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Enhancement of benzene biodegradation by variation of culture medium constituents

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Three different compounds (humic acid, yeast extract and hydrogen peroxide) and two related culture media (mineral salt solution and prokaryotic medium) were evaluated for enhancing biodegradation of benzene under aerobic conditions. The results showed that yeast extract was more effective than humic acid in benzene biodegradation. The presence of both yeast extract and humic acid resulted in increased benzene removal efficiency, depending on the yeast extract concentration. In the presence of hydrogen peroxide, benzene removal efficiency was increased by more than 55%. The benzene removal efficiency in Prokaryotic medium was higher than in the mineral salt medium.

Key words: Benzene, yeast agar, humic acid, prokaryotic medium, hydrogen peroxide.

INTRODUCTION

In addition to many other hydrocarbon constituents, petroleum contains benzene, toluene, ethyl benzene and xylenes (BTEX). Contamination of soil and groundwater by BTEX compounds due to petroleum leaks is a widespread phenomenon. Benzene (C6H6), which typically makes up less than 2% of petroleum, is a colorless liquid at room temperature (melting point 5.5°C) with a density of 0.87 g/cm3 at 20°C.

Although BTEX compounds are degraded naturally in groundwater systems (Johnson et al., 2003), different methods have been described for the degradation of benzene in contaminated water sources, e.g. anaerobic biodegradation, electro- oxidation and Advanced Oxidation Processes (AOPs) (Groher, 2001). Among AOPs, the Fenton's reagent has been used efficiently as a che-mical process for removal of aromatic compounds from aqueous environments. The efficiency of different Fenton-related oxidative processes such as Fenton, solar-Fenton, UV-Fenton and Fenton reactions in differ-rent batch reactors has been examined previously (Sabzali et al., 2005). While benzene is recalcitrant under anaerobic conditions, available evidence shows that this compound is moderately degradable in the presence of oxygen (Johnson et al., 2003). Degradation can proceed via catechol to CO_2 (Ribbons et al., 1992) and it has been reported that 3.08 mg of oxygen is necessary to biodegrade 1 mg of benzene to CO_2 and water (Wiedemeier et al., 1995).

Most of the data obtained regarding benzene degradation under aerobic conditions have been reported for aquifer environments. Field studies at six different locations consistently reported the biodegradation of benzene and yielded half-life values ranging from 58 to 693 days. The longer half-life was associated with an uncontaminated aquifer study. Initial concentrations of up to 25 mg/l were biodegraded under field conditions (Davis et al., 1994). Biodegradation of benzene has also been observed during in situ microcosm studies. Half-lives ranged from 1.4 to 103 days with an average half-life of 4 days. The high half-life value represents biodegradation in the groundwater section only of the in situ microcosm; half-life values obtained in both the aquifer sediment and groundwater section were significantly lower (Holm et al., 1992). Mineralization half-lives for benzene in laboratory microcosm studies, however, ranged from 7 (Kemblowski et al., 1987) to 1195 days (Thomas et al., 1990).

The main objective of this research was to determine the effects of different compounds, as well as related culture media (mineral salt solution and Prokaryotic me-

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dium) on aerobic benzene degradation.

MATERIALS AND METHODS

Culture media

Mineral salts medium (pH 7.0) consisted of 0.694 g of KH2PO4; 0.854 g of K2HPO4; 1.234 g of (NH4)2SO4; 0.46 g of MgSO4.7H2O; 0.176 g of CaCl2.2H2O; 0.001 g of FeSO4.7H2O; 60 mg of H3BO3; 40 mg of CoCl2.2H2O; 20 mg of ZnSO4.7H2O; 6 mg of MnCl2.4H2O; 6 mg of NaMnO4.2H2O; 4 mg of NiCl2.6H2O and 2mg of CuCl2.2H2O in 1000 ml of deionized water.

Prokaryotic medium (4.2) consisted of 0.46 g/l of MgSO4.7H2O; 0.5 g/l of KNO3; 0.009 g/l of CaCl2.2H2O; 0.5 g/l of KH2PO4; 0.5 g/l of K2HPO4; 1 g/l of NaCl and 1 ml of a solution that included 1.5 mg/l of NiCl2.4H2O; 0.015 g/l of CuCl2.2H2O; 0.025 g/l of NiCl2.6H2O; 0.1 g/l of MnCl2.4H2O; 0.12 g/l of CoCl2.6H2O; 0.07g/l of ZnCl2; 0.025g/l of NaMnO4.2H2O; 0.06 g/l of H3BO3 and 5.2 g/l of EDTA.4H2O.

Other culture media that was used in the study included Trypticase soy agar (TSA), Trypticase soy broth (TSB), Agar-agar medium and an Agarose-containing medium.

Analytical techniques

The inoculum used in the study was enriched from oil-contaminated soils obtained from the Asalouye of the Pars Petroleum Company refinery in Booshehr, southern Iran. Initially, 100 g of the mixed soil sample was added to 100 ml of sterilized mineral salts medium and spiked with 15 mg/l of benzene (Merck). The benzene concentration was measured with a PU 4410 Gas Chromatograph (GC) equipped with a packed column and a FID detector. The temperature of injection was 200°C, while the column temperature was typically 50°C and nitrogen was used as the carrier gas. In all experiments, the benzene was injected directly into the samples (the volume of the solution in the broth medium was 10 ml), measured as headspace and directly injected into the GC in a gas form. In order to prevent gas leakage, all sample bottles were properly sealed. All experiments were performed at 23.0 ± 3.0°C.

Benzene removal efficiency in TSB medium

In this experiment, various dilutions (1:8, 1:32, 1:64 and 1:128) of TSB medium were prepared in distilled water, and benzene in various concentrations (1740, 5220, 8700 and 12180 mg/l) was added directly into the dilutions with a syringe. Afterwards, 1 ml of the microbial suspension was inoculated into the medium. As a control, 0.11 g of sodium cyanide (NaCN; Merck) was added into a blank sample to prevent bacterial growth. The properly sealed bottles were incubated on a shaker device (120 rpm) to ensure complete mixing of the reagents. Batch biodegradation experiments were performed in duplicate, with replicate experiments performed at separate times to provide an accurate assessment of variability. The benzene removal efficiency was evaluated in all samples for 21 days.

Cultivation of TSB-positive samples on Agar-agar and agarose media

TSB-positive samples were inoculated using the spread plate technique onto Agar-agar (3.0%) and agarose (1.5%) media with a mineral salt base. The agar plates were placed in a plastic container of which the atmosphere was saturated by benzene vapors using a benzene solution (1 ml benzene in 10 ml distilled water) water) and the container was then closed. After 10 days, visible colonies that had grown on the surface of the respective media at room temperature (20 - 25°C), were characterized and the final microbial consortium was then prepared by inoculating different colonies into the mineral salt solution. Inoculated mineral salt solution, as described here, was used in all experiments indicated below.

Evaluation of mineral salt solution and different medium constituents on benzene biodegradation efficiency

Run 1 (mineral salt solution)

In this instance, 5 µl of pure benzene was injected directly into 15 ml of mineral salt solution (293 mg/l) and then placed on a shaking device (200 rpm). The benzene concentration was measured for 16 days. The benzene concentration was identical (std. 1.2 mg/l) in all of the runs indicated here (run 1 - 5).

Run 2 (mineral salt solution + yeast extract)

Yeast extract in concentrations of 0.25, 0.5 and 1 g/l were added into mineral salt solution in the same condition. Control sample (blank allocated for each concentrations (blank with yeast extract and added bacteria but without benzene).

Run 3 (mineral salt solution + humic acid)

Humic acid, which was prepared by alkali extraction (Tay et al., 1999), was added in concentrations of 0.01, 0.02 and 0.03 g/l into mineral salt solution in the same condition.

Run 4 (mineral salt solution + humic acid + yeast extract)

Humic acid (0.02 g/l) and yeast extract (0.5 g/l) were added simultaneously into mineral salt solution in the same condition.

Run 5 (mineral salt solution + hydrogen peroxide [H_2O_2])

Hydrogen peroxide in concentrations of 40 and 60 ml/l were added into mineral salt solution with same condition.

Evaluation of benzene removal efficiency in prokaryotic medium

The biodegradation efficiencies of three benzene concentrations (351.2, 614.6 and 878 mg/l) were evaluated in prokaryotic medium. All experiments were performed in triplicate for each concentration.

RESULTS

TSB medium

The results of this study showed that culture medium constituents are important for biodegradation of benzene. In TSB medium, as shown in Table 1, for dilutions higher than 1:64 and benzene concentrations higher than 5220 mg/l, benzene removal efficiency was equal to zero. The data also suggests that available nutrient has decreased for microbial metabolism, while benzene concentrations higher than 5220 mg/l may have been toxic to the micro-

Table 1. Benzene removal efficiency in various dilutions of TSB and concentrations of benzene.

	Benzene concentrations(mg/l)			
TSB dilutions	1740	5220	8700	12180
1:8	43%	17%	0	0
1:32	14%	0	0	0
1:64	0	0	0	0
1:128	0	0	0	0

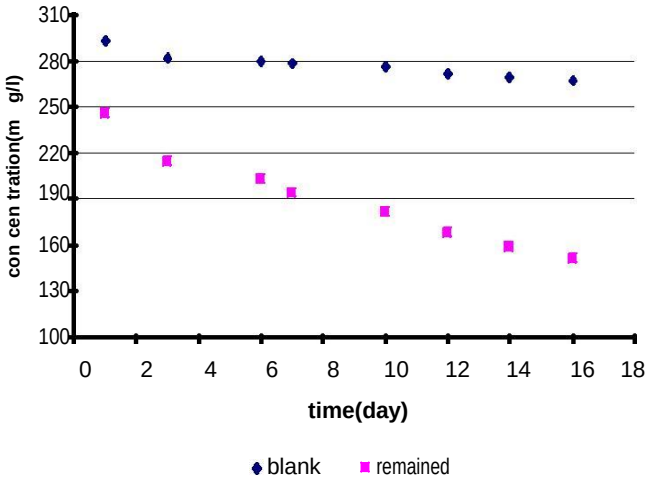

Figure 1. Variation of benzene residual concentration with time in the mineral salt medium without any additive (run1).

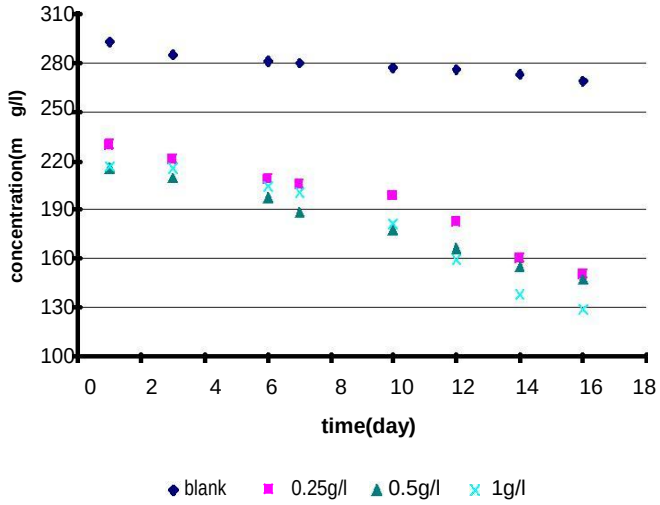

Figure 2. Variation of benzene residual concentrations in the mineral salt medium containing yeast extract with concentrations of 0.25, 0.5 and 1 g/l (run 2).

bial consortium.

In Agar-agar and agarose media, the first colony was observed on the surface of the Agar-agar media after 3

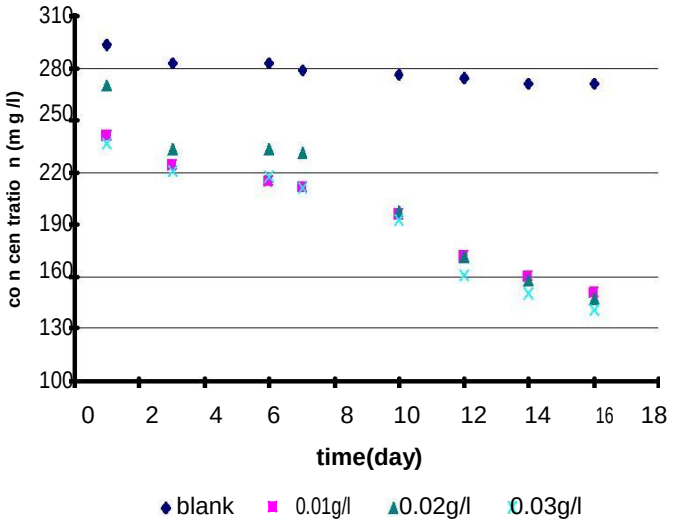

Figure 3. Variation of benzene residual concentrations in the mineral salt medium containing humic acid with concentrations of 0.01, 0.02 and 0.03 g/l (run 3).

days but after 1 week other colonies were observed on the surface of both media. All colonies in both media had the same colony characteristics. Gram staining showed that most of the bacteria were Gram-negative cocci, but a few Gram-positive cocci were also observed. Subsequent examination revealed that the isolated bac-terial consortium produced catalase. The production of catalase, which decomposes potentially toxic hydrogen peroxide to oxygen and water, is related to aerobic growth (Vaccari et al., 2005).

Benzene residual concentrations in batch experiments

The data obtained from run 1 to run 5 are shown in Figures 1 - 5 as residual concentrations of benzene during 16 day experiments. According to results shown in Figure 1, yeast extract was more effective than humic acid for benzene biodegradation. As shown in Figure 1, in mineral salt medium, more than 43% of the benzene was degraded during 16 days. The benzene was biodegraded by more than 50% when the concentration of yeast ex-tract was 1 g/l. Even in the presence of both yeast extract and humic acid, an increase in the benzene removal efficiency could be ascribed to yeast extract (Figures 2 - 4).

The application of ozone and hydrogen peroxide (H_2O_2) prior to biological processes has been reported pre-viously. In this study, the main reason for addition of H_2O_2 to the media was based on the presence of catalase-positive cocci in the mixed microbial consortium. It was evident that in the presence of H_2O_2 (60 ml/l of H_2O_2), the benzene removal efficiency was increased by more than 55%. Higher concentrations of H_2O_2 may have led to cell

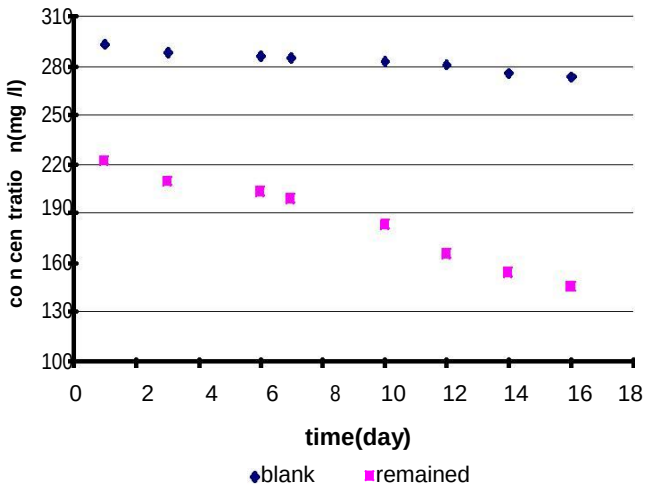

Figure 4. Variation of benzene residual concentrations in the mineral salt medium containing humic acid (0.02 g/l) and yeast extract (0.5 g/l) (run 4).

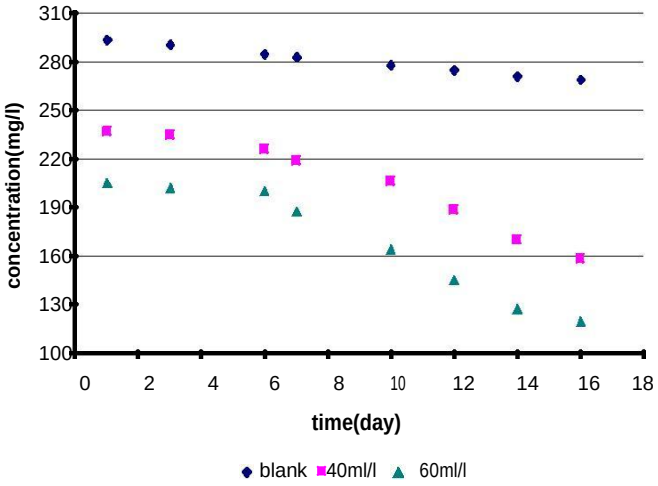

Figure 5. Variation of benzene residual concentrations in the mineral salt medium containing hydrogen peroxide in concentrations of 40 and 60 ml/l (run 5).

wall damage, thus interfering in the biodegradation process (Figure 5).

Biodegradation efficiency of benzene in prokaryotic medium

Figure 6 shows benzene residual concentrations in prokaryotic medium. The biodegradation process was performed with a primary concentration of 878 mg/l during 7 days. The benzene residual concentration was below the detection limit (1 mg/l) of the GC apparatus (Figure 6). However, benzene biodegradation efficiencies at applied primary concentrations of 351.2 and 614.6 mg/l were approximately 100% after 4 days (results not shown).

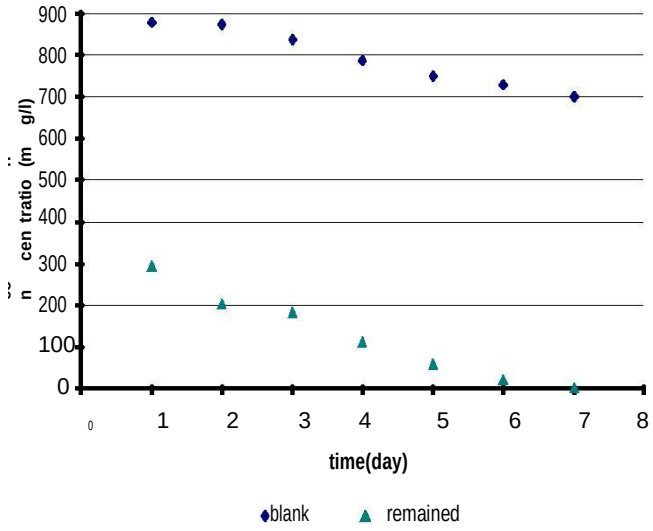

Figure 6. Variation of benzene residual concentrations in prokaryotic medium containing a benzene concentration of 878 mg/l.

Data reported by Kenneth et al. (2000) regarding benzene biodegradation (35 mg/h of benzene fed to culture bioreactor with a working volume of 1.5 L in modified Hutner medium) revealed that benzene was consumed nearly as rapidly as toluene and with a similarly short lag time (6.5 h). In addition, microbial growth stopped at essentially the same time that the benzene was depleted (Kenneth et al., 2000). As shown in Figure 6, the lag phase in Prokaryotic medium at a benzene concentrations of 878 mg/l was approximately 1 day.

DISCUSSION

One feature of benzene common to many other 'xenobiotics', is its toxicity to microorganisms, and the concomitant difficulty in degrading it when present in high concentrations (Yeom and Daugulis, 2001). Studies regarding the biodegradation of benzene have reported the bacteria to be aerobic, rod-shaped, gram-negative, non-motile, catalase-positive, oxidase-positive, with yellow pigments (Tay et al., 1999). In agar-agar medium, several colonies formed in the medium in addition to those present on the agar surface. The presence of some colonies in the agar medium suggest that that they may be related to anaerobic bacteria, because the oxygen concentration in the agar medium may be decreased (anoxic condition). Benzene is biodegraded in the absence of oxygen under a variety of terminal electron-accepting conditions. However, the mechanism by which anaerobic benzene degradation occurs is unclear. Phenol and benzoate have been consistently detected as intermediates of anaerobic benzene degradation, which suggests that the hydroxylation of benzene to phenol is one of the initial steps in anaerobic benzene degradation. The conversion of phenol to benzoate occurs by the carboxylation of phenol to form 4-

hydroxybenzoate, followed by the reductive removal of the hydroxyl group to form benzoate (Coates et al., 2002).

Studies have noted that microbial degradation of a compound present in a mixture can be strongly affected by other constituents of the mixture. This has been observed not only for mixtures of toxic chemicals (bioremediation), but also for mixtures of pollutants and readily degraded compounds (wastewater treatment) and mixtures of sugars (fermentation). To understand these effects, the metabolic role that each compound may play for the microorganisms must be understood (Kenneth et al., 2000). Literature in the fields of bioremediation and ecotoxicology indicates that organic xenobiotic compounds, which have been in contact with soil, display reduced availability and the magnitude of this effect increases over time. Recent models of sequestration relating to contaminant biodegradation recognize that the overall sequestration process most likely encompasses two individual mechanisms, i.e. partitioning into or onto humic matter. Most authors have concluded that interacttions between humic substrates and contaminants such as benzene occur as a two-step process, with adsorption onto the hydrophobic surface material taking place first, and partitioning into pores later (Bogan et al., 2003).

The results obtained in this study revealed that yeast extract was more effective than humic acid in enhancing benzene biodegradation when added to the mineral salt solution. The yeast extract may serve as a source of carbon, nitrogen and energy for the microbial consortium. An increase in the yeast extract concentration resulted in increased biodegradation of benzene. Similarly, biodegradation of 2-methyl, 2-ethyl, and 2-hydroxypyridine by an Arthrobacter sp. isolated from subsurface sediment was reported to increase with a concomitant increase in the yeast extract concentration of the culture medium (Edward et al., 1999). In this study, a noticeable feature was also the enhancing effect of hydrogen peroxide on the biodegradation efficiency of benzene. Benzene removal efficiency in Prokaryotic medium was higher than that in mineral salt medium. In addition to decreasing the lag phase, the prokaryotic medium also aided in increasing the benzene biodegradation efficiency. Three compounds present in the prokaryotic medium (NaCl, KNO_3 and EDTA) may play a role in benzene biodegradation since mineral salt lacked these components. The mechanism by which this may occur is unclear and requires further investigation.

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