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ORIGINAL PAPER

Enhanced degradation of phenol by *Pseudomonas* sp. CP4 entrapped in agar and calcium alginate beads in batch and continuous processes

P. Y. Aneez Ahamad · A. A. Mohammad Kunhi

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Abstract Phenol is one of the major toxic pollutants in the wastes generated by a number of industries and needs to be eliminated before their discharge. Although microbial degradation is a preferred method of waste treatment for phenol removal, the general inability of the degrading strains to tolerate higher substrate concentrations has been a bottleneck. Immobilization of the microorganism in suitable matrices has been shown to circumvent this problem to some extent. In this study, cells of *Pseudomonas* sp. CP4, a laboratory isolate that degrades phenol, cresols, and other aromatics, were immobilized by entrapment in Ca-alginate and agar gel beads, separately and their performance in a fluidized bed bioreactor was compared. In batch runs, with an aeration rate of 1 vol^{-1} vol^{-1} min^{-1}, at 30°C and pH 7.0 ± 0.2, agar-encapsulated cells degraded up to 3000 mg l^{-1} of phenol as compared to 1500 mg l^{-1} by Ca-alginate-entrapped cells whereas free cells could tolerate only 1000 mg l^{-1}. In a continuous process with Ca-alginate entrapped cells a degradation rate of 200 mg phenol l^{-1} h^{-1} was obtained while agar-entrapped cells were far superior and could withstand and degrade up to 4000 mg phenol l^{-1} in the feed with a maximum degradation rate of 400 mg phenol l^{-1} h^{-1}. The results indicate a clear possibility of development of an efficient treatment technology for phenol containing waste waters with the agar-entrapped bacterial strain, *Pseudomonas* sp. CP4.

Keywords Phenol · *Pseudomonas* sp. CP4 · Immobilization · Ca-alginate · Agar gel · Degradation rate

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Introduction

Phenol is one of the 50 major bulk chemicals produced in the world and its annual production reached 6.6 billion pounds in 2004 and expected to grow by 6% per year (CMR 2005). Phenol and its derivatives are also generated by various industries, such as petroleum refining, petrochemical, coke conversion, pharmaceutical, plastic, and resin manufacturing, in the waste effluents and its concentration may vary from 1 to 15000 mg l^{-1}. United States Environmental Protection Agency (USEPA) and Central Pollution Control Board of India (CPCB) have prescribed maximum permissible limits of 3.4 and 5.0 mg l^{-1}, respectively, of phenol in industrial waste discharges. Phenol is highly toxic to flora and

fauna including humans. It also causes taste and odor problems in drinking water at far lower concentrations (Rittmann and McCarty 2001). Hence, it is imperative to eliminate phenol from waste streams before they are disposed off. Several physical methods have been tried to remove phenol which include solvent extraction, adsorption, chemical oxidation, incineration, etc. All these methods, however, are not cost effective and also have the drawback of formation of hazardous by-products (Loh et al. 2000). Biological processes are generally preferred due to their lower operational costs and the possibility of complete mineralization (Agarry et al. 2008; Brar et al. 2006). A number of microorganisms have been shown to utilize phenol and its derivatives as sole sources of carbon and energy at varying concentrations, under aerobic conditions. They include several bacterial cultures (Agarry et al. 2008; Ahamad and Kunhi 1996; Ahamad et al. 1996; Babu et al. 1995b; Chung et al. 2003; El-Sayed et al. 2003; Feitkenhauer et al. 2003; Karigar et al. 2006; Kumar et al. 2005; Liu et al. 2009; Ma et al. 2010; Mordocco et al. 1999; Prieto et al. 2002; Shetty et al. 2007), a few yeast strains (Agarry et al. 2008; Fialova et al. 2004; Yan et al. 2005) and mycelial fungi (Agarry et al. 2008; Anselmo et al. 1989; Santos et al. 2003; Yordanova et al. 2009). Removal of phenol also has been carried out using activated sludge systems for the last several years (Amor et al. 2005; Marrot et al. 2008). But, high phenol loading rates and fluctuations in phenol loads have been reported to cause the breakdown of these systems (Watanabe et al. 2000). Packed-bed reactors of inert support materials have gained interest in recent years, where attached processes take place, improving the system efficiency (Bajaj et al. 2008; Mailin and Firdausi 2007; Shetty et al. 2007; Tziotzios et al. 2007).

The major difficulty encountered in the microbial treatment of phenol-containing wastewater is the substrate inhibition of microbial growth and concomitant hindrance in the biodegradation. To overcome these problems immobilization of the degrading organism(s) has been proposed as an effective strategy (Keweloh et al. 1989). The main advantages of using immobilized cells, rather than free suspended ones, include the retention in the reactor of higher concentrations of microorganisms, protection of cells against toxicity and prevention of suspended microbial biomass in the effluent. Moreover, immobilization of microbial cells provides, in general, high degradation efficiency and good operational stability. Aerobic granulation of the degrading organism(s) as a new form of cell immobilization for exploitation in phenol wastewater treatment in sequencing batch reactors also has been gaining importance, recently (Tay et al. 2004). The aggregation of microbial cells into compact self-immobilized granules can serve as an effective protection against phenol toxicity. However, the traditional immobilization technique of entrapment of microorganisms in gel polymers has been shown to be more efficient in resisting the phenol toxicity and improving the degradation rates. Several different polymeric materials have been tried as matrices for immobilization. Ca-alginate has been used as the gel matrix in several studies for encapsulating strains of bacteria, yeasts, and mycelial fungi (Abd-El-Haleem et al. 2003; Bandhyopadhyay et al. 1999; Bettmann and Rehm 1984; Chung et al. 2003; Dursun and Tepe 2005; Hannaford and Kuek 1999; Kapoor et al. 1998; Karigar et al. 2006; Keweloh et al. 1989; Lakhwala et al. 1992; Mordocco et al. 1999; Santos et al. 2003). Other polymers which were occasionally used for immobilizing phenol-degrading microorganisms were agar, polyacrylamide (PAA), polyacrylamide hydrazide (PAAH), polyvinyl alcohol (PVA), polysulfone hollow fiber membrane, and modified polyacrylonitrile membrane (Bettmann and Rehm 1985; Chen et al. 2002; Juárez-Ramírez et al. 2001; Karigar et al. 2006; Liu et al. 2009; Loh et al. 2000; Yordanova et al. 2009). All the phenol-degrading strains exhibited improved ability, to varying degrees, in the immobilized systems than as freely suspended cells. However, studies on comparison of the suitability and efficiency of different matrices for immobilization of phenol-degrading organisms are very rarely found in literature (Bettmann and Rehm 1984). Particularly, a comparative appraisal of the calcium alginate and agar gel-immobilized microorganisms has been very scarcely found (Karigar et al. 2006).

Pseudomonas sp. strain CP4, a laboratory isolate was found to be a potent degrader of phenol, isomers of cresol, and other aromatics under shake flask conditions (Ahamad and Kunhi 1999; Ahamad et al. 2001; Babu et al. 1995b) and was an efficient partner of a mixed culture with *Pseudomonas aeruginosa* strain 3mT in the degradation of mixtures of 3-chlorobenzoate (3-CBA) and phenol/cresols (Babu et al. 1995a). In the present work it was intended to study the

performance of the strain CP4 as free suspended cells and that of the cells immobilized by entrapment in Ca-alginate and agar gels (separately), for its ability to degrade phenol in a fluidized bed reactor and to also evaluate the comparative efficiency of the two immobilized systems. The study was conducted both under batch and continuous operations and the data obtained are presented here.

Materials and methods

Chemicals

Phenol (A R grade) was procured from Qualigens Fine Chemicals, Bombay, India. Sodium alginate was purchased from Allied and Company, Bombay, India. Agar powder and other chemicals used in the culture media and reagents were of high purity and were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India.

Microorganism and culture medium

Pseudomonas sp. CP4 used in this study was a laboratory isolate (Babu et al. 1995b). The culture was grown in a mineral medium (M3 medium) having composition as described earlier (Babu et al. 1995b). The pH of the M3 medium was maintained at 7.0 ± 0.2. The un-buffered medium used in some of the experiments was devoid of the buffering salts KH_2PO_4 and Na_2HPO_4, but the pH was adjusted by the addition of acid or alkali by the pH control module of the fermenter, as needed. In another experiment the medium devoid of phosphates was buffered with Tris–HCl (200 mM) to maintain pH at 7.2.

Inoculum preparation

Cell biomass was prepared by growing *Pseudomonas* sp. CP4 in a 10 l capacity fermenter (Digi-Ferm fermenter, locally fabricated) in M3 medium containing 500 mg phenol l^{-1} at 30°C with stirring (500 rpm) and aeration at 1 vol^{-1} vol^{-1} min^{-1} which yielded about 15 g wet biomass at mid-log phase (i.e., at 48 h). The cells were harvested by centrifugation at $6000 \times g$ and washed by suspending in sterile saline (0.85%) and re-pelleting by centrifugation. These cells were used for the degradation studies as free cells or as gel-entrapped biomass.

Immobilization of *Pseudomonas* sp. CP4

All the equipments and materials used in the preparation of gel-entrapped cells of *Pseudomonas* sp. CP4 were sterilized and all the operations were carried out under sterile conditions.

Entrapment in calcium alginate gel

The biomass pellet (15 g wet wt.) was suspended in 100 ml of sterile saline and was mixed thoroughly with equal volume of 4% sodium alginate solution (final concentration of alginate being 2%), taking care not to include any air bubbles. The mixture was extruded through a wide needle into a beaker containing 1 l of 0.2 M $CaCl_2$ solution in such a way as to obtain uniform gel beads of an average size of 3 mm dia. The beads were washed thrice with $CaCl_2$ solution and kept for 24 h at 5°C before use.

Entrapment in agar gel

A 4% solution of agar in 100 ml of saline was sterilized by autoclaving and was cooled to 50°C. 100 ml of saline suspension of 15 g (wet wt.) of cells, pre-warmed to about 37°C, was mixed thoroughly with the agar solution, avoiding inclusion of any air bubbles. The gel beads were prepared by dropping the mixture through a wide needle into 1 l of cold 0.2 M KCl in such a way as to get a uniform bead size of 3 mm dia. The beads were washed with M3 medium and stored at 5°C. The beads were washed with the respective medium before their use in different experiments.

Phenol degradation studies

All the degradation experiments were carried out in a Gallenkamp modular fermenter (A. Gallenkamp & Co., London, UK) fitted with a 1 l capacity fermenter jar (bioreactor), air sparger, peristaltic pumps for substrate feeding and drawing samples, reservoir for substrate, pH module for auto-adjustment of the pH of the medium, if necessary. In all the studies including that with free cells and cells entrapped in Ca-alginate/agar beads 500 ml of M3 medium with required

concentration of phenol was taken in the fermenter jar. The medium was sparged with sterile compressed air at the rate of 500 ml min^{-1} (i.e., 1 vol^{-1} vol^{-1} - min^{-1}), through an air sparger fitted at the bottom of the reactor jar, throughout the experiment which was effective in mixing the medium thoroughly besides providing oxygen. No other agitation such as stirring was used. In all the experiments temperature was maintained at 30 ± 1°C.

Degradation by free cells

500 ml M3 medium in the reaction vessel was inoculated with freshly harvested cells of *Pseudomonas* sp. CP4 at a level equivalent of 6 µg cell protein ml^{-1}. Phenol concentrations of 200, 500, 800, 1000, and 1200 mg ml^{-1} was tested in different batches. Samples in triplicate were drawn at required intervals for analysis of residual phenol and growth of the organism.

Degradation by Ca-alginate-entrapped cells

Degradation of phenol in batch was studied in M3 medium (500 ml) containing phenol (500 mg l^{-1}) that was inoculated with 20 g of Ca-alginate beads containing strain CP4 cells. The initial cell content in terms of cell protein in the beads was about 3.3 mg g^{-1} beads (wet wt.), i.e., the total inoculum added was equivalent of 66 mg in 500 ml medium or in other words 0.132 mg ml^{-1}. The reactor was sparged with sterile air at the rate of 1 vol^{-1} vol^{-1} min^{-1}. In other two experiments the phosphates were eliminated from M3 medium as they caused extensive disruption of the Ca-alginate beads releasing the cells to the medium. In one case un-buffered medium, i.e., M3 medium devoid of phosphates was used whereas in the second case it was buffered with Tris–HCl (200 mM). Samples were collected at required intervals and were analyzed for residual substrate and for free cells in the medium which was leached from the beads and grown.

Degradation of different concentrations of phenol from 500 to 2000 mg l^{-1} by Ca-alginate immobilized strain CP4 was studied using un-buffered M3 medium, in batches. Initial inoculum as cell protein in gel beads was 3.6 mg g^{-1} beads (wet wt.). The same beads were used consecutively in batches with 1000, 1500, and 2000 mg l^{-1} phenol, after harvesting by filtration through a sterile muslin cloth and washing them with same medium. Other conditions were same as mentioned above.

Degradation of phenol by immobilized strain CP4 in a continuous process was tried by altering the substrate feed rate from 50 to 200 mg l^{-1} h^{-1} by increasing the dilution rate from 0.2 to 0.8 h^{-1} as described under "Results."

Degradation by agar-entrapped cells

Batch degradation of phenol at 500, 1000, 1500, 2000, 2500, 3000, and 3200 mg l^{-1} by agar-entrapped cells were carried out in M3 medium (with phosphates) under conditions as described above. 20 g beads (wet wt.) having cells equivalent of 2.94 mg protein g^{-1} wet beads was used as inoculum. The same gel beads that were used in the 500 mg l^{-1} phenol batch were used in the subsequent runs with higher concentrations, after washing them with the medium.

In continuous degradation of phenol by the agar-entrapped cells the dilution rate, D of 0.1 h^{-1} was kept constant all through the experiment while the concentration of phenol in the feed solution was varied from 1000 to 5000 mg l^{-1} to obtain a feed rate of phenol of 100–500 mg l^{-1} h^{-1} as described in the "Results." Other conditions were as in the case of Ca-alginate gel.

Quantitative determinations

All the analyses, including the estimation of cell biomass both in the media and the gels, quantification of residual phenol and the HPLC analysis of the cell free extracts for phenol and intermediary metabolites were carried out in triplicate and the results are expressed as average values.

Estimation of growth

Growth of *Pseudomonas* sp. CP4 in the medium, cells grown in or leached from the beads to the medium or the cells entrapped in the gel beads (both agar and Ca-alginate) were estimated as total cell protein as follows: Known quantity of the culture broth was centrifuged at 6000×*g* and the cell pellet was suspended in 3.5 ml of distilled water and 0.5 ml of 20% NaOH was added to it. The solution was

incubated in a boiling water bath for 10 min, cooled and 0.5 ml of this solution was used for protein estimation by the method of Lowry et al. (1951). In the case of gel beads, the beads were blotted on a blotting paper to remove the medium sticking on them and 0.3 g (wet wt.) of beads was macerated well in a test tube by a round tipped grass rod before the addition of alkali. After digestion with alkali as mentioned above the tube was centrifuged and 0.5 ml of the supernatant solution was taken for protein assay. The biomass was expressed as mg cell protein ml^{-1} of culture broth or mg g^{-1} wet wt. of the gel beads.

Determination of phenol

Residual phenol in the feed solution and in the cell-free culture broth was routinely estimated by a 4-amino-antipyrene colorimetric method based on the procedure of Lacoste et al. (1959). The culture filtrate with no or very low amounts of residual phenol as determined by this method was further confirmed by HPLC analysis as described earlier (Ahamad and Kunhi 1999) to ensure that the mineralization was complete.

The intermediary metabolite 2-hydroxymuconic-semialdehyde (2-HMS) was identified as described previously (Babu et al. 1995b).

Results

Phenol degradation by free cells of *Pseudomonas* sp. CP4

Degradation of phenol at 200–1200 mg l^{-1} was studied by inoculating each batch with freshly grown cells at levels equivalent of 0.006 mg ml^{-1} cell protein. Growth started after a lag of varying durations of 2, 6, 10, and 24 h with 200, 500, 800, and 1000 mg l^{-1} of phenol, respectively, and complete disappearance of the substrate was observed at 10, 30, 54, and 82 h, respectively (Fig. 1). Maximum growth of about 0.095, 0.16, 0.20, and 0.19 mg protein ml^{-1} was observed at 24, 30, 54, and 108 h with 200, 500, 800, and 1000 mg l^{-1} substrate, respectively. No growth and degradation occurred at 1200 mg l^{-1} phenol (data not shown in Fig. 1).

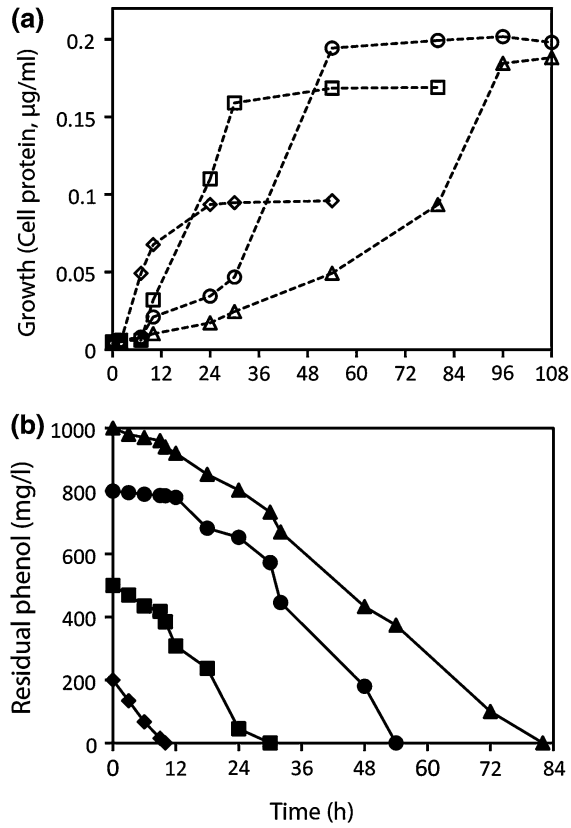

Fig. 1 Growth (**a**) and degradation (**b**) of different concentrations of phenol by suspended free cells of *Pseudomonas* sp. CP4. Experimental details are as in "Methods." [Residual phenol (mg l^{-1}) from 200 (*filled diamond*), 500 (*filled square*), 800 (*filled circle*), and 1000 mg l^{-1} (*filled triangle*), respectively. Growth (cell protein, mg l^{-1}) in 200 (*open diamond*), 500 (*open square*), and 1000 mg l^{-1} phenol (*open circle* and *open triangle*), respectively]

Degradation by Ca-alginate-entrapped *Pseudomonas* sp. CP4

20 g of Ca-alginate beads were inoculated into 500 ml M3 medium containing 500 mg l^{-1} of phenol. Within 2 h the beads started disintegrating releasing the cells from the Ca-alginate gel (Fig. 2). Phenol disappeared within 13 h and the organism grew well, the cell protein content being 1.06 mg ml^{-1} of the medium after 13 h, i.e., a total of 530 mg cell protein in 500 ml medium from the initial 66 mg.

As phosphate-buffered medium caused extensive disintegration of the cell-immobilized beads the M3 medium devoid of phosphates but buffered with Tris–HCl (200 mM, pH 7.2) was tested. No disintegration of the gel beads occurred and the free cells in the medium

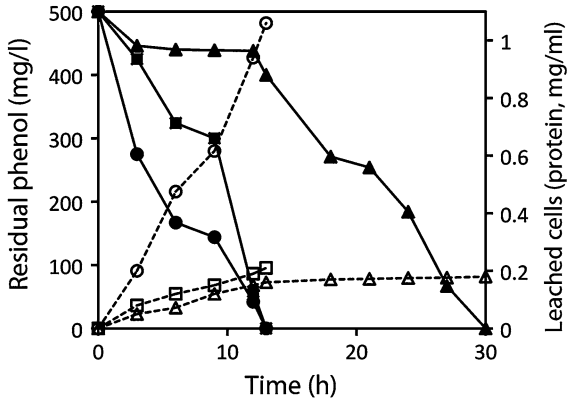

Fig. 2 Degradation of 500 mg l^{-1} of phenol by Ca-alginate-entrapped cells of *Pseudomonas* sp. CP4 in phosphate-buffered, Tris–HCl-buffered and un-buffered M3 medium. Experimental details are as in "Methods." [Residual phenol in phosphate-buffered medium (*filled circle*), un-buffered medium (*filled square*), and Tris–HCl-buffered medium (*filled triangle*). Cells leached out and grown in phosphate-buffered medium (*open circle*), un-buffered medium (*open square*), and Tris–HCl-buffered medium (*open triangle*)]

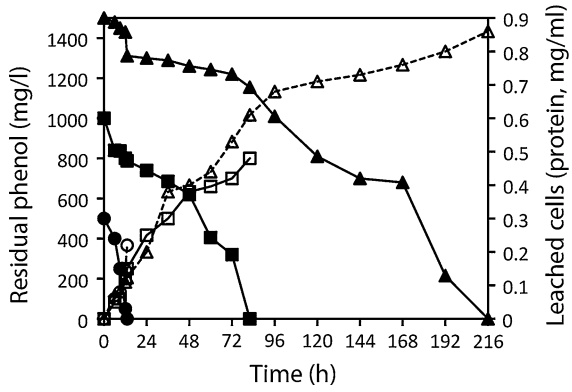

Fig. 3 Degradation of different concentrations of phenol by Ca-alginate immobilized *Pseudomonas* sp. CP4 in batches. Details are as in "Methods." [Residual phenol from 500 (*filled circle*), 1000 (*filled square*), and 1500 mg l^{-1} batches (*filled triangle*), respectively. Cells leached out and grown in the medium with 500 (*open circle*), 1000 (*open square*), and 1500 mg l^{-1} phenol (*open triangle*), respectively]

(leached out to and grown) were equivalent of only about 0.18 mg protein ml^{-1}, after 30 h (Fig. 2). But, the degradation rate was considerably reduced taking 30 h for the disappearance of 500 mg l^{-1} phenol and the mineralization taking much longer as evidenced by the presence of 2-HMS (the yellow colored intermediary metabolite) up to 100 h of incubation. As Tris-buffered medium also was not suitable an un-buffered M3 medium was tested. In this medium 500 mg l^{-1} phenol was degraded in 13 h as was in the case of phosphate-buffered M3 medium (Fig. 2). In un-buffered medium also the cell leaching was comparatively low, a maximum free cells equivalent of only 0.21 mg protein ml^{-1}. All further experiments with Ca-alginate-immobilized system, therefore, were carried out using an un-buffered M3 medium.

Degradation of different concentrations of phenol viz. 500, 1000, 1500, and 2000 mg l^{-1} was studied as follows: 500 ml of un-buffered M3 was inoculated with 20 g of Ca-alginate beads with a cell protein content of 3.6 mg g^{-1} beads (wet wt.). 500 mg l^{-1} of phenol was degraded in 13 h with a final free cell protein content of 0.22 mg ml^{-1} in the medium (Fig. 3). With 1000 mg l^{-1} of phenol the cell protein content in the medium was 0.48 mg ml^{-1} at the end of 82 h, the time taken for complete disappearance of phenol. Disappearance of 1500 mg l^{-1} of phenol took 216 h. The cell protein content observed in the medium at the end of the run was 0.86 mg ml^{-1}. 2000 mg l^{-1} phenol was not degraded at all (data not plotted in Fig. 3). The initial cell protein content of the beads of 3.6 mg g^{-1} was reduced to 3.28, 3.02, and 2.98 mg g^{-1} beads (wet wt.) after the passages through 500, 1000, and 1500 mg l^{-1} phenol, respectively.

The pattern of degradation of phenol by the Ca-alginate-immobilized cells of strain CP4 in a continuously fed system is depicted in Fig. 4. 500 ml un-buffered medium containing 500 mg l^{-1} phenol and 20 g (wet wt.) Ca-alginate beads were taken in the reactor. In about 14 h the phenol concentration came down to zero. After 24 h feeding of fresh medium containing 500 mg l^{-1} of phenol was started so as to obtain a substrate feeding rate of 50 mg l^{-1} h^{-1}, the dilution rate D being 0.2 h^{-1}. Within 3 h the effluent phenol concentration came down to zero. The feeding was continued for 30 h and it was observed that the phenol concentration did not increase in the effluent indicating continuous degradation of the fed substrate. At this stage the feeding rate was increased to 100 mg l^{-1} h^{-1} by increasing the dilution rate to 0.4 h^{-1}. In about 13 h the effluent phenol concentration was almost zero and the feeding was continued up to 102nd h with no accumulation of phenol, indicating efficient degradation. Then the phenol feed rate was increased to 150 mg l^{-1} h^{-1} by enhancing the

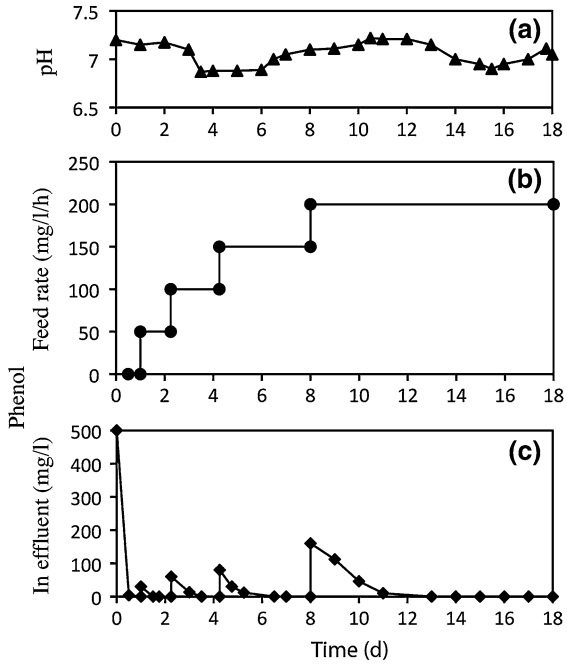

Fig. 4 Continuous degradation of phenol by Ca-alginate-entrapped *Pseudomonas* sp. CP4 in a bioreactor fed with 500 mg l^{-1} phenol at different dilution rates to obtain feed rates of 50, 100, 150, and 200 mg l^{-1} h^{-1}. Other details are as in "Methods" and in "Results." **a** pH of the medium during the run, **b** phenol feed rate, **c** phenol concentration in the effluent

dilution rate to 0.6 h^{-1}. The phenol concentration in the effluent dropped to almost zero after another 18 h (i.e., after 5 days of the run). Feeding at this rate was continued for another 68 h (i.e., till 7.8th days) with no accumulation of phenol in the reactor. At this stage, the dilution rate was increased to 0.8 h^{-1}, which enabled a substrate feeding rate of 200 mg l^{-1} h^{-1}. After 11 days a steady state was attained with no phenol in the effluent and the feeding was continued till 432th h (18 days) with no accumulation of phenol. Further increase in dilution rate (i.e., D = 1.0 h^{-1}) with a feeding rate of 250 mg phenol l^{-1} h^{-1}, resulted in accumulation of phenol (data not shown in Fig. 4). Therefore, the feeding was stopped. The pH of the medium was maintained between 6.8 and 7.2 throughout this continuous operation of the bioreactor. The maximum rate of degradation of phenol was 200 mg l^{-1} h^{-1} as the input phenol of 200 mg l^{-1} h^{-1} was degraded without any accumulation in the reactor after attaining the steady state.

Degradation by agar-entrapped *Pseudomonas* sp. CP4

500 ml M3 medium (with phosphate buffer) was inoculated with 20 g (wet wt.) of the agar beads containing cells equivalent to 2.94 mg protein g^{-1} of beads (wet wt.). Phenol concentrations used were 500–3200 mg l^{-1} and were inoculated with same gel beads consecutively, starting with 500 mg l^{-1} substrate, after washing them thoroughly but gently with M3 medium. The time taken for complete degradation of phenol at 500, 1000, 1500, 2000, 2500, and 3000 mg l^{-1} were 14, 34, 35, 72, 90, and 135 h, respectively (Fig. 5a). Phenol at 3200 mg l^{-1}, however,

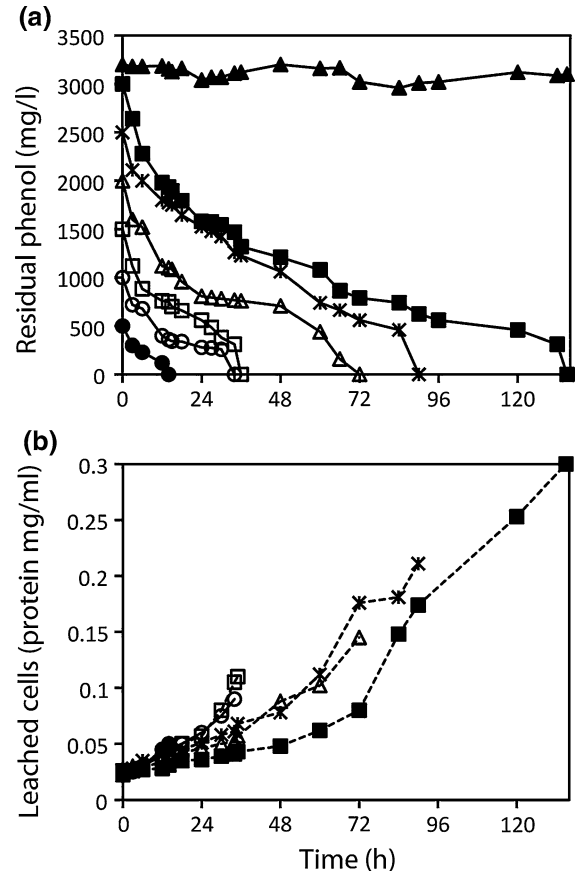

Fig. 5 Degradation of different concentrations of phenol by agar immobilized *Pseudomonas* sp. CP4 in batches. Details are as in "Methods." [**a** Residual phenol (*solid lines*) and **b** cells leached out from the beads and grown in the medium (*dotted lines*) with 500 (*filed circle*), 1000 (*open circle*), 1500 (*open square*), 2000 (*open triangle*), 2500 (*cross sign*), 3000 (*filled square*), and 3200 mg l^{-1} of phenol (*filled triangle*), respectively]

was not degraded. There was an increased lag in the appearance of free cells in the medium with increasing phenol concentrations and the growth also was rather slow (Fig. 5b). At phenol concentrations of 500, 1000, 1500, 2000, 2500, and 3000 mg l^{-1} the maximum cell protein content observed in the medium were only 0.05, 0.09, 0.11, 0.15, 0.20, and 0.30 mg ml^{-1}, respectively. Another interesting observation was that the cell content of the agar beads did not decrease, as happened with Ca-alginate beads, after their repeated use with increasing concentrations of the substrate. There was rather a small gain in cell protein content. From an initial value of 2.94 mg cell protein g^{-1} of agar beads (wet wt.) the protein value increased, after every passage through batches with different phenol concentrations from 500 to 3000 mg l^{-1}, finally to about 3.2 mg g^{-1}.

In the continuous degradation studies with agar-entrapped cells the dilution rate of 0.1 h^{-1} was kept constant all through whilst the concentration of phenol in the feed solution was varied. The results are depicted in Fig. 6. The pH of the medium during the run remained within a range of 6.8–7.2 without any adjustment by the addition of alkali or acid (Fig. 6a), which is the optimal pH range for strain CP4 (Babu et al. 1995b). The initial concentration of phenol taken in the reactor was 500 mg l^{-1} which disappeared within 12 h. At this point, continuous feeding of stock solution containing 1000 mg l^{-1} of phenol was started to provide a feeding rate of 100 mg l^{-1} h^{-1}. Phenol concentration in the effluent was 8 mg l^{-1} after 6 h and was zero within another 6 h. The feeding was continued up to 24th h. There was no increase in phenol concentration in the effluent during this period indicating the complete degradation of the fed substrate. At this point, feeding of 2000 mg l^{-1} phenol was started, i.e., a feed rate of 200 mg l^{-1} h^{-1}. By 36th h the phenol concentration came down to almost 0 mg l^{-1}. Feeding with this concentration was continued for another 18 h (i.e., up to 54th h) without any increase in the effluent phenol concentration. At this point feeding of the substrate at a rate of 300 mg l^{-1} h^{-1} was started. At this feeding rate the effluent phenol level came to zero by 72nd h. The feeding was continued up to 78th h with no accumulation of phenol in the bioreactor. Now, feeding of phenol stock of 4000 mg l^{-1} (i.e., a feed rate of 400 mg l^{-1} h^{-1}) was started. This was again continued until 120th h during which period the effluent phenol concentration came down to almost zero at 102nd h. The feeding at a concentration of 5000 mg l^{-1} at this point resulted in accumulation of phenol in the reactor (data not shown in Fig. 6). Hence, the feeding was stopped. However, a high degradation rate of 400 mg l^{-1} h^{-1} was attained at the steady state when the feed concentration was 4000 mg l^{-1}.

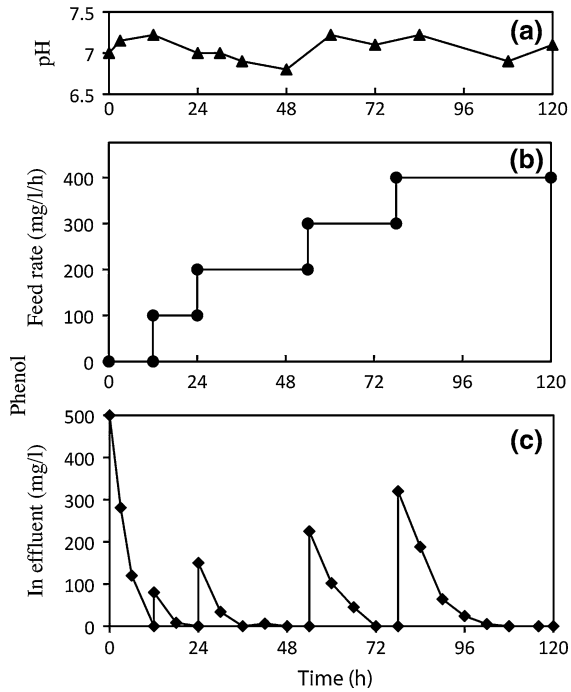

Fig. 6 Continuous degradation of phenol by agar-entrapped *Pseudomonas* sp. CP4 in a bioreactor fed with different concentrations of phenol to obtain feed rates of 100, 200, 300, and 400 mg l^{-1} h^{-1}. Experimental details are as in "Methods" and "Results." **a** pH of the medium during the run, **b** phenol feed rate, **c** phenol concentration in the effluent

Discussion

Pseudomonas sp. CP4 was shown earlier to degrade up to 1500 mg l^{-1} of phenol (Babu et al. 1995b) and up to 1400, 1100, and 2200 mg l^{-1} of *o*-, *m*-, and *p*-cresol, respectively (Ahamad and Kunhi 1999) in shake flasks. Immobilized microbial systems have been known to be of advantage in terms of improved rate of degradation, enhanced tolerance to higher concentrations of the substrate, repeated usability, etc. In the present study it was intended to evaluate the performance of this strain in the degradation of

phenol in batch and continuous modes when immobilized by entrapment in 2 different gel matrices viz. Ca-alginate and agar. In batch degradation, Ca-alginate immobilized strain CP4 mineralized up to a maximum of 1500 mg l^{-1} of phenol in the bioreactor (Fig. 3). Under the same conditions, free cells could degrade only 1000 mg l^{-1} of phenol (Fig. 1). The growth pattern of the free cells of the strain at different concentrations of the substrate was rather interesting. Increased lag periods were observed with increasing concentrations of the substrate and the biomass build up was not proportionate to the substrate concentration, e.g., 0.20 and 0.19 mg protein ml^{-1} for 800 and 1000 mg l^{-1} substrate (Fig. 1). This could be due to the partial inhibitory effect of phenol at higher concentrations. The lower levels of inoculums could also be a reason for this phenomenon, as same amounts of inoculum was added to all concentrations of the substrate. Insufficient oxygen supply also could be yet another reason, but all these need to be verified by experimentations.

Both free cells and immobilized cells took about 82 h for complete degradation of 1000 mg l^{-1}. Degradation of 1500 mg l^{-1} of phenol by the Ca-alginate-immobilized strain CP4 occurred within 216 h (Fig. 3) while the free cells could not degrade even 1200 mg l^{-1}. Babu et al. (1995b) have, however, shown complete mineralization of 1500 mg l^{-1} phenol within about 66 h when strain CP4 was grown in shake flasks. The low rate of degradation in the fermenter by the immobilized cells may be due to various factors including low inoculum levels, oxygen limitation, etc., as mentioned above in the case of growth. In this study with free cells an inoculum level equivalent of only 0.006 mg ml^{-1} of cell protein was used in the bioreactor, whereas in shake flask experiments an inoculum of 0.18 mg ml^{-1} dry cells (i.e., equivalent of about 0.09 mg ml^{-1} of cell protein) was used (Babu et al. 1995b). Moreover, the shake flasks (500 ml capacity flasks containing 100 ml medium) were agitated by gyration at 150 rpm which would be providing more oxygen for the organism. Hannaford and Kuek (1999) have demonstrated a linear response to increasing air flow in a bubble column reactor to the degradation rates of phenol by Ca-alginate-entrapped *P. putida* ATCC 11172.

Several workers have reported improved performance of different microorganisms on their encapsulation in Ca-alginate gel (Abd-El-Haleem et al. 2003; Bandhyopadhyay et al. 1999; Bettmann and Rehm 1984; Chung et al. 2003; Dursun and Tepe 2005; Hannaford and Kuek 1999; Kapoor et al. 1998; Karigar et al. 2006; Lakhwala et al. 1992; Mordocco et al. 1999; Santos et al. 2003). Ca-alginate-entrapped strain CP4 could degrade 1500 mg l^{-1} of phenol in the bioreactor whereas the free cells could not tolerate even 1200 mg l^{-1}. Entrapment of cells of *P. putida* P8 (formerly *Pseudomonas* sp.) in alginate and PAAH resulted in the improved degradation of phenol, i.e., 2 g l^{-1} in less than 2 days whereas free cells did not grow at this concentration at all (Bettmann and Rehm 1984). *Acinetobacter* sp. W-17 degraded 500 mg l^{-1} of phenol from a minimum salts medium within 24 h and the same concentration from simulated waste water within 15 h as against 120 h taken by free cells (Abd-El-Haleem et al. 2003). Chung et al. (2003) have reported that *P. putida* CCRC 14365 immobilized in Ca-alginate could degrade up to 1000 mg l^{-1} of phenol whereas free cells tolerated only 600 mg l^{-1}. Another *P. putida* on immobilization in Ca-alginate reduced the growth inhibition by phenol which was correlated with the formation of colonies in the gel matrix (Keweloh et al. 1989). Toxicity of bacteriostatic concentrations of phenol was shown to be significantly lower towards Ca-alginate-encapsulated mixed bacterial cultures than their free cell counterparts (Kapoor et al. 1998). Alginate-immobilized mycelial mass of *Graphium* sp. F1B4 could effectively degrade 18 mM phenol whereas freely suspended mycelia were not able to tolerate even 14 mM (Santos et al. 2003). As could be seen from the above examples, the improvement in the degrading ability of these organisms was slightly better as compared to alginate-encapsulated strain CP4. This could be because of the use of an un-buffered medium in the present case. This cannot, however, be taken as a drawback of the strain because much improved performance was exhibited by this strain on its encapsulation in agar gel, as discussed later.

In the present study extensive dissolution of the Ca-alginate beads (2% alginate) was observed when used in M3 medium containing KH_2PO_4 (2.72 g l^{-1}) and Na_2HPO_4 (5.0 g l^{-1}) as buffering salts. High phosphate concentration and also, probably, low concentration of alginate may be the reasons for this (Dainty et al. 1985). The bead disruption, however,

was prevented by eliminating phosphates from the medium. Keweloh et al. (1989) have reported dissolution of the beads in a medium containing 2 g l^{-1} of K_2HPO_4 leading to loss of protection to phenol. However, other workers who have used the same matrix were silent about this aspect, probably because they have used media with low concentrations of phosphates. Abd-El-Haleem et al. (2003) have reported that they did not observe any disintegration of the beads containing 4% alginate although their media contained K_2HPO_4 (2.75 g l^{-1}) and KH_2PO_4 (2.25 g l^{-1}). These were the reasons which prompted the authors to try phenol degradation using the alginate-immobilized cells in the normal phosphates-containing medium. By increasing the concentration of alginate in the gel and reducing the phosphate concentration in the medium or/and strengthening the gel structure by chemical modifications may, probably, this problem could be solved; but need to be experimentally verified. In the present study, however, better degradation of phenol by the Ca-alginate-entrapped strain CP4 in an un-buffered medium was observed both in batch mode (up to a concentration of 1500 mg l^{-1}) and in a continuous mode (with a degradation rate of 200 mg l^{-1} h^{-1}) than that by the free cells. Tris–HCl as a buffering agent did not prove to be useful as the degradation of 500 mg l^{-1} phenol itself was very slow and accumulation of the intermediary metabolite 2-HMS was observed indicating incomplete degradation. Accumulation of 2-HMS or any other catabolite, however, was not detected when Ca-alginate entrapped cells were used in M3 medium and un-buffered medium and agar gel-entrapped cells in M3 medium, as analyzed by HPLC. No accumulation of any metabolite was observed in earlier studies of phenol and cresols degradation with free cells in shake flasks also which was established by estimation of total organic carbon (TOC) and HPLC analysis of the culture filtrates which had proved the efficiency of the strain (Ahamad and Kunhi 1999; Babu et al. 1995b).

In the present study 2% alginate was used in the gel beads which were found to be quite efficient in phenol degradation and was sturdy enough to withstand the mixing by air sparging even after repeated uses, except in the case of phosphate-buffered medium, as mentioned above. Concentration of alginate between 2 and 4 was shown to have no effect on degradation of phenol by *P. putida* ATCC11172 (Hannaford and Kuek 1999).

It is known that lower the diameter of the beads better the oxygen transfer rate to the cells (Ogbonna et al. 1991). A bead size of 3 mm was used in the present study for both Ca-alginate and agar beads. Bettman and Rehm (1984) have demonstrated 1.4 times more activity of *Pseudomonas sp*. P8 immobilized in PAAH beads of a size of 2.5 mm diameter than that of 3 mm. This higher activity was attributed to higher surface area of smaller beads. Mordocco et al. (1999) have reported a bead diameter between 1 and 2 mm to be optimal for Ca-alginate beads containing *P. putida* ATCC 11172. However, in majority of reports on phenol-degrading immobilized systems the sizes of the beads were not mentioned. It may, probably, be possible to increase the degrading efficiency of the present strain by decreasing the bead size of the gels; but needs to be verified.

There are only very few reports on the use of agar-entrapped microorganisms for degradation of phenol (Juárez-Ramírez et al. 2001; Karigar et al. 2006). In the present study the strain CP4 immobilized in agar beads were found to be far better than the Ca-alginate immobilized one, in various respects. First of all, the protection provided against substrate toxicity was much higher than that of Ca-alginate. Leaching out of cells was minimal as against what was observed in the case of Ca-alginate immobilized system, because agar beads were highly compatible with phosphate buffered M3 medium and were more rigid. The comparative amounts of cells leached out and grown were equivalent of 0.05, 0.09, and 0.11 mg ml^{-1} of medium from agar beads as against 0.22, 0.48, and 0.86 mg ml^{-1} from Ca-alginate beads when used with 500, 1000, and 1500 mg l^{-1} phenol, respectively. Moreover, the cell content of agar beads did not decrease after several passages through increasing concentrations of the substrate in different batches, contrary to what was found in the case of Ca-alginate beads. There was rather a slight increase in cell population in agar beads. This may be because of the better cell holding capacity and flexibility of agar gel to accommodate more cells that were generated during their growth.

The degradation of phenol by agar entrapped cells of strain CP4 was faster than that by free cells and by Ca-alginate-entrapped cells. The rates of degradation (Q_{max}) of phenol in batch mode were 35.7, 36.6, 31.7, 27.8, 27.8, and 22.1 mg^{-1} l^{-1} h^{-1} when the concentrations were 500, 1000, 1500, 2000, 2500, and

3000 mg l^{-1}, respectively, which are higher and more consistent than that for Ca-alginate system. The comparative Q_{max} values for Ca-alginate immobilized system under batch conditions were found to be 38.5, 18.2, and 8.62 mg l^{-1} h^{-1} at concentrations of 500, 1000, and 1500 mg l^{-1}, respectively. It is quite evident that the protection provided towards higher concentrations of the substrate by entrapment in agar gel was far superior as compared to that by Ca-alginate gel as the latter could tolerate only up to a concentration of 1500 mg l^{-1}. The maximum rates of degradation (Q_{max}) of phenol in the case of free cells grown in fermenter under batch mode, at concentrations of 200, 500, 800, and 1000 mg l^{-1} were 20.6, 21.9, 21.7, and 15.1 mg l^{-1} h^{-1}, respectively, as calculated from the exponential phases of degradation. The Q_{max} reported earlier for shake flask was 57 mg l^{-1} h^{-1} at a phenol concentration of 1500 mg l^{-1} (Babu et al. 1995b). The reasons for this difference may be due to the effect of the less amount of inoculum used and the low rate of aeration provided in the present case, as already discussed above.

In continuous mode Ca-alginate beads of strain CP4 could degrade phenol at a maximum rate of 200 mg l^{-1} h^{-1}, at a dilution rate of 0.8 h^{-1}. With agar-entrapped cells, however, complete mineralization of phenol occurred with a maximum rate of 400 mg l^{-1} h^{-1}, at a steady state which was attained within a short time at an influent concentration of 4000 mg l^{-1}. *This seems to be the highest rate of degradation of phenol by any organism immobilized in any polymeric matrix that has been reported so far.* Phenol degradation at a rate of 7.68 g l^{-1} day^{-1} (320 mg l^{-1} h^{-1}) at an inlet concentration of 5000 mg l^{-1}, in an air-lift bioreactor under continuous operation, by *C. tropicalis* entrapped in PAA beads was the only nearer value found in literature (Chen et al. 2002). *A. citreus* immobilized in agar beads could degrade 22 mM (2070 mg l^{-1}) in batch mode at a maximum rate of 0.47 g l^{-1} day^{-1} (19.58 mg l^{-1} h^{-1}) (Karigar et al. 2006). A strain of *C. tropicalis* entrapped in agar could degrade phenol at 10 times higher rates than by free cells in a fluidized bed reactor with a phenol concentration of 3500 mg l^{-1} (Juárez-Ramírez et al. 2001). A few other polymers were also used for immobilizing microorganisms for phenol removal. PAAH-immobilized *P. putida* P8 in a fluidized bed reactor, at a dilution rate of 0.067, degraded phenol at a rate of 7.2 g l^{-1} day^{-1} (300 mg l^{-1} h^{-1}) under sterile condition and 3.12 g l^{-1} day^{-1} (130 mg l^{-1} h^{-1}) under non-sterile condition from an initial 2 g l^{-1} phenol (Bettmann and Rehm 1985). A mixed culture of *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03 immobilized in PVA degraded 95% of 800 mg l^{-1} phenol within 35 h (Liu et al. 2009). *A. awamori* NRRL 3112 immobilized in polyacrylonitrile with glutaraldehyde as coupling agent showed better degradation rates than by free mycelia (Yordanova et al. 2009). Under optimal conditions *P. putida* ATCC 11172 immobilized in Ca-alginate degraded phenol at a maximum rate of 250 mg l^{-1} h^{-1} (Hannaford and Kuek 1999). Mordocco et al. (1999) have studied continuous degradation of low concentrations of phenol by increasing the dilution rate and at a dilution rate of 0.6 h^{-1} a maximum degradation rate of 108 mg l^{-1} h^{-1} was achieved.

Conclusions

It has been demonstrated, in this study, that *Pseudomonas* sp. CP4 when immobilized in Ca-alginate and agar gels its tolerance to phenol and its degrading ability could be enhanced remarkably. Agar-entrapped cells were far superior to the Ca-alginate-immobilized ones. Agar-encapsulated cells degraded up to 3000 mg l^{-1} of phenol as compared to 1500 mg l^{-1} by Ca-alginate-entrapped cells whereas free cells could tolerate only 1000 mg l^{-1} in batch runs at an aeration rate of 1 vol^{-1} vol^{-1} min^{-1}, at 30°C and pH 7.0, whereas in a continuous process with Ca-alginate entrapped cells a degradation rate of 200 mg phenol l^{-1} h^{-1} was obtained while agar-entrapped cells could degrade up to 4000 mg phenol l^{-1} in the feed with a maximum degradation rate of 400 mg phenol l^{-1} h^{-1}.The strain is capable of degrading phenol, cresol isomers and a number of other aromatic compounds in shake flasks and also is an efficient partner to a 3-CBA-degrading *P. aeruginosa* 3mT in a mixed culture in the simultaneous degradation of phenol/cresols and 3-CBA, as shown in earlier studies. All these positive attributes will make it a very suitable candidate for deployment in the development of treatment technologies for removal of phenol, its derivatives and other aromatics from waste waters. However, more elaborate studies with respect to the longevity and the reusability of the immobilized systems, particularly that of agar gel entrapped ones

and the effect of various physical and chemical parameters and fluctuations in phenol loading rates may have to be conducted.

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