Biodegradation of Phenol and 2, 4 Dichlorophenol : The Role of Glucose In Biomass Acclimatization

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Abstract

Batch experiments were carried out to examine the acclimatization process in the aerobic biodegradation of phenol and 2, 4 dichlorophenol (2, 4 DCP) by Pseudomonas putida immobilized in polyvinyl alcohol (PVA) gel pellets in a bubble column bioreactor. The bacteria were acclimatized to phenol and 2, 4 DCP with concentrations of up to 200 mg/l, with and without the addition of glucose. The experimental results for an initial phenol concentration in the range 25-200 mg/l indicated better performance by the no-glucose acclimatized bacteria, which had higher biodegradation rates for the entire range of initial phenol concentration. For 2, 4 DCP initial concentrations in the range 25-100 mg/l, the rates were either very close or slightly better for the glucose-acclimatized bacteria, but not significantly enough to justify the inclusion of glucose in the acclimatization process. The absence of glucose from the acclimatization process either stimulates the biodegradation capabilities of P. putida or has no significant effect on its performance during biodegradation.

1. Introduction

Chemical and petroleum industries generate a wide variety of highly toxic organic pollutants, which have led to cumulative hazardous effects on the environment. The effluents of these industries often contain aromatic organic compounds that are rather resistant to natural degradation and therefore persist in the environment. This makes them capable of long range transportation and bioaccumulation in human and animal tissues. Organic pollutants represent a potential group of chemicals that can be seriously hazardous to human health [1-3], and many aromatic compounds show carcinogenic, teratogenic or mutagenic properties [4]. Non-biodegradable organic compounds must be pretreated into biodegradable or less toxic compounds.

Contamination of soil, surface water and underground water by aromatic organic pollutants such as phenol and its derivatives has caused great concern worldwide. Phenolic compounds are among the most prevalent forms of chemical pollutants in the industrial wastewater, generated mainly from oil refineries, coal conversion plants, petrochemicals, polymeric resins, pharmaceuticals, smelting and related metallurgical operations [5-7]. Phenols are well known for their high toxicity for human life, aquatic life and others [8-10]. They are considered to be among the most hazardous contaminants, and they are certainly the most difficult to remove [11]. They are not amenable to conventional treatment process and, in the presence of chlorine, can react with chlorine to produce chlorophenols, which are carcinogenic and even more resistant to degradation than phenol itself [12, 13].

Due to these adverse health effects of phenols, the World Health Organization (WHO) has set a guideline of 1 µg/l to regulate the phenol concentration in drinking water [14]. The high-volume use of phenols in the United States and their potential toxicity has led the U.S. Environmental Protection Agency (US EPA) to define them as priority pollutants [16]. Most countries specify the maximum allowable concentration of phenols in the effluent streams to be less than 1 mg/l [16, 17]. The legislations in the UAE limit the total phenols in industrial water discharged to the marine environment to 0.1 mg/l [18].

Therefore, to save the soils and aqueous ecosystems, it has been mandatory worldwide for industries to treat their wastewater effluents before safe disposal to the environment. Biological treatment of phenols has been an increasingly important process in pollution control [2, 19, 20]. Moreover, compared with physico-chemical methods, the biodegradation method of phenols removal is a more environmental friendly and cost effective alternative, universally preferred, because of the possibility of complete mineralization of phenol [2, 11], which results in complete conversion of a compound to its inorganic mineral constituents [1]. It is

considered a favorable and most promising approach [10].

Many types of aerobic bacteria, including *Pseudomonas putida*, are believed to be capable of consuming aromatic compounds as the only source of carbon and energy [11, 20, 22]. In recent years, the strain of *P. putida* has been the most widely used type of bacteria for phenols biodegradation. It has been studied by many researchers in free and immobilized forms, using different types of bioreactors [11]. Biomass immobilization is an important and effective technique that is usually employed to protect the bacteria from high phenol concentrations and allow reutilization of the biomass [11, 20, 21]. During biological treatment, however, the bacteria must first be adapted to the phenolic compound, which is known to be toxic to microorganisms. It has been reported that phenol is inhibitory to bacteria growth at concentrations above 0.05 g/l [21, 23]. Therefore, in order to obtain efficient biodegradation, microbial acclimatization (adaptation) to phenol or its derivatives is necessary [11, 21, 23-25].

The effect of lag times at the beginning of the process is specially recognized when the biomass cells are not pre-adapted to the contaminant medium. The lag time increases with increased initial concentration of phenol [24]. This process has often been carried out by utilizing glucose as an easily biodegradable source of carbon [6, 23, 26-33]. Based on the same concept, Contreras et al. [34] used cheese whey instead of glucose, whereas Tziotzios et al. [22] used sodium acetate as an initial carbon source. In studying the biodegradation of a mixture of chlorophenols, Sahinkaya and Dilek [35, 36] replaced glucose with peptone in the acclimation process. In previous work [11, 20, 21], the bacterial cells were activated by gradually increasing the concentration of glucose over a period of few days. Once activated, the bacterial cells were then slowly acclimatized to phenol by gradual increase in the phenol concentration accompanied with a gradual reduction in the glucose concentration. It may be more stimulating for the bacteria to remove glucose from the acclimatization process and start directly with low concentration of phenol or its derivative followed by a gradual increase to its targeted maximum [37]. The aim of this current study, therefore, is to assess the justification of glucose addition during biomass acclimatization for the biodegradation of phenol and 2, 4 DCP by PVA-immobilized *P. putida* in a bubble column bioreactorAll manuscripts must be in English. These guidelines include complete descriptions of the fonts, spacing, and related information for producing your proceedings manuscripts.

2. Materials and methods

Detailed description of the bacteria preparation, acclimatization and immobilization as well as the analytical techniques can be found elsewhere [11]. However, for the sake of clarity and completeness, brief descriptions are repeated here.

2.1. Reagents

Analytical grade phenol was purchased from BDH Chemicals, UK. 2, 4 DCP was obtained from Sigma-Aldrich, Germany with purity greater than 99%. Synthetic phenol or 2, 4 DCP solutions were prepared in the desired concentrations by dissolving predetermined amount of the organic substance in nutrient solution (Section 2.4). The prepared solutions were always kept in dark containers in closed cabinets to avoid light oxidation of phenol or 2, 4 DCP. All other chemicals and PVA powder were of analytical grade and were also obtained from BDH.

2.2. Preparation and microbial culture

A special strain of the bacterium *P. putida* was obtained in cereal form (AMNITE P300) from Cleveland Biotech Ltd., UK. A 100 g portion of the cereal was mixed in a 1000 ml solution of 0.22% sodium hexameta phosphate buffered with Na_2CO_3 to a pH of 8.5. The mixture was homogenized in a blender for about one hour, decanted and kept in the refrigerator at 4 °C for 24 h. Bacteria slurry was prepared by four consecutive steps of low speed centrifugation at 6000 rpm for 15 min. The supernatants were collected and centrifuged again at 10,000 rpm for 20 min. The biomass attached to the walls of tubes was re-suspended as slurry in distilled water and kept in the refrigerator for subsequent immobilization.

2.3. Immobilization

PVA gel was used for immobilizing the bacteria cells. A homogenous 10 wt% PVA viscous solution was prepared by mixing 100 g of PVA powder with 900 ml of distilled water at about 70–80 °C. The 10% mixture is known to result in good quality polymer matrix with high porosity [11]. PVA, which is a synthetic polymer, has better mechanical properties, and it is more durable than Ca-alginate which is biodegradable and can be subject to abrasion [11]. The formed mixture was allowed to cool to room temperature before adding 10 ml of the bacterial suspension prepared as in Section 2.2, then well stirred for 10–15 min to ensure homogeneity of the solution. The solution was then poured into special molds and

kept in a freezer at −20 °C for 24 h, then transferred to room temperature and allowed to thaw at about 4 °C for three hours. The freezing–thawing process was repeated 4 times. The frozen molds were cut into the specified size of 1 ml cubes, washed with distilled water to remove any uncross-linked chains, and sent for acclimatization.

2.4. Acclimatization of bacteria

The study was performed in two parts. In the first part, *P. putida* was acclimatized to phenol and the study carried out with phenol as a substrate and then continued with 2, 4 DCP as a substrate. In the second part, the study was carried out with freshly-prepared bacteria adapted directly to 2, 4 DCP and the study carried out with 2, 4 DCP as a substrate.

For the first part, a portion of 40 ml of the immobilized bacteria, prepared as in Section 2.3, was suspended in a measuring cylinder, to a total volume of 135 ml in solution containing 1000 mg/l of glucose as an easy biodegradable source of organic carbon in addition to 825 mg/l of other essential mineral nutrients with concentrations shown in Table 1. The bacteria were then slowly acclimatized to phenol concentrations by increasing the phenol concentration from zero to 50, 100, 150, up to 200 mg/l over a period of 5 days. At the same time, glucose concentration was gradually reduced from 1000 mg/l to zero (1000, 750, 500, 250, 0 mg/l). Another portion of 40 ml of the immobilized bacteria was suspended to a total volume of 135 ml in solution containing 25 mg/l of phenol as a source of organic carbon dissolved in the nutrient solution. The bacteria were then slowly acclimatized to phenol concentrations by increasing the phenol concentration from 25 to 50, 100, 150, up to 200 mg/l over a period of 5 days.

Table 1. Composition of nutrient mineral medium

Component	Concentration, mg/l
$MgSO_4.7H_2O$	300
K_2HPO_4	250
$CaCl_2.2H_2O$	150
$(NH_4)2CO_3$	120
$FeSO_4.7H_2O$	3.5
$ZnSO_4.7H_2O$	1.3
$MnCl_2.H_2O$	0.13
$CuSO_4.5H_2O$	0.018
$CoCl_2.6H_2O$	0.015
$Na_2MnO_4.2H_2O$	0.013
Total	824.98

In the second part, the two portions of bacteria were 90 ml each, suspended in a measuring cylinder to a total volume of 300 ml. The two portions were acclimatized to 2, 4 DCP up to 200 mg/l in exactly the same way, but starting with 25 mg/l for both of them (25, 50, 100, 150, 200). In all runs, the portion involving glucose in the acclimatization process is referred to as A, while that with no-glucose is referred to as B.

2.5. Analytical methods

Phenol and 2, 4 DCP concentrations in the biomass-free samples were determined quantitatively using a Shimadzu UV Spectrophotometer, Model UV-1800, Japan at 270 and 243 nm, respectively. Measurements were made after filtering the samples through 0.45 µm GHP Acrodisc filter. Residual concentration was determined against a calibration curve of standard solutions of known concentrations and a standard solution was used to recheck the accuracy of the spectrophotometer every day. All experimental results reported in the study were based on averaging results of repeated experimental runs (duplicates or triplicates), with the standard deviation ranging from 2 to 7% of the reported average.

2.6. Batch biodegradation of phenol or 2, 4 DCP

The procedure in the first part is the same as that in the second part of the study, except for the slight differences mentioned above in section 2.4. After acclimatization, and before starting the runs, the PVA gel cubes were soaked in the substrate solution of required concentration for 10 minutes, then the soaking solution was decanted and the fresh solution added. All phenol biodegradation experiments were performed in 250 ml measuring cylinder containing a total volume of 135 ml, whereas the 2, 4 DCP biodegradation was performed in 500 ml measuring cylinder containing a total volume of 300 ml. The cylinder was initially filled with standard mineral medium, prepared according to Table 1, which contained the required concentration of the substrate and 30 vol% PVA gel cubes with immobilized bacteria. The cylinder was immersed in a water bath to control the temperature at 30 °C. Air was

continuously introduced into the cylinder to enhance mixing and at the same time provide excess oxygen to sustain aerobic condition. The initial pH ranged from 8.1-8.5 and the initial substrate concentration ranged from 25 to 200 mg/1 for phenol and from 25 to 100 mg/1 for 2, 4 DCP. Samples were withdrawn at regular time intervals, and analyzed for residual phenol or 2, 4 DCP concentrations.

3. Results and discussion

3.1. Acclimatization to phenol

Biodegradation of phenol: The experimental results for the biodegradation of phenol for 25 and 50, 75 and 150, 100 and 200 mg/l are shown in Fig. 1, 2 and 3, respectively. It is clear that the degradation by bacteria acclimatized without glucose was faster for all concentrations. It is noticeable that the reduction in phenol concentration is practically linear with time, and therefore, the degradation rate represented by the slope of each curve is constant for all initial concentrations of phenol. However, the biodegradation rate increases with increasing the initial concentration of phenol, reaching a maximum of 40 mg/l.h at 75-100 mg/l for the glucose-acclimatized bacteria (A) and 47 mg/l.h at 100 mg/l for the no glucose-acclimatized bacteria (B), as shown in Fig. 4. The maximum rate for the group B is comparable to the result of previous work by El-Naas et al. [11], which was obtained by glucose-acclimatized bacteria at 75 mg/l. The degradation rate changes slightly in the range of 100-150 mg/l for both bacteria and then continues to increase.

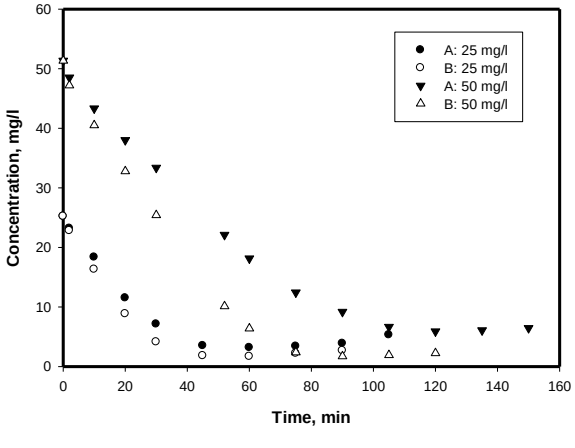

Figure 1. Variation of phenol concentration with time, initial concentration 25, 50 mg/l

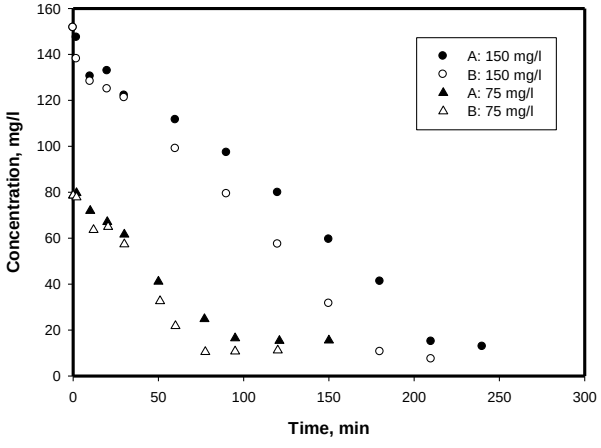

Figure 2. Variation of phenol concentration with time, initial concentration 75, 150 mg/l

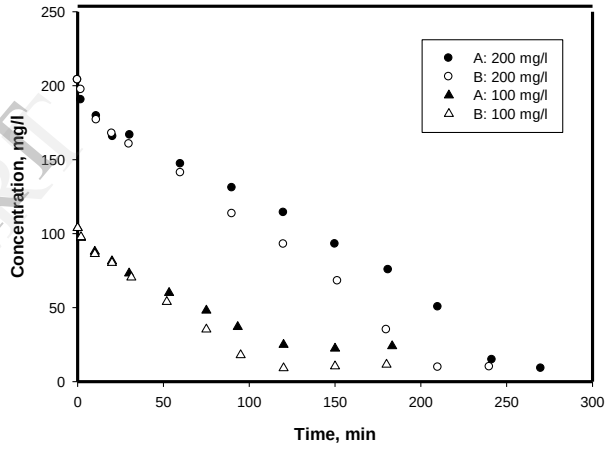

Figure 3. Variation of phenol concentration with time, initial concentration 100, 200 mg/l

It can be justified that substrate inhibition was not encountered in the range of substrate concentration studied mainly due to the immobilization of the bacteria within the PVA matrix that shielded them from direct contact with the high phenol concentration. As depicted in Fig. 4, this is more noticeable in the case of the no-glucose acclimatized bacteria, which emphasizes its better performance.

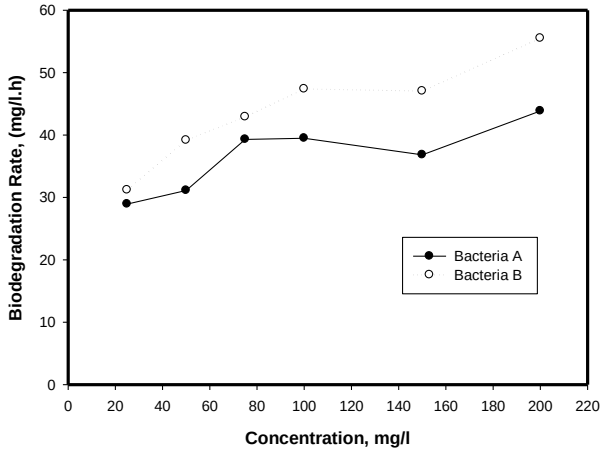

Figure 4. Biodegradation rate at different phenol concentrations

Biodegradation of 2, 4 DCP: Experimentation was continued with the same bacteria that were used for phenol biodegradation, for initial 2, 4 DCP concentrations of 25 to 100 mg/l. For comparison purposes, the results for the four concentrations are shown in Fig. 5. It is noticeable that the reduction in 2, 4 DCP concentration is much lower than that of phenol, mainly because 2, 4 DCP is much less biodegradable and the bacteria have not been acclimatized to it. As depicted in the figure, the performances of the portions A and B are almost identical.

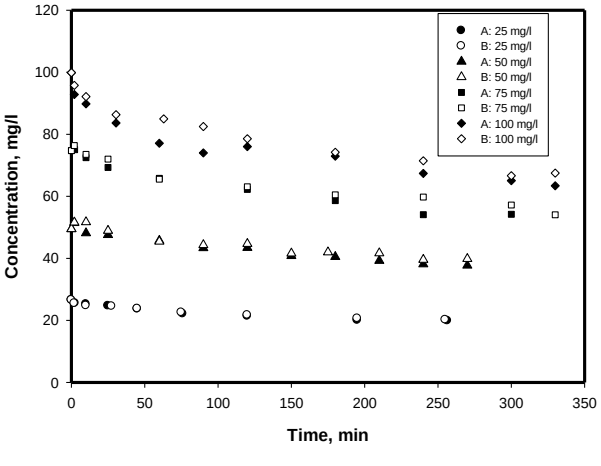

Figure 5. Variation of 2, 4 DCP concentration with time, initial concentration 25-100 mg/l

3.2 Acclimatization to 2, 4 DCP

Freshly prepared bacteria, directly acclimatized to 2, 4 DCP up to 200 mg/l as explained in Section 2.4, were used for biodegradation of 2, 4 DCP with initial concentrations of 25, 50, 75 and 100 mg/l. As mentioned in Section 2.4, the acclimatization of the two portions (the one with glucose A and the no-glucose B) followed the same procedure as for phenol in section 3.1 but starting with a 2, 4 DCP concentration of 25 mg/l for the two portions A and B (25, 50, 100, 150, 200 mg/l). During the acclimatization process, the concentration was monitored with time and the results for 150 and 200 mg/l are shown in Figures 6 and 7, respectively. Although the performance of the glucose-acclimatized bacteria is slightly better at 150 mg/l, they are quite close at 200 mg/l.

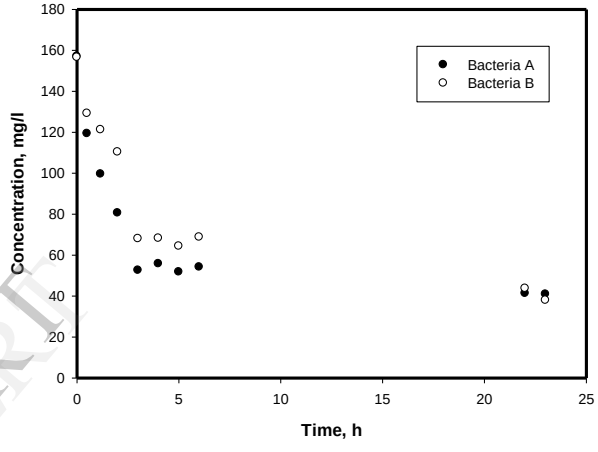

Figure 6. Acclimatization, initial concentration 150 mg/l

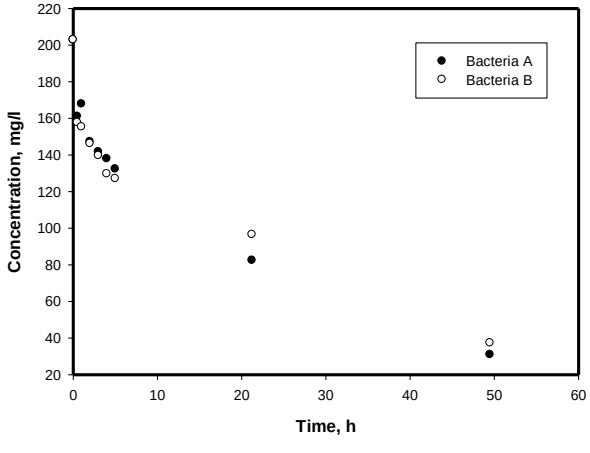

Figure 7. Acclimatization, initial concentration 200 mg/l

The results of 2, 4 DCP biodegradation with initial concentration of 25, 50, 75 and 100 mg/l are shown in Fig. 8, 9, 10 and 11, respectively. It is noticeable that the degradation rate by the no-glucose bacterial portion (B) is slightly lower than the rate by the glucose-acclimatized portion (A), opposite to what was observed in Section 3.1 for phenol biodegradation. This can be justified by the fact that 2, 4 DCP is not as easily biodegradable as phenol, and the addition of glucose in the acclimatization process slightly enhances the performance of bacteria.

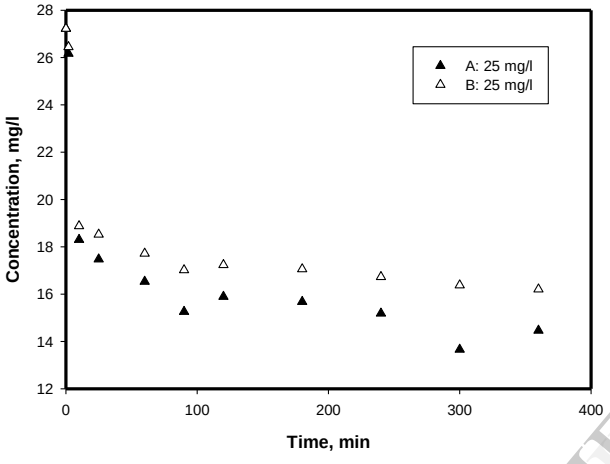

Figure 8. Variation of 2, 4 DCP concentration with time, initial concentration 25 mg/l

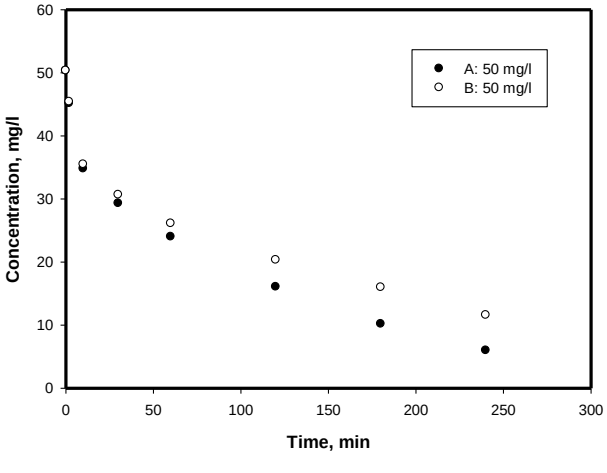

Figure 9. Variation of 2, 4 DCP concentration with time, initial concentration 50 mg/l

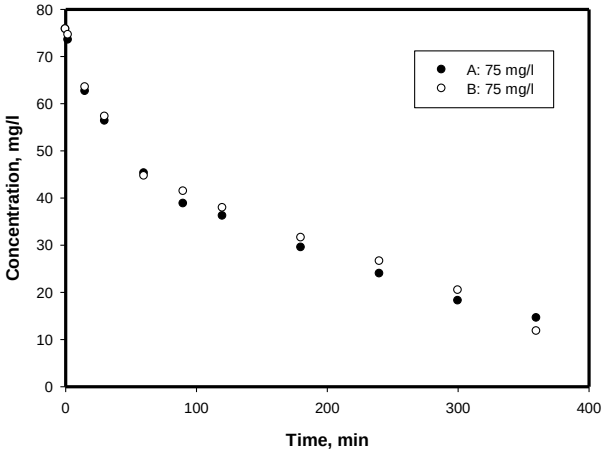

Figure 10. Variation of 2, 4 DCP concentration with time, initial concentration 75 mg/l

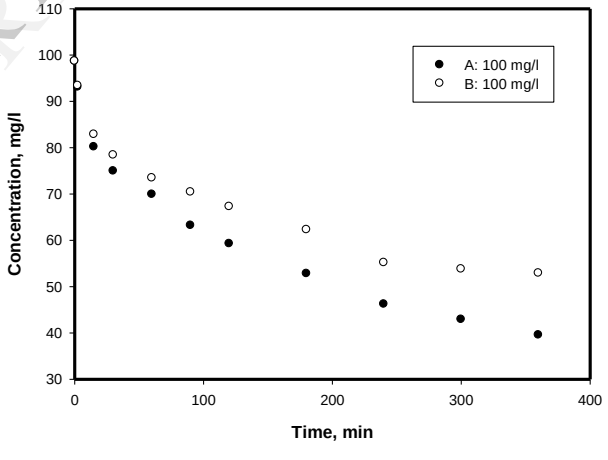

Figure 11. Variation of 2, 4 DCP concentration with time, initial concentration 100 mg/l

The biodegradation rates are depicted in Fig. 12. It is evident that the biodegradation rate reached a maximum at an initial 2, 4 DCP concentration of about 75 mg/l and that both acclimatization schemes A and B had almost the same biodegradation rate. From these results presented in Figures 8 to 12, it can be concluded that though the biodegradation rate is slightly higher for the glucose-acclimatized bacteria, the difference is not significant enough to justify the glucose-based acclimatization.

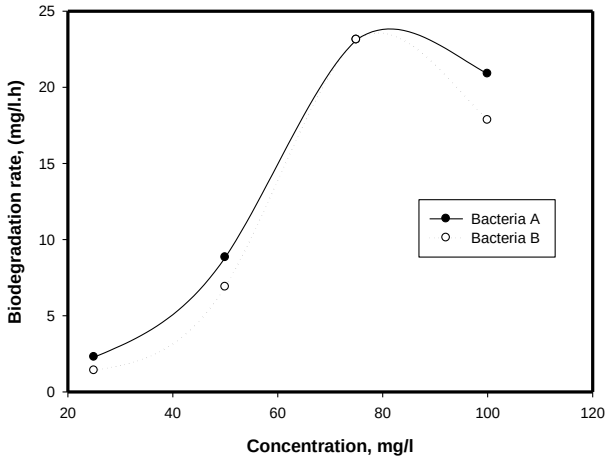

Figure 12. Biodegradation rate at different 2, 4 DCP concentrations

4. Conclusions

Batch experiments were carried out in a bubble column bioreactor to examine the acclimatization process with and without glucose for the biodegradation of phenol and 2, 4 DCP by P. putida immobilized in PVA gel pellets at different initial substrate concentrations. As for phenol, the biodegradation rates continuously increased with increasing phenol concentration up to 200 mg/l for both types of acclimatization regimes. However, glucose has a negative effect in acclimatization process, making the bacteria less active. This was confirmed by better performance in terms of higher degradation rates for the bacteria acclimatized without glucose. For 2, 4 DCP, there was no significant difference in the biodegradation rates for experiments carried out as a continuation after phenol runs by the same bacteria acclimatized to phenol. When the biodegradation was examined by bacteria acclimatized to 2, 4 DCP, the biodegradation rates by both portions of bacteria (A, and B) were either very close or slightly higher by the glucose-acclimatized portion but not significant enough to justify the inclusion of glucose in the acclimatization process. As the two types of acclimatized bacteria are expected to reach the same performance after long operation, there is no point in including glucose in the acclimatization process.

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