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Immobilization of *Pseudomonas putida* in PVA gel particles for the biodegradation of phenol at high concentrations

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ABSTRACT

Pseudomonas putida, immobilized in polyvinyl alcohol (PVA) particles, has been successfully utilized for the bioremoval of phenol from simulated wastewater, using two immobilization techniques and two types of bioreactors. The biodegradation efficiency of *P. putida* immobilized within the PVA gel before the cross-linking stage of the polymer (T1) was compared to that of the same bacteria immobilized by soaking blank PVA particles in bacterial suspension (T2), a procedure that avoids subjecting the bacteria to sub-freezing temperature during the cross-linking stage. The effects of nutrient deprivation and exposure to high phenol concentrations on the activity of *P. putida* were also evaluated. The experimental results indicated that the immobilized bacteria remained active for a period of 72 h, even without the addition of nutrients. Subsequently the activity gradually decreased, but the bacteria easily regained their original activity with the addition of nutrients. Sudden exposure to high phenol concentrations resulted in immediate decline in the biodegradation activity, but the bacteria adapted to the new concentrations and regained their activity within 24 h. The biodegradation experiments were carried out in two types of bioreactors namely, bubble column and spouted bed bioreactor (SBBR). Both reactor configurations and both immobilization techniques proved to be effective in the biodegradation of phenol.

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1. Introduction

Phenol, which is a major constituent in the wastewater of most chemical and petroleum industries, is known to be carcinogenic and toxic even at relatively low concentrations of 5–25 g m^{-3} [1]. Biodegradation is an environmentally friendly and cost effective treatment alternative to the conventional techniques that do not degrade phenol, but rather remove it from the wastewater and pass it to another phase [2]. A considerable amount of research has been carried out in recent years on the biodegradation of phenols by *Pseudomonas putida* because of its high removal efficiency and low cost [3–5]. Under aerobic conditions, the bacterial biomass converts phenol to carbon dioxide and other intermediates such as benzoate, catechol, cis–cis-muconate, β-ketoadipate, succinate and acetate [6]. Analysis of the products suggests that these intermediates are present in negligible amounts, which indicates the degradation proceeds to completion [7]. Immobilization of bacterial biomass is an effective technique, usually employed to protect the bacteria from high phenol concentrations, which cause substrate inhibition, and to allow reutilization. *P. putida* has been studied by many researchers in free and immobilized forms in different types of bioreactors. The authors of this work used *P. putida* immobilized in polyvinyl alcohol (PVA) gel particles for the bioremoval of phenol in a spouted bed bioreactor (SBBR) in both batch [7] and continuous [8] operations. PVA was a preferable immobilization matrix due to its porous structure that allows the substrate and oxygen to diffuse into the internal pores, where biodegradation takes place. The main drawback of the typical biomass-entrapment immobilization technique is subjecting the bacteria to sub-freezing temperature during the cross-linking stage. Although this did not affect the activity of *P. putida*, as previous results have shown [7,8], other types of bacteria could be destroyed during this step. It has been reported in the literature that some unicellular microorganisms, including bacteria, are destroyed by rapid chilling. For example, it was found that 99.98% of *Bacterium typhosurn* (Salmonella typhi) were killed after freezing at −17.8 °C [9]. Therefore, it is important to assess the effectiveness of another immobilization technique that avoids subjecting the bacteria to subfreezing conditions. The effectiveness of the new immobilization technique has been evaluated using different bioreactor configurations. The results of this work provide useful information for effectively using immobilized bacteria and optimizing bioreactor design.

2. Materials and methods

2.1. Chemicals

Analytical grade phenol was purchased from BDH Chemicals, UK. Synthetic phenol solutions were prepared for the desired

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concentration in distilled water before each experimental run. The solutions were always kept in a brown flask to avoid light oxidation of the phenol. All other chemicals and PVA powder were of analytical grade and were also obtained from BDH, UK.

2.2. Bacterial suspension

A special strain of the bacterium *P. putida* (A300) was obtained in an AMNITE cereal form from Cleveland Biotech Ltd., UK. A 100 g sample of the cereal was mixed in a 1 L of 0.22% sodium hexametaphosphate buffered with Na_2CO_3 to a pH of 8.5. The mixture was homogenized in a blender for about 1 h, decanted and kept in the refrigerator at 4 °C for 24 h. Bacteria slurry was prepared by first low speed centrifugation at 6000 rpm for 15 min. Then, the supernatant was collected and centrifuged again at 10,000 rpm for 20 min. Harvested bacteria cells were collected and kept in the refrigerator for immobilization. This extraction procedure was found to be effective for the extraction of microorganisms from the same cereal [10] and from soil [11].

Nutrient mineral medium solution was prepared by dissolving in 1 L of distilled water 825 mg of mineral salt mixture consisting of: 299.58 mg $MgSO_4{\cdot}7H_2O$, 249.65 mg K_2HPO_4, 149.80 mg $CaCl_2{\cdot}2H_2O$, 119.83 mg $(NH_4)_2CO_3$, 3.50 mg $FeSO_4{\cdot}7H_2O$, 1.30 mg $ZnSO_4{\cdot}7H_2O$, 1.30 mg $MnCl_2{\cdot}4H_2O$, 0.018 mg $CuSO_4{\cdot}5H_2O$, 0.015 mg $CoCl_2{\cdot}6H_2O$ and 0.013 mg $Na_2MoO_4{\cdot}2H_2O$. To precisely determine the added small masses, larger amounts were first dissolved in 100 ml of distilled water. A 5 ml sample was then added to the rest of the salts and diluted to a total volume of 1 L.

2.3. Immobilization of bacteria in PVA gel

2.3.1. Immobilization technique 1

A homogeneous PVA solution was prepared by mixing 100 g of PVA powder with 250 ml of distilled water and 50 ml of bacterial suspension (prepared as explained in Section 2.2) at about 70–80 °C. The biomass concentration in the 50 ml bacterial suspension was determined using the dry weight analysis to be 2333 g m^{-3} [7]. This mixture is known to result in a good quality polymer matrix with high porosity [12]. It was then well stirred for 10–15 min to insure homogeneity of the whole solution. The mixture was then poured into special molds and kept in a freezer at −20 °C for 24 h, before it was transferred to the refrigerator and allowed to thaw at about 4 °C. This gives the gel a lower thawing rate and enhances the crystalline area formation, which enhances the mechanical strength of the formed polymer. The freezing–thawing process was repeated three to four times for 5 h for each cycle. This improves the cross-linking in the polymerized PVA gel structure [10]. The frozen molds were then cut into 1 cm × 1 cm × 1 cm cubes. The immobilized bacteria using this approach are referred to as T1.

2.3.2. Immobilization technique 2

As mentioned earlier, the main drawback of immobilization technique 1 (T1) is that the bacteria are subjected to sub-freezing temperature during the cross-linking stage, which may destroy them. Therefore, an alternative immobilization technique that does not involve subjecting the bacteria to sub-freezing temperature has been proposed in this work. Bacteria-free PVA gel particles were prepared following similar approach to the one described in Section 2.3.1. The only difference is that the 50 ml bacterial suspension was replaced with 50 ml of nutrient mineral medium, free of bacteria. All other steps are identical to those mentioned in Section 2.3.1. The formed bacteria-free PVA particles were then soaked in 1 L solution, made up of 900 ml bacterial suspension, 100 ml mineral medium solution containing 82.5 mg of salts mixture as given in Section 2.2, and 150 mg of phenol, to bring the initial phenol concentration to 150 g m^{-3}. The system was kept under continuous aeration for two weeks. On a daily basis, a 100 ml sample was withdrawn from the solution for analysis and replaced with 100 ml of nutrient mineral medium solution containing 82.5 mg of salts mixture and 150 mg of phenol to bring their respective concentrations back to their original values. The bacteria immobilized using this approach are referred to as T2. Sections in the PVA particles, with and without bacteria, were examined under a transmission light microscope (ZEISS, Germany) that is connected to KS300 Kontron Electronic software. Images for the matrix structure of the PVA with and without the immobilized bacteria are presented in Fig. 1(a) and (b), respectively. The magnification in Fig. 1(b) has been enlarged to clearly show the bacterial colonies present in the PVA particles.

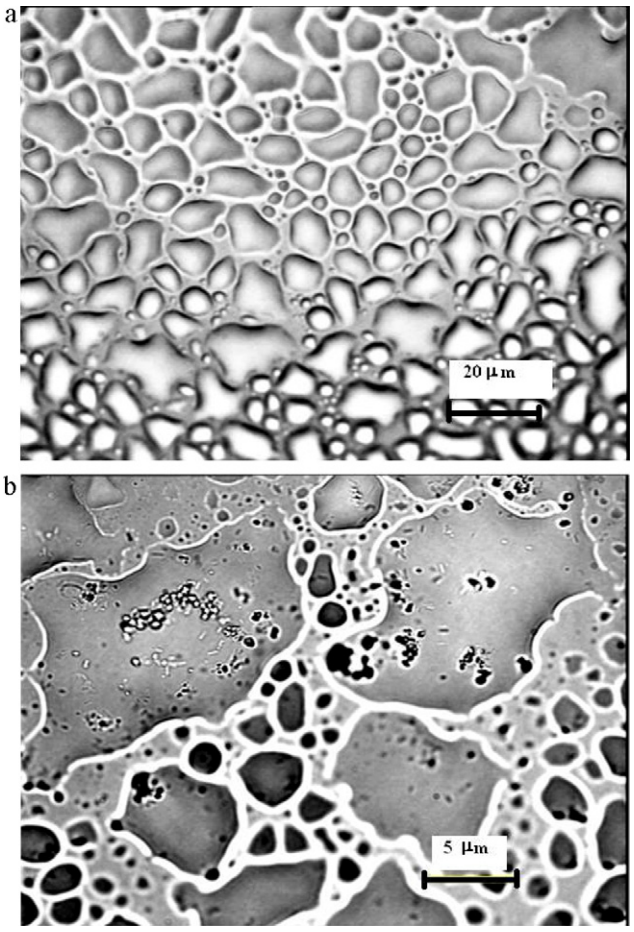

Fig. 1. Light scattering microscopic image of the PVA section: (a) free of bacteria (b) with bacteria.

2.4. Phenol removal in bioreactors

2.4.1. Bubble column

The bubble column bioreactor was made of Plexiglas with a total volume of 1.1 L and was equipped with a surrounding jacket for temperature control. The temperature of the reactor system was controlled by circulating water into the reactor jacket from a water bath set at the desired value. A schematic diagram showing the dimensions of the bubble column reactor is shown in Fig. 2(a). Air was continuously introduced into the reactor at a constant flow rate of 1 L min^{-1} to induce mixing and, at the same time, provide excess oxygen to sustain aerobic condition. The reactor was initially filled with standard nutrient mineral medium solution prepared as given in Section 2.2, which contained 30 vol% PVA gel cubes with immobilized bacteria prepared by either T1 or T2. The initial phenol concentration was 150 g m^{-3}, chosen to be higher than the sub-

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