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Enhanced phenol degradation by *Pseudomonas* sp. SA01: Gaining insight into the novel single and hybrid immobilizations

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ABSTRACT

In this work, *Pseudomonas* sp. SA01 cells were immobilized in a series of singular and hybrid immobilization techniques to achieve enhanced phenol removal. The singular immobilization strategies consisted of various concentrations of alginate (2–4%) and pectin (3–5%), while the hybrid strategies incorporated polyvinyl alcohol (PVA)–alginate and glycerol–alginate beads and alginate–chitosan–alginate (ACA) capsules. Immobilization protected cells against phenol and resulted in remarkable reduction (65%) in degradation time by cells immobilized in either alginate (3%) beads, in a hybrid PVA–alginate beads, or in ACA capsules compared to freely suspended cells. Cells immobilized in PVA–alginate and ACA provided the best performance in experiments using elevated phenol concentrations, up to 2000 mg/L, with complete degradation of 2000 mg/L phenol after 100 and 110 h, respectively. Electron microscopy examination indicated that cell loading capacity was increased in PVA–alginate hybrid beads through reduced cell leakage, resulting in higher activity of PVA–alginate hybrid beads compared to all other immobilization methods.

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1. Introduction

Phenol is a toxic organic component often found in wastes from oil refining, plastics, coke, petroleum refining, and pharmaceutical processing. Being water-soluble, phenolic effluents contaminate nearby watersheds. The fate of phenol and its derivatives is of serious environmental concern since they persist as toxic species. The U.S. Environmental Protection Agency (EPA) has listed phenol as a major pollutant [1] with toxicity to aquatic microorganisms and malodors imparted at very low concentrations (0.005 mg/L) [2]. Phenol is toxic upon ingestion, contact or inhalation and it is recommended that human exposure to phenol not exceed 20 mg in an average day [3]. Also, phenol is toxic to fish and can be lethal at concentrations of 5–25 ppm. Additionally, concentrations as low as 0.1 ppm can taint the taste of fish. Due to their toxicity to microorganisms, phenol and its derivatives may often cause the breakdown of wastewater treatment plants by inhibition of microbial growth [4]. Therefore, treatment of phenol effluents is critical to maintaining both human and wildlife environments. Among the methods for removing phenol and its homologues from polluted environments, biological treatment is generally preferable due to its effectiveness and innocuous end products [5,6]. However, microorganisms suffer from substrate inhibition at higher concentrations, slowing their growth [7]. Several strategies have been proposed to overcome substrate inhibition, including cell acclimation to higher phenol concentrations [8], use of genetically engineered microorganisms (GEMs) [9], and cell immobilization [10,11]. Acclimation to higher pollutant concentrations by exposure to successively increasing phenol concentrations is not always feasible and usually requires long lag-times. The method demonstrated by Masque required times as high as 20 days to degrade 1000 mg/L phenol [8]. Currently, there is controversy over the use of wild type or GEMs in bioremediation. Thus, government agencies are often unwilling to release GEMs to the environment given the potential for unforeseen ecological impacts [12]. This leaves cell immobilization on various supports as an attractive and promising strategy to overcome substrate inhibition. The degradation of aromatic compounds by immobilized cells has been reported previously, including immobilized cell reactors using phenol as the model toxic compound [13]. Since the immobilized cells remain viable for a considerable duration, they are a better alternative than free cells for the biore-

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mediation of toxic organics in effluents. Moreover, immobilized cells offer the possibility of degrading higher concentrations of toxic pollutants than can be achieved with free cells [14,15]. In the present investigation, a pure culture of phenol degrading bacteria, *Pseudomonas* sp. SA01, was immobilized on different matrices using different immobilization methods to compare the phenol degradation activity of free and immobilized cells. The mechanical strength of the immobilization beads was also investigated through single and hybrid immobilization techniques. Moreover, the performed investigation includes the assessment of density, distribution and morphological statues of immobilized cells by light and transmission electron microscopy. We hoped to decrease detrimental effects of elevated concentrations of phenol by improving the immobilization conditions. The improved immobilization efficiency presented here can be applied for effective degradation of various mono- and poly-aromatic pollutants and may help satisfy industrial bioremediation requirements for eliminating toxic wastes which further emphasize its potential application in environmental biotechnology.

2. Materials and methods

2.1. Microorganisms and media

The bacterial strain was isolated from a pharmaceutical contamination site and identified to be *Pseudomonas* sp. SA01; it was able to grow on phenol as a sole source of carbon and energy. For phenol degradation studies using free cells, the mineral salt medium (MSM) contained (g/L): Na_2HPO_4 (3), KH_2PO_4 (1.5), NH_4Cl (1), NaCl (0.5), $MgSO_4{\cdot}7H_2O$ (0.2), $CaCl_2$ (0.02), and thiamine (0.006). For immobilized cells, the degradation medium contained (g/L): Na_2HPO_4 (0.5), KH_2PO_4 (0.5), NH_4Cl (1), NaCl (0.5), $MgSO_4{\cdot}7H_2O$ (0.2), $CaCl_2$ (0.02), and thiamine (0.006). All the chemicals in the growing medium were analytical grade from Aldrich, USA. To improve bead stability, the phosphate level of the medium was reduced by a factor of three for the immobilized trials medium. The pH for all solutions was adjusted to 7.0 and phenol was added to the media at various concentrations under vigorous agitation to obtain a homogenous mixture. MSM and apparatus were autoclaved at 121 °C for 15 min, and phenol was sterilized by membrane filter.

2.2. Inoculum development

A loop of pure *Pseudomonas* sp. SA01 culture was inoculated into 100 mL MSM containing 500 mg/L phenol in 250 mL Erlenmeyer flasks, incubated at 30 °C and agitated at 150 rpm until optimum growth was achieved. This culture was then used for inoculums.

2.3. Cell harvesting for immobilization

The phenol degrading bacterium, grown in MSM containing phenol as the sole source of carbon, were harvested at the stationary phase by centrifugation at 5000 rpm for 10 min at 4 °C and subsequently immobilized. For all immobilization procedures, the final cell concentration was 1.2×10^8 cfu/mL, matched with a McFarland standard (0.5).

2.4. Entrapment of whole cells in alginate

A 10 mL bacterial cell suspension was added to an alginate (Sigma, USA) solution at 40 °C and mixed on a magnetic stirrer to obtain final alginate concentrations of 2%, 3% and 4% (w/v). These alginate-cell suspensions were extruded into a 0.2 M $CaCl_2$ solution to form beads with a diameter of roughly 2 mm. After gelling for 1 h, the beads were washed three times with normal saline and used for experiments.

2.5. Encapsulation of whole cells in alginate–chitosan–alginate

Encapsulation was done according to Oi et al. [16]. A 10 mL bacterial cell suspension was added to an alginate solution to obtain a 1.5% (w/v) alginate concentration. The cell-alginate suspension was extruded into a 0.1 M $CaCl_2$ solution and allowed to gel for 30 min to obtain cell containing beads. A 0.5% chitosan (Sigma, USA) solution was then added to the beads at a volume ratio of 1:5 (beads:solution) to form a membrane. The membrane was then rinsed with normal saline to remove excess chitosan. A 0.15% alginate solution was subsequently added to counteract remaining chitosan charges on the membrane. Finally, the alginate–chitosan–alginate (ACA) membrane was disrupted with 55 mmol/L sodium citrate to obtain capsules having liquid cores.

2.6. Entrapment of whole cells in polyvinyl alcohol (PVA)–alginate and glycerol–alginate

PVA (Sigma–Aldrich, USA) (1 g) and sodium alginate (0.16 g) were mixed in distilled water at 80 °C. The final concentrations of PVA and sodium alginate were 5% and 0.8% (w/v) respectively (PVA:alginate ratio 6.25:1). The PVA–alginate solution was then cooled to 40 °C and mixed thoroughly with a cell suspension to obtain. Two additional ratios of PVA to alginate of 8:1 (1.28 g PVA–0.16 g sodium alginate) and 10:1 (1.6 g PVA–0.16 g sodium alginate) were also prepared. The PVA–alginate mixtures were then extruded in a 0.2 M $CaCl_2$ solution. The resulting beads were washed with normal saline and used for experiments. Cell preparations and immobilization in glycerol (Merck, Darmstadt, Germany)–alginate and the ratio of glycerol to alginate were similar to those used for PVA–alginate entrapment.

2.7. Entrapment of whole cells in pectin

Pectin (Sigma, USA) solutions were prepared by drop wise addition of 2N NaOH with stirring to achieve pH 7.0 and mixed with 5 mL of bacterial cell suspension to obtain final pectin concentrations of 3%, 4% and 5% (w/v). Afterwards, bead preparation was done in the same manner as described for alginate. Briefly, the pectin-cell suspensions were extruded in a 0.2 M $CaCl_2$ solution to form beads with a diameter of about 2 mm. After gelling, the beads were washed in normal saline and used for experiments.

2.8. Shake flask culture experiments

2.8.1. Free cells

Batch fermentations of free cells (1.2×10^8 cells) were performed in 1000 mL conical flasks containing 200 mL MSM supplemented with phenol at different initial concentrations (500, 700, 1000 and 1200 mg/L). Fermentations were carried out at 30 °C on a rotary shaker at 150 rpm for the desired incubation periods. Samples of the culture broth were taken at the indicated times for phenol analysis. Control experiments were also carried out to evaluate abiotic degradation in sterile medium.

2.8.2. Immobilized cells

For immobilized *Pseudomonas* sp. SA01 cells, 50 g of wet biocatalyst was placed in 1000 mL conical flasks containing 200 mL MSM and the desired amount of phenol (1000, 1200, 1600, or 2000 mg/L). Due to immobilized cells high efficiency in phenol degradation, concentrations lower than 1000 mg/L were not tested. Fermentations were carried out at 30 °C on a rotary shaker at 150 rpm for the desired incubation periods and sampled at the indicated times

for phenol analysis. During all immobilization experiments, control tests were also carried out using polymer beads without cell to evaluate if the polymer beads contribute to phenol removal.

2.8.3. Repeated batch fermentations

Repeated batch fermentations were carried out to establish the long-term and operational stability of phenol degradation by immobilized cells. After complete phenol degradation, the used medium was decanted and fresh medium containing phenol at two different initial concentrations (1000 and 1200 mg/L) was added to the flasks. The degradation processes were carried out under identical conditions until the biocatalysts lost their degradation efficiency.

2.9. Analysis of phenol

Phenol (Merck, Darmstadt, Germany) concentrations were determined using the 4-aminoantipyrine method [17]. A reaction mixture containing 9.8 mL distilled water, 100 μL sample, 25 μL 2% 4-aminoantipyrene, and 50 μL 2 M ammonia were mixed well before adding 25 μL 8% potassium hexacyanoferrate (III). The absorbance at 500 nm was measured and correlated to a calibration curve. All samples were analyzed three times to calculate an average value.

2.10. Biomass estimation

To verify the efficiency of biocatalysts in the reaction system, the biomass estimation method of Song et al. [18] was modified and employed that is based on the cell protein content in the gel beads. To this end, 1 g of gel beads was put on a clean glass plate and sectioned into a number of pieces using a surgical knife. The sectioned gels were then placed in a test tube and incubated in sodium dodecyl sulphate (SDS) solution (10%, 3 mL) for 2 h to extract cell protein. After centrifugation, the protein content of the gel beads was assayed using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA), according to manufacturer's instruction. Bovine serum albumin (BSA) (Sigma, USA) was used as a calibration standard.

2.11. Morphological observation

To assess the density, distribution and morphological status of immobilized bacteria, individual beads from different experiments were exposed to 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer and kept at 4 °C for 24 h to fix the beads. The fixative solution was then carefully removed and the beads were washed three times for 10 min with 0.1 M cacodylate washing buffer. The beads were then incubated in 1% osmium tetroxide (OsO_4) in a 1.5% potassium ferrocyanide (KFeCN) solution at 4 °C for 30 min. OsO_4 was then removed and the beads were again washed with 0.1 M cacodylate washing buffer. After 10 min, the beads were dehydrated with ethanol in increments of 10% from 30% up to 90%, allowing five-minute intervals between each increment. The beads were then further dehydrated twice with 100% ethanol over 10 min intervals. Following dehydration, the beads were infiltrated by adding increasing amounts of epon to ethanol (from 1:1 to 3:1), allowing 30 min intervals between each subsequent addition. Pure epon, deaerated under vacuum (constant pressure <25 psi), was then added to the beads, whereupon the beads were allowed to sit for 1 h under vacuum to remove ethanol residue and air bubbles in the epon. Finally, the bead containing wells were refilled with new epon and placed in an oven at 60 °C for 48 h to polymerize the epon.

For light microscopy (LM) investigation, semi-thin sections (500 nm thick) were prepared using an Ultracut-E ultramicrotome (Reichert-Jung, Leica Microsystems, Austria), placed on glass slides, and stained with Toluidin Blue for transmission electron microscopy (TEM) observation, ultrathin sections (70 nm thick) were cut and transferred to TEM grids (200 mesh copper grids, EMS Sciences, USA). Digital images were acquired using a Gatan 792 Bioscan 1k × 1k Wide Angle Multiscan CCD camera in Tecnai 12 FEI TEM at 120 kV.

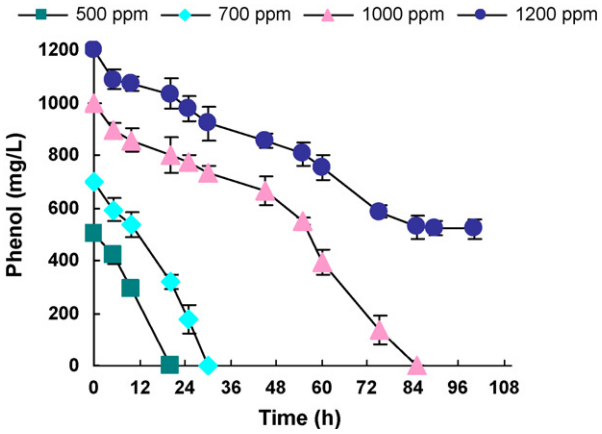

Fig. 1. Phenol degradation by freely suspended cells in batch culture.

3. Results

3.1. Phenol degradation by freely suspended cells

Pseudomonas sp. SA01 bacteria isolated from a pharmaceutical contamination site (registered with accession number EU315700 in NCBI) and were capable of utilizing phenol as a sole source of carbon and energy. Batch degradation results for different initial concentrations using freely suspended bacteria are presented in Fig. 1. The data show that degradation for initial concentrations of 500 and 700 mg/L occurred completely without a significant lag phase after 20 and 30 h respectively, indicating good acclimation of the bacteria to the phenol source and medium. However, when the initial concentration increased to 1000 mg/L, complete degradation required 84 h. Further, this strain was not able to fully degrade 1200 mg/L over the 8 days monitoring cycle.

3.2. Phenol degradation by cells immobilized in alginate beads and ACA capsules

The control studies using sterile immobilized bacteria indicated no significant phenol losses due to evaporation or adsorption on the sterile support. Results for degradation by cells entrapped in alginate (2–4%) beads and in ACA capsules are given in Fig. 2. Using an initial phenol concentration of 1000 mg/L, the time for complete degradation by cells immobilized in alginate (3%) and ACA was 30 h. This shows that the degradation time was dramatically decreased by entrapping the cells in alginate, allowing degradation to occur in roughly 35% of the time required by freely suspended cells for an equivalent initial concentration. Increasing the alginate concentration from 2% to 3% led to a reduction in degradation time; however, there was not a considerable difference between the performances of cells immobilized in 3% and 4% alginate. Cells immobilized in ACA were found to be as capable as 3% alginate entrapped cells for phenol degradation.

3.3. Phenol degradation by cells immobilized in PVA–alginate

A combination of PVA–alginate and a gelling solution of $CaCl_2$ to prevent agglomeration of the PVA beads were also studied using

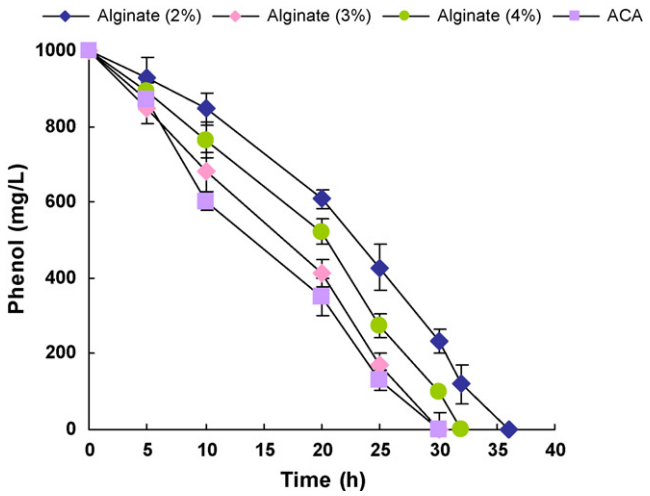

Fig. 2. Phenol degradation by cells immobilized in alginate (2–4%) and ACA (alginate–chitosan–alginate) in batch culture.

PVA to alginate ratios of 6:1, 8:1, and 10:1. It was thought that calcium alginate would improve the surface properties of the beads which would be helpful in preventing agglomeration and producing spherical beads. Results obtained using PVA–alginate hybrid beads showed that this biocatalyst is as capable as alginate (3%) and ACA immobilized cells in phenol degradation (30 h for an initial concentration of 1000 mg/L) (Fig. 3). While agglomeration was prevented for all ratios of PVA to alginate, better phenol degradation resulted from higher alginate ratios which may be resulted from better surface properties that reduced mass transfer resistance.

3.4. Phenol degradation by cells immobilized in glycerol–alginate

The time course of phenol degradation by cells immobilized in glycerol–alginate is shown in Fig. 4; the ratios of glycerol to alginate were same as those of PVA–alginate. The glycerol–alginate immobilized cells showed considerable phenol degradation activity, with a ratio of 6:1 being the best combination. Similar to PVA–alginate hybrid beads, more rapid phenol degradation was achieved with higher concentrations of alginate.

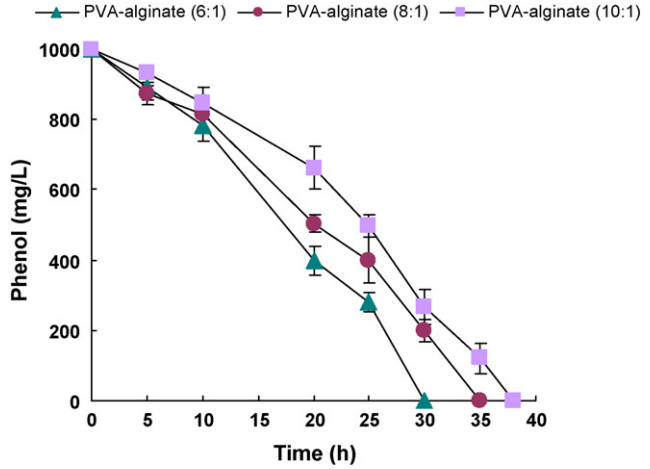

Fig. 3. Phenol degradation by cells immobilized in PVA–alginate (6:1), (8:1) and (10:1) in batch culture.

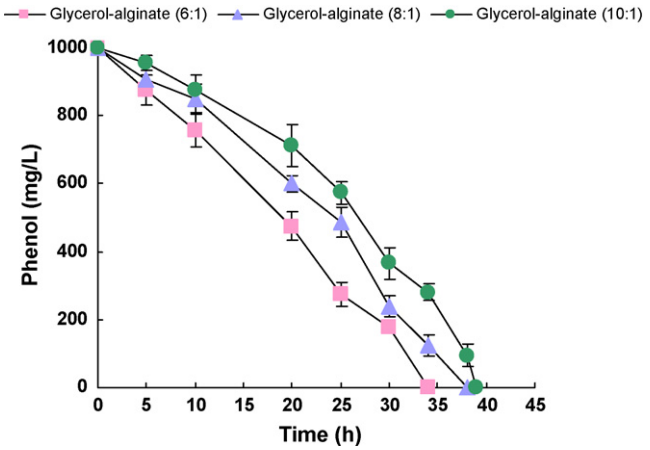

Fig. 4. Phenol degradation by cells immobilized in glycerol–alginate (6:1), (8:1) and (10:1) in batch culture.

3.5. Phenol degradation by cells immobilized in pectin

Results obtained for phenol degradation by cells immobilized in pectin are shown in Fig. 5. The degradation activity of cells immobilized in pectin was less than with cells immobilized in all other matrices studied. However, it remains evident that even the best degradation time (40 h with 5% pectin) was much better than was achieved with freely suspended cells (84 h) for an initial phenol concentration of 1000 mg/L.

3.6. Degradation of higher phenol concentrations

To assess the phenol tolerance of immobilized cells and to evaluate degradation at higher initial concentrations, batch cultures using the various immobilizations studied were conducted using initial concentrations of 1200, 1600 and 2000 mg/L. The results revealed that cells immobilized in PVA–alginate and ACA provided the best performance for all concentrations tested, with complete degradation of 2000 mg/L phenol degraded after 100 and 110 h respectively (Figs. 6 and 7). Other biocatalysts, including glycerol–alginate, alginate, and pectin immobilized cells, were unable to degrade phenol completely at an initial concentration of 2000 mg/L. Further, cells immobilized in pectin had the lowest phenol degradation ability among all biocatalysts at all initial concentrations, including the elevated concentrations.

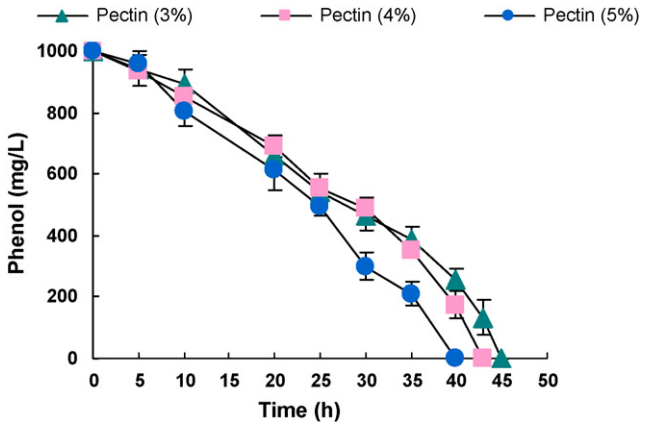

Fig. 5. Phenol degradation by immobilized cells in pectin (3%, 4% and 5%) in batch culture.

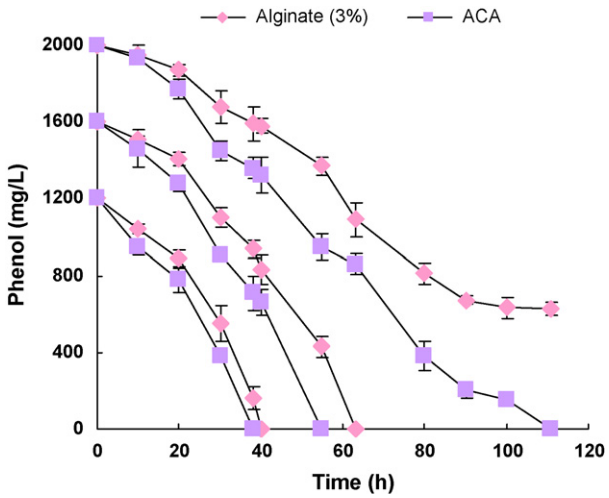

Fig. 6. Degradation of higher phenol concentrations by cells immobilized in alginate (3%) and ACA in batch culture.

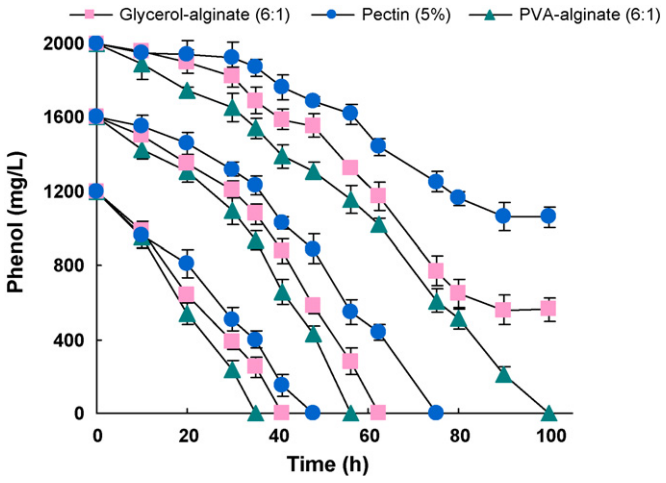

Fig. 7. Degradation of higher phenol concentrations by cells immobilized in PVA–alginate (6:1), glycerol–alginate (6:1) and pectin (5%) in batch culture.

3.7. Semi-continuous degradation by immobilized cells

To assess the stability and durability of immobilized biocatalysts, attempts were made to reuse the beads and capsules under repeated batch cultures. Table 1 details the performance of different biocatalysts under repeated batch degradation at two different loadings (1000 and 1200 mg/L). For an initial concentration 1000 mg/L, up to 15 degradation cycles could be cultured over a period of 35.5 days using PVA–alginate hybrid beads, after which cells continued to degrade phenol at a decreased rate. In comparison, ACA encapsulated cells were reused 14 times, alginate entrapped cells 12 times, glycerol–alginate entrapped cells 11 times, and pectin entrapped cells 8 times. However, when the initial phenol concentration was increased to 1200 mg/L, the PVA–alginate entrapped and ACA encapsulated cells could only be reused for 11 cycles. Similar cycle reductions were noted for the other immobilization matrices (Table 1).

3.8. Biomass estimation

To verify the long-term stability of biocatalysts, cell protein content of the beads and capsules was monitored as an indication of biomass variation during semi-continuous degradation of 1000 mg/L phenol. As shown in Table 2, the PVA–alginate hybrid beads had the highest cell loading capacity both in middle and last runs. Due to high stability and improved surface properties, these hybrid beads had the lowest cell leakage and highest biomass content. In comparison, ACA encapsulated cells ranked second for biomass content. There were no considerable differences in the performance of alginate and glycerol–alginate immobilized cells, further emphasizing the lack of benefit in glycerol addition to alginate for phenol degradation. Pectin immobilized cells had the lowest protein content, which was consistent with the results of the degradation and batch trials.

3.9. Morphological observation

Light microscopy (LM) images from all samples revealed that the morphological features of the beads were well preserved after embedding in epon resin. The polymeric matrices of the beads were uniform, with no lamination or layered structures observed. Clusters of bacteria were observed within the polymer matrix of beads obtained from each of the experiments. The density and distribution of the bacteria, however, varied extensively depending on the composition of the beads and the time point of the experiment. For example, an increase in bacteria was observed in alginate beads until batch 9, thereafter decreasing until the end of experiment (Fig. 8A–C). In contrast, a gradual and continuous increase in bacteria was observed in the PVA–alginate beads (Fig. 8D–F).

The higher magnification obtained in the TEM investigation revealed that the bacteria grew in colonies within the polymeric bead matrices (Fig. 9). There were distinct morphological differences in the structure of the polymeric networks surrounding the bacterial inclusions and the internal areas of the colonies (Fig. 9B and D). Specifically, there was a sharp boundary between polymeric networks and the space occupied by the bacteria, indicating that there is no direct contact between polymers and the cell walls of individual bacteria. Higher bacteria concentrations near bead sur-

Table 1
Repeated batch culture phenol degradation by cells immobilized in different matrices.

Matrix	Initial phenol (mg/L)	Phenol degraded (mg/L)	Incubation period (days)	Number of batches
Alginate	1000	1000	28.5	12
	1200	1200	27	10
ACA	1000	1000	33.5	14
	1200	1200	31.5	11
PVA–alginate	1000	1000	35.5	15
	1200	1200	32.5	11
Glycerol–alginate	1000	1000	30	11
	1200	1200	28	10
Pectin	1000	1000	23.5	8
	1200	1200	22.5	5

Table 2
Comparison of cell protein content of entrapped and encapsulated cells.

Supports	Cell protein content (mg/bead)		
	First batch	Middle batch	Last batch
Alginate	0.7	1.1	0.9
ACA	0.7	1.3	1
Glycerol–alginate	0.7	1.1	0.8
PVA–alginate	0.7	1.5	1.1
Pectin	0.7	1	0.8

faces and increasing volume occupied by bacteria over time also suggest that the alginate beads prepare better surroundings for growing bacteria and their metabolic processes. As there were no isolated single bacteria within the polymeric networks, it is evident that bacterial activity is limited to the microenvironment of colonies. In comparing the different matrix compositions, it was evident that bacteria were better preserved in PVA–alginate beads compared to bacteria entrapped in alginate beads (Fig. 9B and D); however, bacterial colonies were larger in alginate entrapped beads.

4. Discussion

Degradation of phenol by freely suspended and immobilized *Pseudomonas* sp. SA01 cells isolated from a pharmaceutical dispensation waste water plant was assessed to ascertain which matrices and immobilization methods were best suited for bioremediation. In treating phenolic effluents, a major constraint is the availability of suitable microorganisms that can be cultured in effluent conditions. The strain isolated for this work showed a high capacity for phenol degradation, with the phenol degradation rate of 25 mg L^{-1} h^{-1} which was higher compared to the previous reports by Margesin et al. [39] (8.33 mg L^{-1} h^{-1}) and Karigar et al. [23] (19.58 mg L^{-1} h^{-1}) in batch cultures. Complete degradation of phenol by freely suspended cells at concentrations ranging from 500 to 1000 mg/L over periods ranging from 20 to 85 h, respectively, indicated that the time required for degradation is directly proportional to the initial phenol concentration (Fig. 1). However, at concentrations above 1000 mg/L, neither cell growth nor phenol degradation accrued considerably, suggesting that the toxicity of phenol at high concentrations may inhibit biodegradation through lower efficiency or culture death. Chung et al. [10] similarly reported severe inhibition for *P. putida* ATCC 49451 at phenol concentrations above 1000 mg/L. These effects are likely due to phenol's ability to damage microbial cells through alteration of the selective permeability of the cytoplasmic membrane and enzyme deactivation [19].

To improve cell tolerance to severe substrate inhibition, and to improve substrate utilization, cells in this study were immobilized using different matrices. Various methods of bacterial entrapment have been described previously [20] and entrapment within polymeric gel matrices has often been successful. A fundamentally important point in cell entrapment is the creation of a favorable microenvironment for entrapped cells. An ideal gel carrier should possess a highly porous structure to facilitate the diffusion of solutes and dissolved gases [21].

Alginate is one of the most frequently used matrices for this purpose. It is a highly porous and biodegradable polymer that provides rapid and simple aqueous immobilization [22]. As the immobilization matrix of choice for environmental practice, alginate entrapped cells have been used extensively for the bioremediation of numerous toxic chemicals, including phenol [23], naphthalene [24], benzoate [25], and ethylbenzene [26].

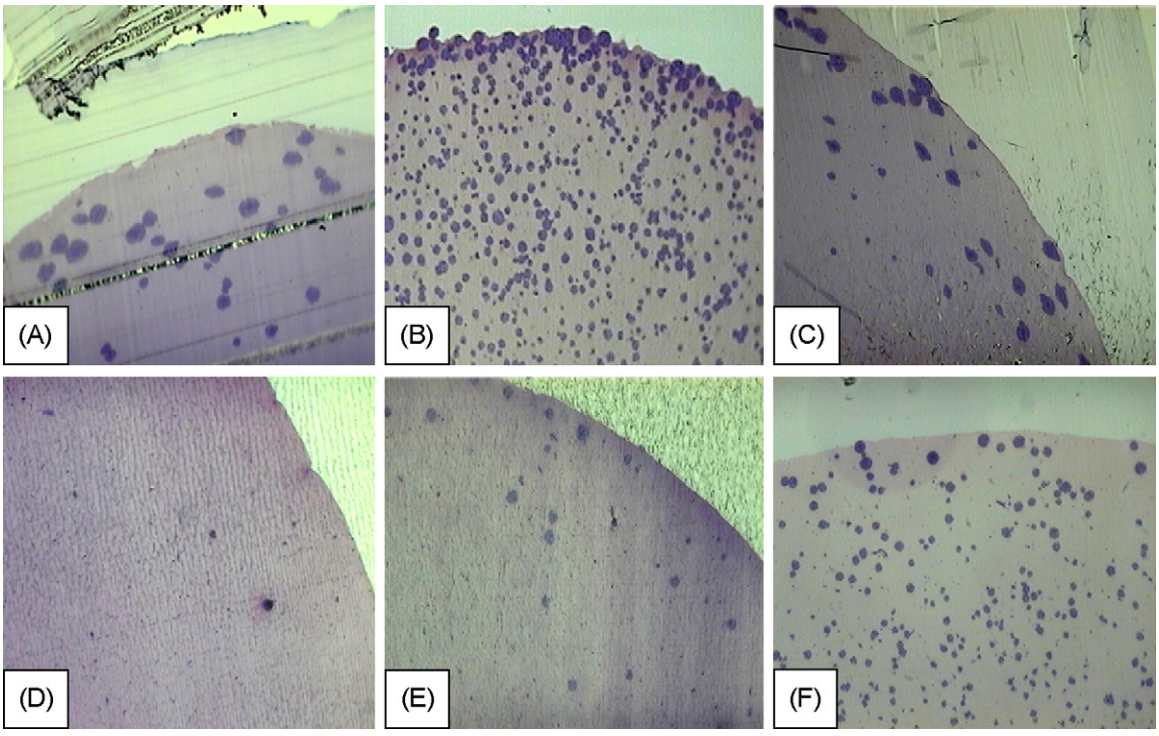

Fig. 8. Light microscopy images of alginate immobilized beads: (A) batch 6; (B) batch 9; (C) batch 12. Images of PVA–alginate beads: (D) batch 1; (E) batch 3; (F) batch 15.

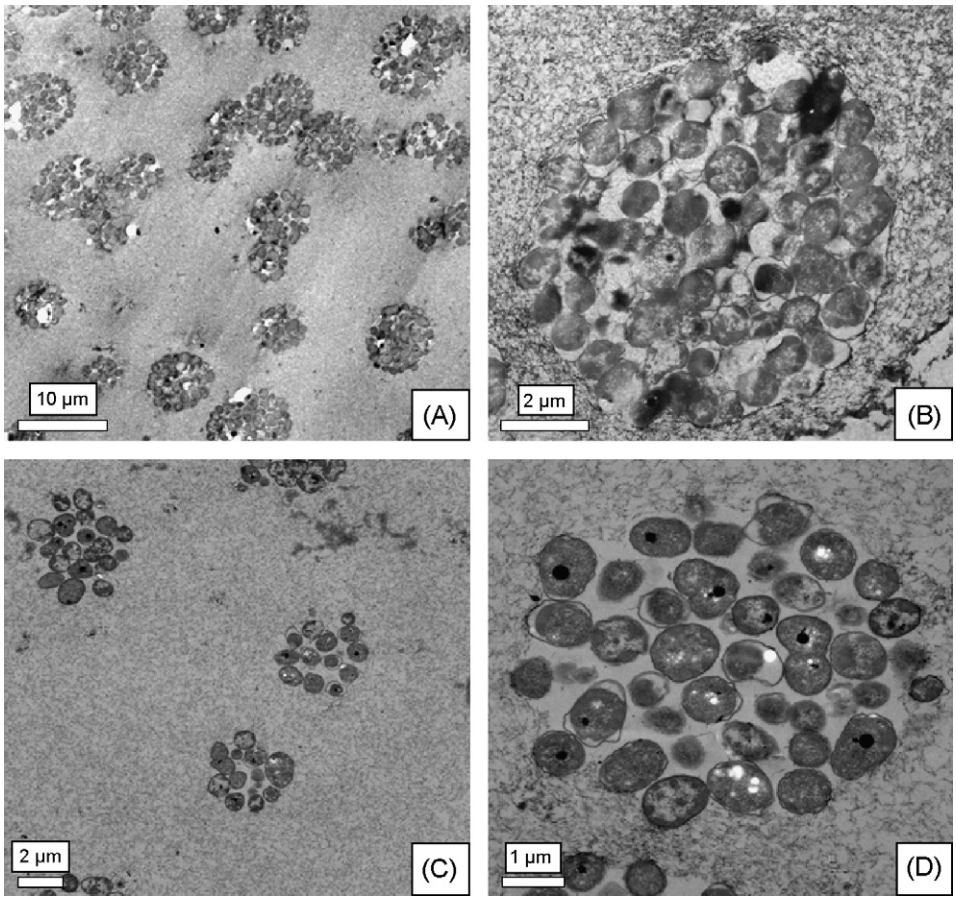

Fig. 9. TEM images of cells immobilized in alginate: (A) batch 9, scale bar 10 μm; (B) batch 9, scale bar 2.0 μm. Images of PVA–alginate beads: (C) batch 15, scale bar 2 μm, (D) batch 15, scale bar 1.0 μm.

In optimizing the entrapment matrix, the stability of the matrix needs to be considered. Although entrapment in conventional alginate beads has been a popular method for cell immobilized, there are two disadvantages to using alginate beads. One is the limitation of space for cellular growth due to the gel–core structure. The second limitation is low physical stability of the matrix in the presence of chelating agents [27]. While the concentration of $CaCl_2$ has little effect on the diffusion characteristics of the beads [28], the presence of chelators in treated effluent can disrupt the alginate matrix.

To overcome these disadvantages of the method of entrapment in gel matrices, an encapsulation method has been developed as an alternative cell immobilization technique. In the encapsulation method, cells can be immobilized within a liquid core surrounded by a thin layer of alginate gel. In a liquid core that has more space for cellular growth; cells can exist at a high density without the carrier [40]. In order to improve alginate bead stability, coating beads with polycations can improve their chemical and mechanical stability, consequently improving the effectiveness of encapsulation. The coating of alginate beads has been extensively studied in pharmaceutics and biotechnology [29–31]. Polycations such as chitosan and poly-L-lysine form strong complexes with alginate, making them stable in the presence of chelators. However, the high cost of poly-L-lysine may limit large-scale use of alginate-poly-lysine-alginate microcapsules for bioremediation. With advances in the study of chitosan as a biomaterial, ACA capsules have been developed, where positively charged chitosan is substituted for poly-lysine. Given its favorable biocompatibility, low cost, abundance in nature, and bioremediation performance demonstrated in this study, ACA encapsulation provides a viable method of protecting cells during phenol degradation. The amino group of chitosan is easily protonated at moderate pH (<6.3) to form a cationic polyelectrolyte, and the carboxylic acid group of alginate is easily ionized in water to form an anionic polyelectrolyte [32]. The polycation and polyanion, carrying electrostatically complementary ionizable groups, form a stable polyelectrolyte complex. The liquid core of the capsules formed gives a larger space for cell encapsulation and reduces mass transfer resistance inside the capsule. As it was evident for phenol degradation, this low mass transfer resistance and stability of encapsulated cells may help satisfy industrial bioremediation requirements for eliminating toxic wastes.

As a promising affordable and nontoxic synthetic polymer, PVA, has been used for cell immobilization. PVA is a carrier with high porosity that favors substrate/product diffusion while providing considerable mechanical, thermal, chemical, and biological stability [33]. Three methods of PVA immobilization have been reported: the UV light method [34], the freeze-thaw method [35], and the boric acid method [36]. The boric acid method is the simplest and most economical technique. However, two potential problems with this technique are the agglomeration of PVA gel beads (PVA is extremely sticky), and the acidity of boric acid; these factors could cause difficulty in maintaining cell viability.

In the present study, a combination of PVA–alginate was used to prevent bead agglomeration. Additionally, mechanical stability was imparted by gelling the beads using $CaCl_2$ instead of boric acid. In this way, calcium alginate improved the surface properties of the beads, reducing the tendency to agglomerate while PVA conferred durability and strength to the beads. It also made it possible to prepare spherical beads, which is the preferred shape in contin-

uous wastewater treatment. Wu and Wisecarver [37] entrapped phenol degrading *Pseudomonas* sp. in PVA gel beads using the PVA–boric acid method, with the addition of calcium alginate to eliminate bead agglomeration. The beads showed high elasticity, degrading phenol in a fluidized bed reactor over 2 weeks. They also reported that the lowest concentration of sodium alginate that would prevent bead agglomeration was 0.02% (a PVA to alginate ratio of 12.5:1). Here, agglomeration and boric acid toxicity were prevented for all ratios, while mechanical strength and good spherical shape increased with increasing alginate ratios. Additional advantages of this method include simplicity of method, one step gelation, affordable precipitation solutions, short immersion time, and high mechanical strength. These added benefits enabled the immobilized bacteria to degrade phenol over more than 15 repeated batches over a period of 35 days.

Unlike the durability provided by PVA–alginate, cells entrapped in pectin had the lowest mechanical strength of all systems studied. Phenol degradation was similarly the worst for all tested concentrations. Although increasing the pectin concentration improved the performance and mechanical strength of the pectin beads, the beads remained softer than other entrapment beads. Wu and Yu [38] similarly found that the adsorption efficiency and mechanical strength of pectin beads were the lowest among all systems studied for bioresorption of 2,4-dichlorophenol by entrapped white-rot fungus *Phanerochaete chrysosporium*.

In this study, morphological investigation of beads composed of different polymeric compositions and collected at different time points suggest that bacteria entrapped within a polymeric gel matrix grow in colonies within a confined area. The number of bacteria increased with time and over repeated batches. However, the density and distribution of bacteria differed among the different compositions; bacteria better preserved in PVA–alginate hybrid beads which further emphasizes that PVA–alginate is the best matrix for immobilization of *Pseudomonas* sp. SA01 cells for enhanced phenol removal.

5. Conclusions

Regardless of the matrix employed, cell immobilization provides increased degradation through accelerated reaction rates that are caused by high local cell densities in or on the matrix. Further, immobilization provides membrane stabilization and increases cell protection. Here, various experimental procedures of single and hybrid immobilization with different polymeric matrices were used to enhance phenol degradation efficiency by *Pseudomonas* sp. SA01 cells. For all entrapment conditions, cells demonstrated a considerable increase in the phenol degradation efficiency compared with freely suspended cells. Taken as a whole, the results obtained here provide a powerful demonstration of the benefits of taking the sort of various single and combined matrices for reducing the detrimental effects of elevated concentrations of mono and poly-aromatic pollutants on bacterial cells. The high stability and the durability of the immobilized cells serve to improve their performance in biodegradation processes, and consequently their potential application in environmental biotechnology cycle, however, further investigation is required to assess their performance in real wastewater treatment plants. Another challenge is the production of the immobilized biocatalysts at industrial scales. In spite of growing trend and numerous reports in the filed of cell immobilization, no technology has reported simultaneous production of beads with a narrow size distribution, a high production rate for industrial applications, and a satisfactory level of material utilization under mild and nontoxic conditions. Therefore such challenges are hotspots for future investigations in the field of cell immobilization for application at industrial scales.

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