African Journal of Biotechnology Vol. 2 (1), pp. 8–12, January 2003
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2003 Academic Journals

Effects of mixed nitrogen sources on biodegradation of phenol by immobilized *Acinetobacter* sp. strain W-17

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Accepted 11 December 2002

Using Ca-alginate immobilized cells of *Acinetobacter* sp. strain W-17, the effects of ammonium-N and nitrate-N on the biodegradation **of** phenol were investigated**. Degradation experiments in three different culture media; minimal salts medium (MSM), simulated (SW) and modified simulated wastewater (MSW) were performed. With the freely suspended cells (cell dry weight 0.2 g/l), complete phenol (500 mg/l) degradation was achieved after incubation for 120 h. Using the immobilized cells, the time was reduced to 24 h in MSM medium, and 15 h in the MSW. The results also indicate that strain W-17 can tolerate to high concentrations of NH_4^+-N (63 mg/l) and NO_3^--N (1000 mg/l) without a significant loss in the phenol biodegradation rate. Moreover, the presence of 500 mg/l phenol in the MSW had no considerable effect on the removal of both ammonium-N and nitrate-N. Repeated use of immobilized cells revealed that they could be used as much as five times without loss of activity. Our findings could be extended to enhance biotreatment of phenol contamination in a variety of biological treatment processes.**

Key words: Phenol, biodegradation, immobilization, *Acinetobacter*, Ca-alginate, ammonium, nitrate.

INTRODUCTION

Phenols are distributed either as natural or artificial mono-aromatic compounds in various environmental sites. As major pollutants, their existence in industrial wastewater treatment plants, such as oil refineries, petrochemical plants, coking plants, and phenol resin industry plants, has been well established (Watanabe et al., 1996). Phenol and its derivatives are among the most frequently found pollutants in rivers, industrial effluents, and landfill runoff waters (Prasad and Ellis, 1978). The toxicity of phenolic compounds often results in the reduction of wastewater biotreatment even at relatively low concentrations (Hinteregger et al., 1992). In phenol-contaminated sites, phenol toxicity studies have shown that bacteria can adapt to low phenol concentrations, but increasing phenol concentrations appear to decrease the overall phenol biodegradation (Dean-Ross, 1989). In municipal wastewater treatment plants, the microorganisms selected for certain phenol bioremediation application have to be adapted also for the high concentrations of ammonium, nitrite and nitrate. It is possible that biodegradation rate of phenol can be significantly affected by the presence of a high load of nitrogen pollutants.

Ammonia is known to be toxic to aquatic life and creates a large oxygen demand in receiving waters (Arthur et al., 1987). Nitrite is a highly toxic compound not only for bacteria but also for wide spectrum of other organisms (De Beer et al., 1997) The higher nitrite concentration levels (over 8 mg NO_2^--N/l) inhibits anoxic phosphate uptake (Meinhold et al., 1999). Nitrates are toxic to humans, especially infants. The presence of more than 45 mg/l of nitrate, the maximum concentration level for drinking water, may cause methemoglobinemia (Salvato, 1994).

Recent studies showed that optimizing the culture medium could enhance the biodegradation of xenobiotics. A positive impact on biodegradation of some aliphatic chlorinated xenobiotics has been observed when the culture medium supplemented with minerals (Henery and Grbic-Galic, 1995). It was also showed that

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Abbreviations: MSM, minimal salts medium; SW, simulated wastewater; MSW, modified simulated wastewater.

yeast extracts, vitamins, and trace elements could significantly enhance the aerobic degradation rate of chlorobenzoic acid isomers (Armenante et al., 1995; Fava et al., 1995).

To improve the efficiency of biological treatment of municipal wastewater, including *in situ* remediation, the present study focused on the optimization of bioremediation process using Ca-alginate immobilized *Acintobacter* sp. strain W-17. The effect of NH_4^+ and NO_3^- on the biodegradation of phenol in wastewater was monitored.

MATERIALS AND METHODS

Microorganism

The phenol-degrading *Acinetobacter* sp. strain W-17 used in this study was previously isolated from a wastewater treatment plant at Alexandria, Egypt as described by Abd-El-Haleem et al. (2002).

Growth media and conditions

To achieve enough cell biomass required for phenol biodegradation, strain W-17 was grown aerobically at 30°C (200 rpm) overnight in LB-liquid medium pH 7.0 containing (per liter) 5 g yeast extract, 10 g peptone and 10 g NaCl. For optimizing the biodegradation rate of phenol, MSM medium containing (per liter) 2.75 g of K_2HPO_4, 2.25 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of NaCl, 0.02 g of $FeCl_3 \cdot 6H_2O$, and 0.01 g of $CaCl_2$ (pH 6.8 to 7.0) was prepared. Phenol was added to 500 mg/l. To study the adaptation of strain W-17 to phenol under wastewater conditions, simulated wastewater (SW) described previously by Martin et al. (2000) was prepared. The SW was prepared in distilled water containing (per liter) 40 mg of K_2HPO_4, 10 mg of KH_2PO_4, 50 mg of $(NH_4)_2SO_4$, 25 mg of KNO_3, 25 mg of $MgSO_4 \cdot 7H_2O$, 2 mg of $FeSO_4 \cdot 7H_2O$ and 0.01 g of $CaSO_4$ (pH 7.2). In addition, the effects of both low and high concentrations of NH_4^+-N (added as ammonium sulfate) and NO_3^--N (added as potassium nitrate) on phenol biodegradation rate were examined.

Immobilization

Encapsulation of strain W-17 into Ca-alginate beads was performed. Liquid cultures were centrifuged in a 50-ml plastic centrifuge tube (2,500 g) at room temperature for 10 min. and the supernatant was discarded. The pellet was resuspended with a previously autoclaved solution of sodium alginate to a final concentration of 4% (w/v) and 10% (v/v) bacterial biomass. The alginate-bacterial mixture was added dropwise with sterile syringe (20 ml) fitted with a wide bore needle (1 mm diameter) from a height of about 20 cm into an autoclaved solution of calcium chloride (3% (w/v), adjusted to pH 7.0), where beads formed immediately. The beads were left in this hardening solution overnight at 4°C before being harvested by filtration.

Degradation experiments and analytical methods

Phenol degradation experiments were performed in shake flasks with immobilized and free cells. Undegraded phenol was estimated by the method of Yang and Humphrey (1975) based on rapid condensation with 4-amino-antipyrene followed by oxidation with alkaline potassium ferricsyanide giving a red color detected by UV spectrophotometery. Controls (unimmobilized Ca-alginate beads in MSM phenol medium) demonstrating that phenol was not adsorbed by the immobilising agent were included with all experiments. Ammonium, nitrite and nitrate were measured calorimetrically according to the standard methods (APHA, 1995).

Scanning Electron Microscope

The Scanning Electron Microscopy (SEM) photographs were carried out using Philips XL30 attached to an EDX unit, with accelerating voltage 30 k.v, magnification 10x up to 400,000x and resolution 3.5 nm. For SEM the samples were fixed by the protein cross linking agent glutraldehyde then washing in mixtures containing increasing concentrations of alcohol in water, finally in absolute alcohol and dried by the critical-point method. After that, samples were coated with a thin layer of gold to make the surface more efficient in electron scattering.

Repeated use

After the first cycle (24 h), the bound cells in alginate beads were filtered, washed and used in second cycle of biodegradation of phenol at a concentration of 500 mg/l. Five repeated batch cycles were performed.

RESULTS

Phenol degradation by free and immobilized cells in MSM medium

Phenol biodegradation experiments using strain W-17 showed that with the freely suspended cells (cell dry weight 0.2 g/l) about 30% of the phenol was degraded within 72h of incubation. Then degradation of the rest of the phenol increased dramatically and complete degradation was achieved after incubation for 120 h. On the other hand, the time needed for complete degradation of the total amount of phenol was reduced to 24 h by

using Ca-alginate immobilized cells in MSM medium (Figure 1). No absorbed phenol was detected on the immobilising agent (data not shown).

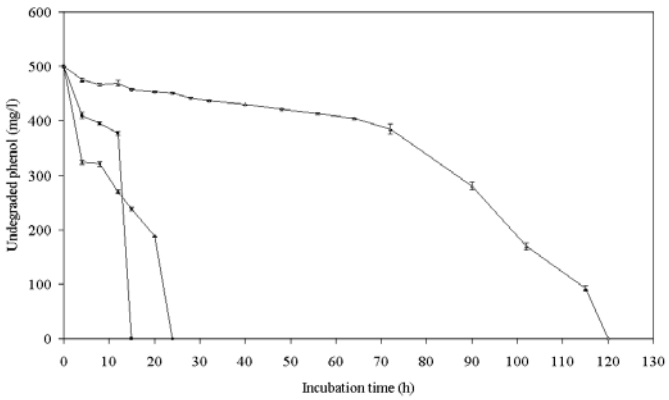

Figure 1. Phenol biodegradation profile by free and immobilized cells of *Acinetobacter* sp. strain W-17 (Free cells, 120 h; immobilized cells into Ca-alginate beads in MSM medium, 24 h; immobilized cells into Ca-alginate beads in MSW medium, 15 h).

Phenol degradation under stress of ammonium and nitrate in SW medium

In SW medium using Ca-alginate immobilized cells, the effects of ammonium-N and nitrate-N on the biodegradation rate of phenol were investigated. A range of ammonium-N concentrations between 1.33 and 256 mg/l NH_4^+-N was tested. After incubation for 24 h, complete degradation of phenol was observed at concentrations ranging from 1.33 to 15.9 mg/l NH_4^+-N. The results also revealed that phenol biodegradation rate was reduced significantly by increasing the concentration of NH_4^+-N to 256.2 mg/l, where 90% of phenol did not undergo degradation (Figure 2A).

Phenol biodegradation rate was also studied under different concentrations of nitrate ranging from 1 to 2000 mg/l NO_3^--N. The complete degradation of phenol occurred at nitrate concentrations ranging between 1 and 300 mg/l, whereas, 95-96% degradation was found at 500 to 1000 mg/l NO_3^--N. Only 36% degradation of phenol took place at concentration of 2000 mg/l NO_3^--N (Figure 2B).

Degradation of phenol under non-inhibitory concentration of NH_4^+ and NO_3^-

Based on the above results, the concentration of NH_4^+-N and NO_3^--N were adjusted to 63 and 1000 mg/l, respectively, to give the modified simulated wastewater (MSW). These concentrations were selected of each component to represent the highest non-inhibitory

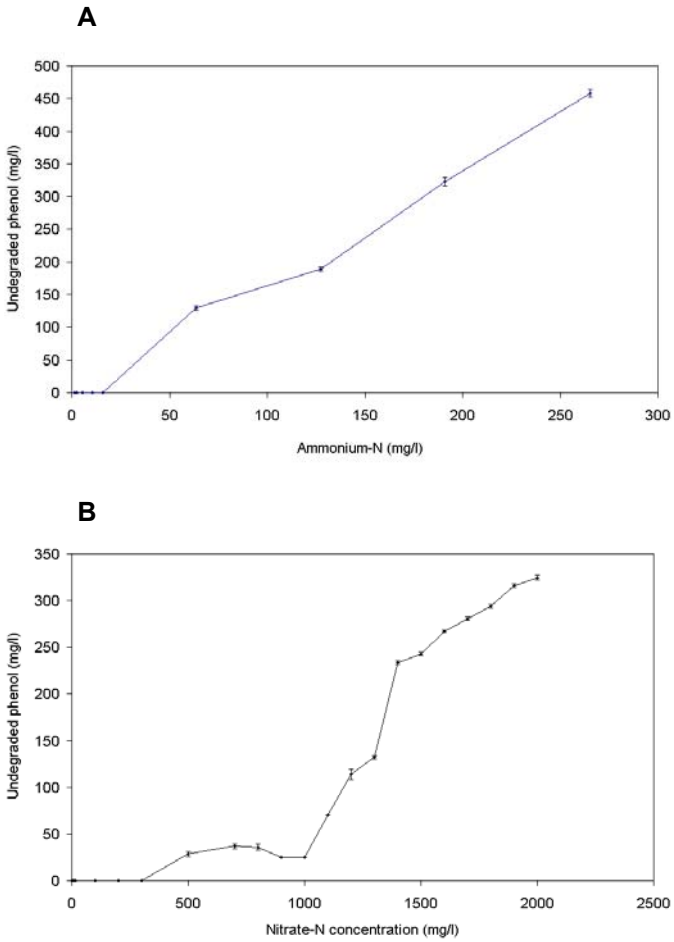

Figure 2. Effect of ammonium-N (A) and nitrate-N (B) on phenol biodegradation by Ca-alginate immobilized cells of *Acinetobacter* sp. strain W-17 in simulated wastewater medium (SW).

concentration to phenol degradation (see Figures 2A and 2B). Biodegradation experiments using Ca-alginate immobilized cells showed that complete degradation of phenol was reduced in MSW medium to 15 h instead of 24 h of incubation in MSM medium (Figures 1 and 3A).

Effect of phenol on the removal of ammonium and nitrate in MSW medium

The removal of both ammonium and nitrate in the presence of phenol in MSW medium was investigated. It was observed that the concentration of NH_4^+-N continuously decreased for 18 h (Figure 3A), after which no significant change in the concentration of NH_4^+-N was observed.

In the first three hours of incubation the concentration of NO_3^--N dropped off dramatically from 1000 to 540 mg/l, and become stable at 530 to 540 mg/l from 3 to 15 hours. The concentration decreased again to 96-110 mg/l between 18 and 48 h of incubation (Figure 3B).

In contrast, the concentration of NO_2^--N increased in the first three hours to 6 µg/l, and continuous increase was observed till 9 h of incubation (Figure 3B).

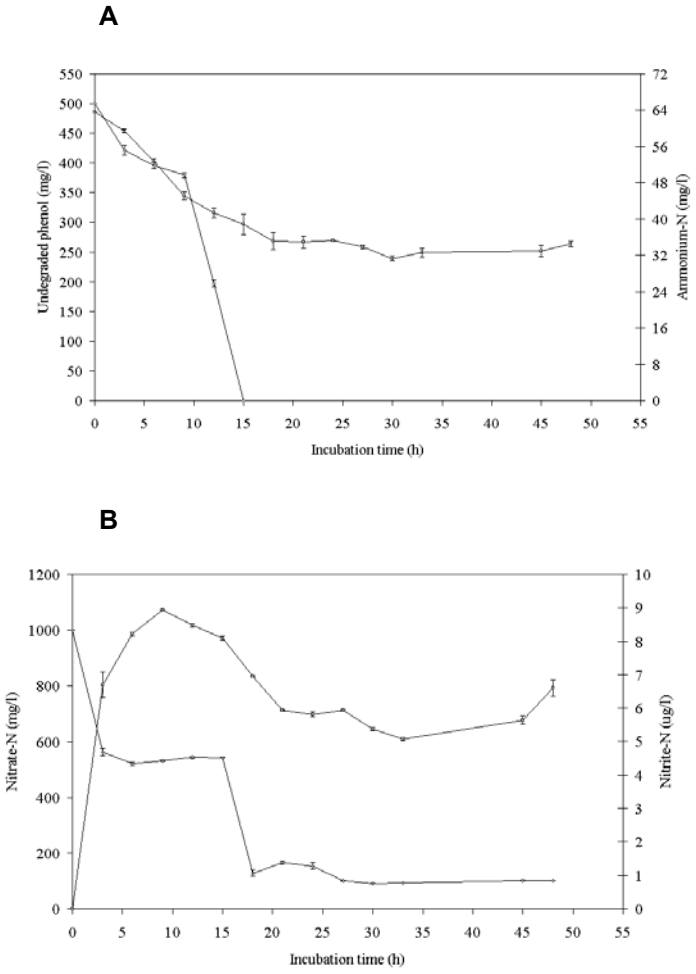

Figure 3. Biodegradation of phenol under non-inhibitory concentration of ammonium and nitrate in MSW medium using Ca-alginate immobilized cells of *Acinetobacter* sp. strain W-17. A) Biodegradation of phenol (completed in 15 h) and ammonium transformation. B) Nitrate reduction and nitrite production.

Reuse of immobilized *Acinetobacter* sp. strain W-17 for phenol biodegradation

Ca-alginate immobilized bacterial cells were tested in several consecutive phenol-degradation experiments to determine if there was deactivation of cells with repeated use. It was observed that immobilized cells of *Acinetobacter* sp. strain W-17 could be reused five times (24 h incubation each) without loosing their phenol-degrading activity. The capacity of the immobilized cells to degrade phenol was equally high in the first five cycles. However, a 10% decrease in phenol biodegradation rate was observed in the 6th cycle.

Determination of bacterial immobilization sites on Ca-alginate beads

The immobilized cells were investigated by scanning electron microscopy to determine the nature of cell attachment. The SEM photographic plates clearly showed that the cells were randomly distributed in the carrier matrix (Figure 4). Plates A and B shows the immobilized W-17 cells onto Ca-alginate beads immediately before and after the phenol biodegradation experiments.

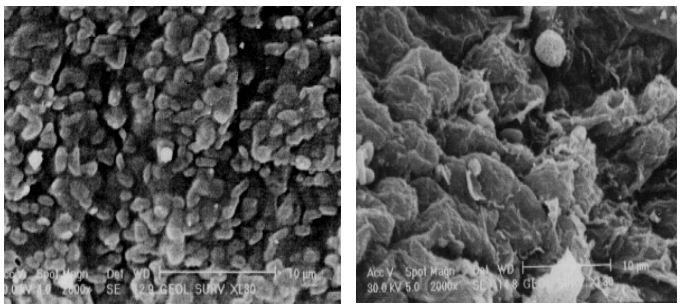

Plate A Plate B

Figure 4. Immobilized *Acinetobacter* sp. strain W-17 onto Ca-alginate beads immediately before (A) and after (B) incubation with phenol.

DISCUSSION

Several *Acintobacter* isolates have been reported to degrade phenol, benzoate, oils, cresols, and other substituted aromatics (Abd El-Haleem et al., 2002; Beshay et al., 2002; Ehrt et al.,1995; Gralton et al., 1997). Introduction of *Acintobacter* as a biocatalyst into a wastewater stream for the bioremediation of some dangerous aromatic hydrocarbons like phenols requires thorough investigation. The economical value of an immobilized biocatalyst depends on its specific potential stability and its capacity to process large amount of substrate. Our results indicate that the free *Acinetobacter* cells in MSM medium were able to degrade phenol at a concentration of 500 mg/l within 120 h of incubation. The same bacterium immobilized onto Ca-alginate was however, able to reduce the time needed for complete degradation of the same amount of phenol to 24 h only. Entrapment in insoluble Ca-alginate gel is a rapid, nontoxic, inexpensive, versalite and the most often used method for immobilization of cells. More than 80% of cell immobilization processes are still carried out using alginate (Thu et al., 1996).

In domestic wastewater there is a great concern about the presence of nitrogen components such as NH_4^+ and NO_3^- since some of these components represents serious health hazard for different forms of life including human.

The presence of these components with phenol in the wastewater may interfere with the phenol biodegradation. In the present study combined effect of these components on phenol biodegradation and the effect of phenol on their removal was investigated. These experiments indicate that immobilized *Acinetobacter* cells, can tolerate high concentrations of NH_4^+-N (63 mg/l) and NO_3^--N (1000 mg/l) without loosing capacity of phenol biodegradation.

Interestingly, it was observed that the time needed for complete degradation of phenol (500 mg/l) reduced from 24 h in MSM medium to 15 h in MSW medium. Therefore, it seems that wastewater containing phenol has no inhibitory effect on nitrate or ammonium removal. Infact, nitrate or ammonium enhances phenol biodegradation. This supports the idea of using *Acinetobacter* strain W-17 for biotreatment of phenol in the presence of high concentrations of nitrogen components present in wastewater.

In addition, the results showed that transformation of ammonium-N to nitrate was not inhibited by the presence of phenol. Hence, it is possible to conclude that nitrification and phenol biodegradation may have independent pathways, as previously reported by Kostyal et al. (1997). Other investigators have shown that oxidation enzymes (such as oxygenases) responsible for the degradation of phenol exhibit non-specific activity to methane and ammonium (Kim And Hao, 1999).

The reduction of nitrate-N from 1000 mg/l at the start point of the experiment to 96 mg/l after 18 h of incubation is of much interest. It may be due to the fact that many heterotrophic bacteria are able to denitrify aerobically under low oxygen concentration. In the presence of high input of ammonium, bioxidation of NH_4^+-N increase the oxygen uptake and create conditions suitable for denitrification (Helmer et al., 1999).

One of the main advantages of the immobilized cells is the possibility for repeated use (Bandhyopadhyay et al., 1999). Our data revealed that up to the fifth cycle the cells function well and the degree of biodegradation remained 100%. This indicates the suitability of the Ca-alginate beads for several consecutive batches of biodegradation.

In conclusion, phenol biodegradation by *Acinetobacter* sp. strain W-17 in the presence of high concentration of nitrogen components (NH_4^+ and NO_3^-) is enhanced. This have the potential application to improve industrial processes wastewaters and the *in situ* bioremediation of phenol, ammonia and nitrate contaminated soils.

ACKNOWLEDGEMENTS

This work was completely funded by the Genetic Engineering & Biotechnology Research Institute (GEBRI), Mubarak City for Scientific Research & Technology Applications.

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