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Biodegradation of carbazole by *Pseudomonas* sp. GBS.5 immobilized in polyvinyl alcohol beads

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Abstract

The *Pseudomonas* sp. GBS.5 cells were immobilized in calcium alginate (2%): PVA (6%) matrix and the carbazole degradation was studied after dissolving the alginate linkages. This matrix was chosen for immobilization based on mechanical stability and immobilized cell viability. The maximum carbazole degradation rate achieved by PVA-alginate immobilized cells was 52.62 ppm h^{-1} as opposed to 39.06 ppm h^{-1} by cells immobilized in calcium alginate beads. The effect of cell loading capacity and initial carbazole concentration on carbazole degradation were also studied. The cells immobilized in PVA beads could be stably stored and be repeatedly used for eight cycles at 100 ppm carbazole concentration with high degradation activity. The immobilization process described in the paper resulted in increased carbazole degradation activity and hence the decrease in the cost of operations.

Keywords: Biodegradation, Calcium alginate, Carbazole, Poly vinyl alcohol, *Pseudomonas* sp.

Introduction

Carbazole (CAR) is nitrogen containing heterocyclic compound and is chiefly found in the waste generated by various pharmaceutical, petrochemical and dye industries (Benedik et al. 1998). It is reported to be carcinogenic and mutagenic and can also undergo radical chemistry to generate more genotoxic compound (Jha and Bharti 2002). Bioremediation is considered as the most effective and environmentally benign cleanup technology for the degradation of xenobiotic compounds. Several bacteria have been reported to utilize CAR viz. *Acinetobacter*, *Enterobacter*, *Gordonia*, *Klebsiella*, *Pseudomonas*, *Sphingomonas* etc. (Santos et al. 2006; Li et al. 2008; Larentis et al. 2011; Singh et al. 2011a, 2011b). They follow similar biochemical pathway and convert CAR to anthranilic acid which is then mineralized. Genes and enzymes involved in the conversion of CAR to anthranilic acid are also well established (Nojiri 2012).

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Bioremediation can be carried out by both free and immobilized cells but immobilized cell system shows several unique advantages over free cells such as prolonged activity and stability of the biocatalyst, feasibility of continuous processing, increased tolerance to high substrate concentration, easier recovery with no need for separation and filtration steps, regeneration and reuse of the biocatalyst for extended periods in batch operations, reduction of microbial contamination risk and ability to use smaller bioreactors with simplified process designs (Siripattanakul et al. 2008; Ahamad and Kunhi 2011). The key for immobilization is the choice of the support material. Each support has its own advantage and disadvantage in terms of the microorganism used and the compound to be degraded. PVA is the most promising matrix as it is cheap, non toxic and mechanically robust. However, the biggest limitation with the use of PVA is the formation of sheets rather than beads resulting in the decrease of surface area. It has been reported that PVA beads are formed by the addition of sodium alginate along with PVA to the saturated boric acid and calcium chloride solution (Zain et al. 2011). PVA contribute to the durability and strength of the bead while sodium alginate helps in improving surface property and preventing agglomeration. Although, the addition of sodium alginate is important for the stability of the beads but it also contributes to the decrease in the pore size, leading to mass transfer limitation and ultimately decrease in the degradation activity of the microorganism immobilized (Phisalaphong et al. 2007). The pore size of the bead is decided by three different factors i.e. concentration of gelling solutions (PVA, sodium alginate), concentration of cross linking agents (boric acid, calcium chloride) and the time of incubation for crosslinking.

The main focus of the present study was to determine the most suitable condition for the synthesis of the PVA beads for carbazole degradation. The beads were evaluated in terms of stability, viability of immobilized cells and CAR degradation activity. The degradation studies of CAR were carried out by immobilization of *Pseudomonas* sp. GBS.5. This microorganism is reported to have the ability to convert CAR into anthranilic acid (Singh et al. 2013).

Materials and Methods

Chemicals

All the chemicals used were of highest purity grade. CAR was purchased from Acros Organics (New Jersey, USA). Sodium alginate and polyvinyl alcohol were purchased from Hi-media Laboratory (Mumbai, India). Calcium chloride, sodium nitrate, sodium sulfate, boric acid and components of phosphate buffer were supplied by Qualigens Fine Chemicals (Mumbai, India). Organic solvents used (acetonitrile, acetone, and ethyl acetate) were of HPLC grade from Qualigens Fine Chemicals.

Microorganism and Culture conditions

CAR degradation was studied by *Pseudomonas* sp. strain GBS.5 which was previously isolated in our laboratory, from the soil contaminated with dye. Luria broth solidified with 1.5% (w/v) agar was used for maintenance (at 30ºC). The basal salts medium used for pre-cultivation of microorganism contained (g l^{-1}) KH_2PO_4, 2.44; Na_2HPO_4, 5.57; Na_2SO_4, 2.0; KCl, 2.0; $MgSO_4.7H_2O$, 0.2; $MnCl_2$, 0.02; $FeCl_3$, 0.001; $CaCl_2$, 0.003. The medium was supplemented with CAR (500 ppm) and kept at 30˚C on a rotary shaker at 180 rpm for the desired incubation period. For degradation studies, mineral salts medium containing (g l^{-1}) $MgSO_4.7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2$, 0.2; K_2HPO_4, 0.15; NH_4Cl, 1.0 was used. The pH of the medium was adjusted to 7.0 ± 0.05. CAR at various concentrations (100, 300 and 500 ppm) was used. The medium was kept at 30˚C on a rotary shaker at 180 rpm for degradation studies.

Immobilization of Pseudomonas sp. GBS.5

During immobilization studies *Pseudomonas* sp. strain GBS.5 was grown in the mineral salts medium containing CAR (500 ppm) as sole source of carbon and energy. Cells were harvested during late logarithmic phase (40 h) by centrifugation at 6000 rpm for 10 min at 4°C. The pellet obtained was used for immobilization. For immobilization in calcium alginate beads, alginate (2% w/v) was dissolved in boiling water and autoclaved at 121˚C for 15 min. The bacterial pellet obtained was suspended in alginate solution (30˚C), and mixed by mild vortexing. This alginate/ cell mixture was extruded drop by drop into a cold, sterile 0.2M $CaCl_2$ solution through a syringe with needle. Gel beads of approximately 2 mm diameter were obtained. The beads were hardened in the $CaCl_2$ solution for 2 h. Finally these beads were washed and stored in distilled water at 4ºC till further studies. For immobilization in PVA-alginate beads, sterilized solution containing PVA and sodium alginate was prepared by dissolving the compounds in boiling water and autoclaving at 121°C for 15 min. The bacterial pellet obtained was suspended in PVA-alginate solution (30°C), and mixed by mild vortexing. This solution was extruded through a syringe into a beaker containing the gelling solution with known concentration. The beads were kept in the cross-linking agent for the desired time period. These beads were washed with distilled water to remove any remaining gelling solution and incubated in phosphate buffer for six hours to dissolve the calcium alginate linkages.

Test for mechanical stability of beads

To determine the mechanical stability of various bead prepared for this study, 50 beads of each type were taken separately in 50 ml beakers with 10 ml distilled water and sonicated in a sonicating water bath. At 30 min interval, beads were collected and counted to determine the number of beads remaining after the mechanical stress. The beads were also air dried and its weight was measured to calculate percentage weight loss.

Test for viability of immobilized cells

For calculating the viability of cells immobilized in PVA beads, samples of respective bead (ten each) were washed with sterile distilled water and disintegrated in buffer solution containing 0.05M Na_2CO_3 and 0.02M citric acid. The buffer solution was then plated onto agar plates and CFU was calculated after incubating the plates at 30°C for 16 h.

Carbazole degradation studies

Based on the characterization results beads prepared using 6% PVA and 2% sodium alginate were used for immobilizing *Pseudomonas* sp. GBS.5. CAR degradation efficiency was assessed in 250 ml flask with 50 ml media containing the desired amount of CAR (100, 300 or 500 ppm). The beads with cells were added to the flask and incubated in orbital shaker at 30°C and 180 rpm. Samples of the medium were collected at the indicated times for the analysis of CAR and acidified using 2N HCl to stop any residual activity. For establishing the long-term stability of CAR degradation by entrapped cells, repeated batch processes were carried out. After every incubation period, the used medium was decanted, beads were washed with autoclaved water and transferred into a fresh mineral salts medium containing CAR. The degradation process was carried out under identical fermentation conditions. To calculate the loss of CAR by absorption, control experiments with beads without any bacterial culture were carried out in sterile medium.

Analytical method

Residual CAR was quantified after extraction with ethyl acetate using High Performance Liquid Chromatography (HPLC; Waters Associates, Milford, MA). Separation was achieved using a reverse phase C8 column (Waters RP 8, 3.3 µm, 4.6 x 150 mm) and extracts were analyzed using acetonitrile and water (80:20 v/v) as mobile phase, at a flow rate of 0.5 ml per min. CAR detection was performed at 233.7 with a photodiode array detector (PDA 2996; Waters).

Kinetic analysis

Kinetics of CAR biodegradation was calculated by first-order equation:

$$\ln \frac{C}{C_0} = -kt$$

Where C_0 and C are the concentration of CAR at time zero and t (h), respectively, and k_1 is the first order rate constant. The results of biodegradation studies were plotted using natural logarithm of CAR concentration as a function of time. Rate constant (k_1) was calculated by regression analysis. Half-lives of CAR ($t_{1/2}$) were determined by equation:

$$t_{1/2} = \ln 2/k$$

Results and Discussion

Preparation and characterization of the beads

The most suitable bead type was initially screened based on the mechanical stability of the bead and the viability of the immobilized cells. In order to maximize the pore size, different

bead types (Table 1) were prepared by varying concentration of PVA, boric acid and the time of cross linking with fixed sodium alginate concentration. The beads formed were then incubated in phosphate buffer to dissolve the calcium alginate bonds. Fig. 1 represents the appearances of the beads formed using varying concentration of PVA during the study. It can be seen in the figure that bead type 2 was most stable. All other beads either showed agglomeration or disintegration after incubation in phosphate buffer. Mechanical stability of the beads obtained with varying boric acid concentration and the time of crosslinking at fixed 6% PVA concentration (Bead type 7, 8 and 9) was studied. The percentage disintegration decreased with increasing crosslinking time. Bead type 7, 8 and 9 when each crosslinked for 1 h, 3 h, 6 h or 12 h showed complete disintegration after sonication. However, bead type 8 and 9 when crosslinked for 24 h were stable and the percentage weight loss was only 5%. Thus these beads were selected for checking the number of viable cells immobilized inside the beads. Bead prepared using 3% and 4% boric acid showed 10% and 30% decrease in the viability of the cells, respectively (Table 2). Hence, based on mechanical stability and the viability of the immobilized cells inside the beads, bead prepared using 6% PVA and 3% boric acid was selected for further biodegradation experiments.

Table 2: Effect of boric acid concentration on the viability of the immobilized cells.

Bead Type (Cross linking Time 24 h)	Cells/bead	
	Initial	Final
8 (3% Boric Acid)	10×10^9	9×10^9
9 (4% Boric Acid)	10×10^9	7×10^9

The number of beads (n) used for calculating the number of cells per bead was constant in both the cases (n = 50). The beads were dissolved and the supernatant was used for calculating the number of cells per bead.

Effect of cell concentration and carbazole concentration on carbazole degradation

Feasibility of the beads for industrial purpose was calculated by studying the effect of the number of cells immobilized per bead and effect of carbazole concentration on degradation. The initial number of cells used for immobilization was varied and the degradation activity was calculated in the first cycle, second cycle and the fifth cycle (Table 3). Result shows there was no increase in the rate of degradation with the increase in the number of cells. Moreover, the CAR degradation activity of PVA-alginate beads was more as compared to calcium alginate immobilized cells (Table 4). Table 5 shows reuse studies of immobilized cells at different initial CAR concentration i.e. 100 ppm, 300 ppm and 500 ppm. The degradation activity for the immobilized cells increased with

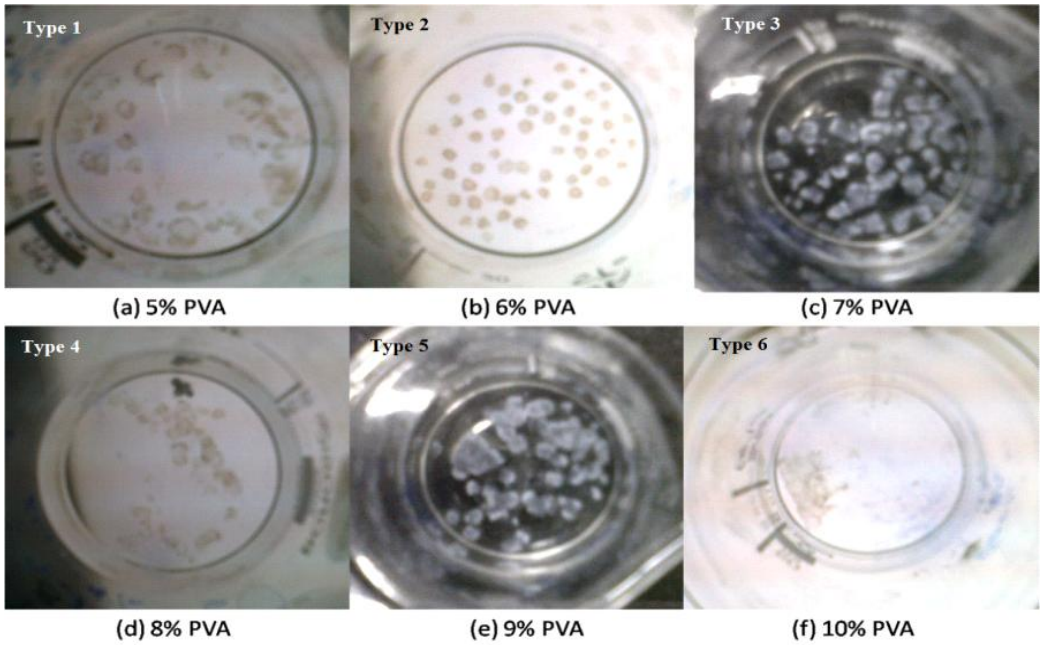

Figure 1: Appearance of the various beads prepared for immobilization using different concentration of PVA (5% - 10%). The concentration of boric acid, sodium alginate and calcium chloride was fixed as 3%, 2% and 0.2 M, respectively. The beads with 5% PVA disintegrated after incubation in phosphate buffer, where as beads with higher PVA concentration (7%-10%) showed agglomeration.

Table 1: Composition and incubation time of various beads prepared and tested for carbazole degradation studies

Bead Type	Immobilization material	Gelling Solution
1	PVA 5% + Alginate 2%	Boric Acid 3% + calcium chloride 0.2 M
2	PVA 6% + Alginate 2%	Boric Acid 3% + calcium chloride 0.2 M
3	PVA 7% + Alginate 2%	Boric Acid 3% + calcium chloride 0.2 M
4	PVA 8% + Alginate 2%	Boric Acid 3% + calcium chloride 0.2 M
5	PVA 9% + Alginate 2%	Boric Acid 3% + calcium chloride 0.2 M
6	PVA 10% + Alginate 2%	Boric Acid 3% + calcium chloride 0.2 M
7	PVA 6% + Alginate 2%	Boric Acid 2% + calcium chloride 0.2 M (Incubation time varied for 1 h, 3 h, 6 h, 12 h and 24 h)
8	PVA 6% + Alginate 2%	Boric Acid 3% + calcium chloride 0.2 M (Incubation time varied for 1 h, 3 h, 6 h, 12 h and 24 h)
9	PVA 6% + Alginate 2%	Boric Acid 4% + calcium chloride 0.2 M (Incubation time varied for 1 h, 3 h, 6 h, 12 h and 24 h)

increasing concentration of CAR during the first cycle. However, by the end of the fifth cycle the degradation activity decreased to one third the corresponding degradation activity in the first cycle for 300 ppm and 500 ppm but the percentage decrease in the degradation activity with 100 ppm was less. The same trend was observed for the calcium alginate immobilized cells. Immobilized cells are preferred for process development as they are easy to operate and have good storage stability in comparison to free cells. However, the biggest problem associated with immobilization is the decrease in the degradation activity due to mass transfer limitation (Phisalaphong et al. 2007; Suttinun et al. 2010). Cells continuously grow and die inside the beads during cultivation.

cases, the ability of the cells to maintain the same degradation activity for the number of cycles decreased with increasing concentration of CAR suggesting that the increased concentration of CAR is toxic to the cells. However, the degradation activity of the PVA-alginate beads was more as compared to calcium alginate beads during the initial cycles at all the different CAR concentration. This decrease in degradation activity of the calcium alginate vs PVA-alginate immobilized cells can be attributed to the mass transfer limitation. This limitation was successfully overcome by removing the calcium alginate linkages in case of PVA-alginate beads. Biodegradation Kinetic analysis showed that

Table 3: The biomass and degradation rate changes in PVA-alginate bead after repeated use

Matrix	Cell content (Number of cells per bead)			Carbazole degradation rate (ppm h^{-1})		
	Initial number of cells (I)*	II	V	I	II	V
PVA-Alginate I	8×10^9	14×10^9	14×10^9	8.50	11.25	9.62
PVA-Alginate II	12×10^9	16×10^9	16×10^9	8.25	10.75	9.75
PVA-Alginate III	15×10^9	17×10^9	17×10^9	8.62	10.25	9.75

*Initial number of cells represents the number of cells at the starting of cycle-I.
The number of cells was calculated at the start of the respective cycles and the carbazole degradation activity was calculated, taking these numbers of cells in to consideration. Initial carbazole concentration was 100 ppm. The values are means of three independent replicates. SD was within the acceptable range.

Table 4: The biomass and degradation rate changes in alginate bead after repeated use

	Cell content (Number of cells per bead)			Carbazole degradation rate (ppm h^{-1})		
	Initial number of cells (I)*	II	V	I	II	V
Alginate I	9×10^9	13×10^9	13×10^9	5.43	10.12	6.56
Alginate II	10×10^9	15×10^9	15×10^9	5.81	11.43	8.75
Alginate III	14×10^9	16×10^9	16×10^9	6.25	11.62	8

*Initial number of cells represents the number of cells at the starting of cycle-I.
The number of cells was calculated at the start of the respective cycles and the carbazole degradation activity was calculated, taking these numbers of cells in to consideration. Initial carbazole concentration was 100 ppm. The values are means of three independent replicates. SD was within the acceptable range.

Table 5: Degradation activity at different carbazole concentration for cells immobilized in alginate and PVA-alginate beads

Initial concentration of carbazole (ppm)	Carbazole degradation rate (ppm h^{-1})					
	Alginate			PVA-Alginate		
	I	II	V	I	II	V
100	5.87	11.50	8.75	7.00	9.25	9.75
300	19.87	28.12	7.75	25.12	28.50	9.37
500	35.00	36.25	6.25	40.00	50.62	13.75

The growth pattern is distributed with more cells growing on the edges where as less number of cell grow in the centre of the bead. It has been proposed that such a difference in the growth pattern is observed because of the limitation of the oxygen and the nutrients in the centre of the bead (Adinarayana et al. 2004). The resultant degradation activity is because of such changes. The degradation activity can be increased by decreasing the mass transfer limitation and thus resulting in the increase in the amount of oxygen and nutrients in the centre of the bead and subsequently increase in the number of immobilized cells. In this study, *Pseudomonas* sp. GBS.5 was immobilized inside PVA-alginate bead and in order to increase the permeability of nutrients, the alginate linkages were removed. The suitability of PVA-alginate bead was compared with those of alginate bead via calculating the effect of cell loading capacity and the effect of different CAR concentration on the degradation activity. CAR is the only carbon source provided in the media and is utilized by the microorganisms for their growth. The increased number of cells results in less concentration of CAR.

The effect of different concentration of CAR (100 ppm, 300ppm and 500ppm) was also studied on the degradation activity. Cells immobilized in PVA-alginate as well as calcium alginate bead were able to degrade all the different concentration of CAR. In both the

Table 6: Carbazole biodegradation rate constants (k_1) and half-lives ($t_{1/2}$)

Immobilization matrix	Kinetic parameters		
	k_1 (h^{-1})	$t_{1/2}$ (h)	R^2
PVA-alginate	0.237	2.92	0.98
Calcium alginate	0.127	5.45	0.97

CAR degradation by *Pseudomonas* sp. GBS.5 immobilized in PVA-alginate and calcium alginate beads follows first order reaction kinetics. Data obtained by kinetics studies (for initial concentration of 500 ppm) are shown in table 6.

Conclusions

Pseudomonas sp. GBS.5 was successfully immobilized with significant CAR degradation activity. The higher CAR degradation activity of PVA-alginate cells as compared to alginate immobilized cells at different CAR concentration and the use of less number of cells suggest that PVA-alginate is a better matrix as compared to the conventional matrices (calcium alginate). This matrix can be exploited to immobilize bacteria for obtaining better degradation activity for other xenobiotic compounds as well.

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