4 M CaCl_2 solution for stabilization. The beads were washed twice with sterilized distilled water before use. The bacterial cell density in a single bead was determined following the method described by Chibata and Tosa (1977).

MSM was supplemented with 180 μ M of PNP throughout the present study. About 180 beads with a stocking density of 3×10^6 cells bead⁻¹ of *Bacillus* species were transferred to 100 ml MSM supplemented with PNP contained in 250 ml Erlenmeyer flasks. A set of flasks was incubated at 37 °C while another set was subjected to shaking in an orbital shaker maintained at 37 °C. The nitrite released from PNP was assayed by withdrawing 0.5 ml aliquots of the culture medium in triplicate flasks at desired intervals using sulphanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride as reagents in the modified Griess–Ilosvay colorimetric method as described by Barnes and Folkard (1951). PNP disappearance from the medium was also monitored by measuring the absorbance at 410 nm (Adhya et al., 1981).

In order to determine the impact of alginate percentage in beads on PNP degradation by *B. pantothenicus*, beads with varying concentrations of 3, 4, and 5% (w/v) calcium alginate were stocked with 3×10^6 cells of per bead as described above. To assess the influence of stocking density of the cells of *B. Pantothenicus* on PNP removal from the medium, stocking densities of 1.5 and 4.5×10^6 cells bead⁻¹ were also included besides 3×10^6 cells bead⁻¹. To study the PNP-degrading potential of immobilized cells of *B. pantothenicus* over time, the beads with a stocking density of 3×10^6 cells were stored up to a period of two years at 4 °C in MSM supplemented with PNP, withdrawn periodically and transferred to fresh MSM containing 180 μ M PNP. The

removal of nitro group as nitrite from PNP by the immobilized cells was monitored.

The average data values ($n = 3$) were analyzed for significant differences ($P \leq 0.05$) using Duncan's multiple range (DMR) test (Duncan, 1955) as adapted by Megharaj et al. (1993b).

3. Results and discussion

The data on PNP-degrading potential of immobilized cells of the two species of *Bacillus*, *B. pantothenicus* and *B. aminovorans* when immobilized in 3% calcium alginate are presented in Figs. 1 and 2. PNP degradation following oxidative release of nitro group as nitrite by immobilized cells of *B. pantothenicus*, with a stocking density of 3×10^6 cells bead⁻¹, in a static culture was very slow; PNP was degraded completely by the end of 30 h incubation as opposed to 26 h incubation period required for the complete removal of PNP by immobilized cells in the shake culture. *B. aminovorans*, on the other hand, with the same stocking density of 3×10^6 cells bead⁻¹ completely degraded PNP only after 48 h when the beads were shaken at 200 rpm during incubation, and the time taken for the removal of PNP in the static culture was about 60 h. Evidently, aeration, provided by shaking, effected more rapid biodegradation of PNP by immobilized cells. In a laboratory scale immobilized bacterial column, Heitkamp et al. (1990) demonstrated about 99% removal of 18,000 mg/L PNP by a mixed culture of three PNP-degrading *Pseudomonas* sp. immobilized by adsorption onto diatomaceous earth bioreactor in a 1.75 L plexi glass column. Beunink and Rehm (1990) observed both reductive and oxidative degradation of 4-chloro-2-nitrophenol by co-immobilization of *Enterobacter cloacae* and *Alcaligenes* sp. TK-2 in 3-mm calcium alginate beads.

Although PNP biodegradation by immobilized cells of both *B. pantothenicus* and *B. aminovorans* resulted in the release of nitrite, no stoichiometric amounts of nitrite accumulated in the culture medium. Presumably, some amounts of nitrite may have been retained inside the beads. It has been shown earlier that PNP-grown cells of *Bacillus sphaericus* JS905, isolated from an agricultural soil in India, released stoichiometric amounts of nitrite into the medium containing PNP or 4-nitrocatechol (Kadiyala and Spain, 1998).

Preliminary studies indicated that *B. pantothenicus*, with a cell density of 1.36×10^6 CFU ml⁻¹, in MSM completely degraded

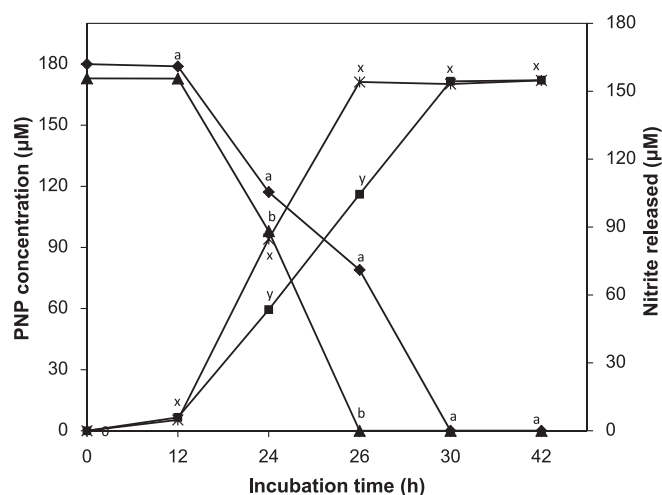


Fig. 1. Degradation of PNP by immobilized cells (3×10^6 cells bead⁻¹) of *B. pantothenicus* in static (◆ PNP, ■ NO₂) and shake cultures (▲ PNP, * NO₂). PNP added to the medium, 180 μ M. Mean values ($n = 3$) for a chemical (PNP or NO₂) at each sampling indicated by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple range (DMR) test.

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