Süleyman Demirel Üniversitesi
Fen Bilimleri Enstitüsü Dergisi
Cilt 27, Sayı 2, 213-218, 2023

Süleyman Demirel University
Journal of Natural and Applied Sciences
Volume 27, Issue 2, 213-218, 2023

DOI: 10.19113/sdufenbed.1187346

An Investigation on the Removal of High Concentrations of PAHs Using Two-Liquid Phase System

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(Alınış / Received: 11.10.2022, Kabul / Accepted: 15.03.2023, Online Yayınlanma / Published Online: 25.08.2023)

Keywords
Two-liquid phase systems, PAH biodegradation, Removal of hydrocarbons, Oily sludge

Abstract: Two-liquid phase systems consisting of two insoluble liquids can be effective in removing high concentrations of hydrocarbons from aqueous environments. In this study, the removal efficiencies of Naphthalene (Nap), Acenaphthene (Acn), Fluorene (Flu), Fluoranthene (Flr), Anthracene (Ant), and Pyrene (Pyr) at high concentrations in the two-liquid phase system were investigated. Two-liquid phase systems were constituted using Bis (2-Ethylhexyl) sebacate (BES) and aqueous fermentation media. Nutrient Broth (NB) and Bushnell Haas Yeast (BHY) medium were used as aqueous fermentation media. Acn, Flu, Flr, Ant, Pyr, and Nap were degraded at a rate of 93.1%, 80.8%, 57.6%, 68.5%, 63.8%, and 100%, respectively with BES/NB system. In the BES/BHY system, Acn, Flu, Flr, Ant, Pyr, and Nap, were degraded at a rate of 29.6%, 44.3%, 22.8%, 68.1%, and 19.7%, 45.4%, respectively. When both systems are compared, it has been shown that the BES/NB system can be effective under specified conditions.

İki-Sıvı Fazlı Sistem Kullanılarak Yüksek Konsantrasyonlardaki PAH'ların Giderilmesi Üzerine Bir Araştırma

Anahtar Kelimeler
İki-sıvı fazlı sistemler, PAH bioparçalanması, Hidrokarbon giderimi, Yağlı çamur

Öz: Birbirinde çözünmeyen iki sıvıdan oluşan iki-sıvı fazlı sistemler, sulu ortamlardan yüksek konsantrasyonlarda hidrokarbonların uzaklaştırılmasında etkili olabilir. Bu çalışmada yüksek konsantrasyonlardaki Naftalen (Nap), Asenaften (Acn), Floren (Flu), Floranten (Flr) Antrasen (Ant) ve Piren'in (Pyr) iki-sıvı fazlı sistemde giderim verimleri incelenmiştir. İki-sıvı fazlı sistemler Bis (2-Etilheksil) sebakat (BES) ve sulu fermentasyon ortamları kullanılarak oluşturulmuştur. Sulu fermentasyon ortamları olarak Nutrient Broth (NB) ve Bushnell Haas Yeast (BHY) besiyerleri kullanılmıştır. BES/NB sistemiyle Acn, Flu, Flr, Ant, Pyr ve Nap sırasıyla %93,1, %80,8, %57,6, %68,5, %63,8, %100 oranında parçalanmıştır. BES/BHY sisteminde Acn, Flu, Flr, Ant, Pyr ve Nap'nin sırasıyla %29,6, %44,3, %22,8, %68,1, %19,7 ve %45,4 oranında parçalanmıştır. Her iki sistem karşılaştırıldığında, BES/NB sisteminin belirlenen koşullar altında etkili bir sistem olabileceği gösterilmiştir.

1. Introduction

PAHs are organic molecules that contain carbon and hydrogen atoms in their structure. They are typically characterized by the formation of two or more benzene rings, which may be linked together by linear, cluster, and angular arrangements [1, 2]. While these compounds can occur naturally, they are also produced by some industries. PAHs enter the environmental systems as a result of pyrogenic, petrogenic, and biological activities. Thus, they cause environmental pollution such as air, soil, and sediment [1, 2]. Besides, many aromatic hydrocarbons are resistant to degradation and can be stable in nature for many years [3]. It is known that the hydrophobicity of PAHs increases with the increase in the number of aromatic rings; accordingly, their solubility in water decreases. In this case, the bioavailability is reduced [4]. Moreover, aromatic compounds can be teratogens, mutagens, carcinogens, and powerful immunosuppressants for living beings [2, 5-10]. Because of all these negative properties, the fate of PAHs in nature creates a great environmental concern [1]. It has been given priority for 16 PAHs listed by the US Environmental Protection Agency (EPA) to remove them from environmental systems [11-15].

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Abiotic and biotic methods are used to eliminate pollutants from the environment. Chemical oxidation, evaporation, combustion, photocatalytic oxidation, and applications with immobilized enzymes are classified as abiotic methods, whereas microorganisms are mostly used in biotic methods [2, 10]. Thanks to the natural process called biodegradation in biotic methods, in which microorganisms mostly work, it is possible to remove pollutants from the environment and restore contaminated areas by rehabilitating them. With the studies in the field of biodegradation, many bacteria, fungi, and algae strains that can decompose various petroleum hydrocarbons have been reported so far [15-17]. The ability and stability of a microbial community to break down or mineralize pollutants are closely related to the resident microorganisms present in that environment. It is known that microbial consortia can achieve efficient hydrocarbon degradation in industrial wastewater [18].

Two-liquid phase systems are being developed for the biodegradation of a variety of poorly soluble and/or toxic chemicals [4]. Two-liquid phase systems are systems based on using an organic solvent phase with a lower molecular weight and biocompatible properties on an aqueous phase. While the microorganisms are located in the lower aqueous phase, a high concentration of xenobiotic substrates (toxic organic compounds) is found in the upper organic phase [19, 20]. Applying the high-speed agitation forms small-sized organic phase droplets containing the substrate and dispersed in the aqueous phase with a large surface area. Thus, the substrate in small droplets becomes usable by microorganisms in the aqueous phase [20]. Although the bioreactor is loaded with a high concentration of toxic organic matter, microorganisms in the underlying aqueous phase only encounter toxic substances at very low concentrations in this way [19]. The system provides appropriate concentrations of the xenobiotic substrate to microorganisms, while the substrate distribution into the aqueous phase continues until the overlying organic phase is completely depleted [19]. The rate of bioavailability from the substrate in the upper phase is closely related to the metabolic activity of the microbial cells in the system [19, 21]. Bioreactor systems constructed with liquid solvents have been successfully used for the degradation of different xenobiotics such as PAHs, phenol, benzene, toluene, and xylene. These were achieved by enrichment with either known bacterial cultures or unidentified microbial cultures [4, 20, 22].

In this study, the degradation capacity of PAHs at high concentrations was investigated using the two-liquid phase systems with microorganisms isolated from oily sludge and wastewater samples of the petrochemical industry.

2. Material and Method

2.1. Chemicals and media

Naphthalene (Nap), Acenaphthene (Ace), Fluorene (Flu), Fluoranthene (Flr), Anthracene (Ant), Pyrene (Pyr), Dimethyl sulfoxide (DMSO), and Acetonitrile (ACN), Bis(2-Ethylhexyl) sebacate (BES), Acetone, Bushnell Haas (BH) Medium, Luria-Bertani (LB) Broth, and Yeast Extract were provided from Sigma-Aldrich. PAH stock solution was prepared in DMSO. Bushnell Haas Yeast (BHY) medium was prepared by adding 0.025 g/L Yeast Extract to the BH medium.

2.2. Analytical measurements

Analytical measurements were made by HPLC (Agilent 1100, USA), equipped with a vacuum degasser, a quaternary pump, an autosampler, and a diode array detector system. ZORBAX Eclipse PAH column (4.6mm × 150 mm, 3.5 µm) (Agilent, USA) was used. The mobile phase consisted of ACN (A) and H_2O (B). The optimized gradient elution was performed at a flow rate of 1 µL/min with 1 µL injection volume using the following linear gradient: 0–16 min: 50–50% to 50–100% of A. The column temperature was set to 25°C before starting the analysis. Detection was carried out at 220 nm, 240 nm, and 254 nm using a UV-visible detector. Data were collected using Chem-Station (Agilent, USA).

2.2.1. Analysis of PAHs in oily sludge

The oily sludge sample was taken from a sludge pile in a wastewater treatment plant in a petrochemical industry. The amounts of PAHs to be used in the study in oily sludge were determined by HPLC. 5 g of oily sludge was taken into 20 mL of hexane and mixed by vortexing at full speed for 5 min. Subsequently, the hexane phase was transferred into HPLC vials. PAH contents of oily sludge were determined according to the retention times of the selected PAHs. Concentrations were calculated using standard graphs for each PAH.

2.3. Acclimation of microorganisms

2.5 g of analyzed oily sludge samples and 2.5 mL of wastewater samples were transferred into sterile 50 ml BHY media in 250 ml flasks. The acclimation was started at 200 rpm, at 25°C for 7 days under dark conditions. At the end of the incubation, inoculations were repeated into new sterile BHY media, and acclimation was continued for 21 days by making a total of three passages. On the 21st day, 2 mL of the 14000 mg/L PAH stock solution (contains Nap, Ace, Flu, Flr, Ant, and Pyr) was transferred into new sterile 50 mL BHY media and mixed homogeneously. Then 2.5 mL of sample from the previous flasks was added. The flasks were incubated at the same conditions.

From the 3rd day of the incubation period, PAH analyzes were performed by HPLC from the samples.

To control microbial viability in the flasks, 1 mL of media contents were transferred into 1.5 mL sterile microcentrifuge tubes, and microbial cell pellets were obtained by centrifuging the microcentrifuge tubes at 6000 rpm for 10 min. Pellets were inoculated on LB (Luria Bertani) Agar and PC (Plate Count) Agar by streaking method and incubated for 24-48 hours at 27°C.

2.4. Constitution of two-liquid phase systems and degradation of PAHs

BES was used for the upper organic phase to form a two-liquid phase system. The upper phase was prepared as a constitution of 10 mL BES, 20 mL PAH stock solution, and 10 mL acetone in a 40 mL total volume for each reactor. An equal volume of the mixture was added to each 250 mL screw cap bottle. The bottles were shaken at 200 rpm for 24 hours under dark conditions in a fume hood to allow the stock solution to pass into BES and evaporation of acetone. Two different fermentation liquids (BHY and NB) were selected to observe the effect of the medium in the two-liquid phase systems.

Acclimated microorganisms were combined to prepare a microbial consortium. For this, microorganisms were adjusted to 0.5 OD at 540 nm wavelength by a densitometer. Subsequently, a microbial consortium was constituted and inoculated as 5% (v/v) into 50 mL sterile BHY and 50 mL sterile NB in 250 mL screw cap bottles. The aforementioned upper organic phase mixture was added as 40 mL to each culture media. 90 ml total working volume two-liquid phase system in each bottle was incubated at 25°C at 200 rpm under dark conditions. PAH removal rates in both systems were observed for 33 days.

3. Results

According to HPLC analysis, the amounts of Ace, Flu, Flr, Ant, and Pyr, in oily sludge were calculated as 59.66 mg/L, 87.83 mg/L, 38.92 mg/L, 34.65 mg/L, and 92.79 mg/L, respectively. Nap was not detected in oily sludge. These values provided insight regarding PAH concentrations for degradation experiments. Initial concentrations of PAHs, the amount of PAHs in the flask after 7 days, and the PAH removal rates are shown in Table 1. Accordingly, the degradative activities of microorganisms in oily sludge and wastewater on PAHs were determined at the end of the 4-week acclimation period.

The effect of two different fermentation liquids was observed. The amounts of PAH in two-liquid phase systems were determined every two days for 33 days. The degradation kinetics in both two-liquid phase systems are shown in Figure 1 and Figure 2.

Table 1. Degradation of PAHs and removal rates in the flasks after acclimation with oily sludge and wastewater samples (OS: Oily sludge, WW: Wastewater).

	Initial Concentration (mg/L)		Final Concentration (mg/L)		Removal Rate (%)	
	OS	WW	OS	WW	OS	WW
Ace	44.0	44.0	17.14	24.49	61.0	44.4
Flu	47.0	47.0	29.17	36.06	37.9	23.3
Flr	11.01	11.01	7.5	10.29	31.9	6.5
Ant	77.14	77.14	52.82	69.59	31.5	9.8
Pyr	42.12	42.12	28.78	41.29	31.7	2.0
Nap	12.13	12.13	0.74	1.19	93.9	90.2

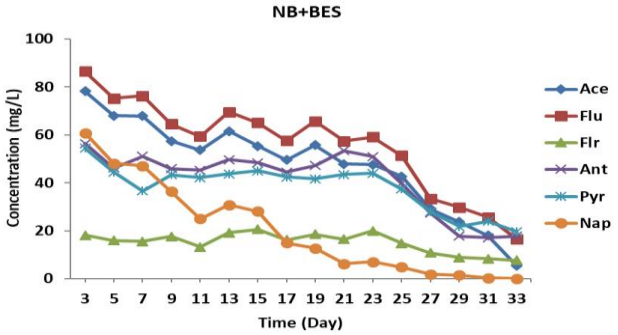

Figure 1. The graph shows the degradation behavior of the microbial consortium in the BES/NB.

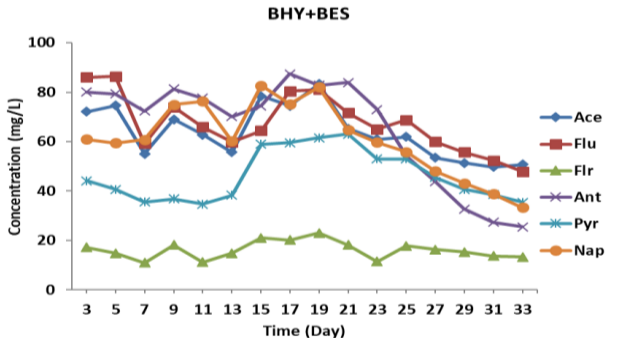

Figure 2. The graph shows the degradation behavior of the microbial consortium in the BES/BHY.

At the end of 33 days, the PAH removal rates of both systems were calculated. Accordingly, the removal rates of Ace, Flu, Flr, Ant, Pyr, and Nap in the BES/NB system are 93.1%, 80.8%, 57.6%, 68.5%, 63.8%, and 100%, respectively (Figure 3). In the BES/BHY system, the removal rates of Ace, Flu, Flr, Ant, Pyr, and Nap were calculated as 29.6 mg/L, 44.3 mg/L, 22.8 mg/L, 68.1 mg/L, 19.7, and 45.4 mg/L, respectively (Figure 3).

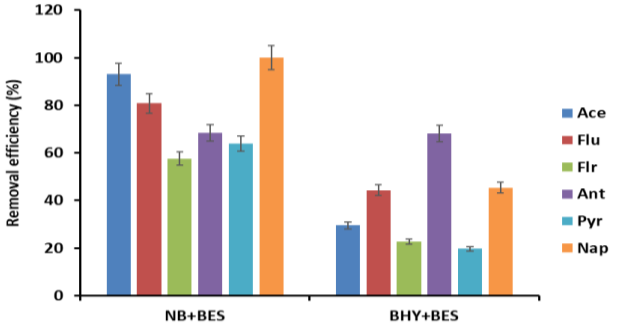

Figure 3. Removal efficiencies of two different two-liquid phase systems.

4. Discussion and Conclusion

Researchers have mentioned that it may not be possible with a single isolate to degrade all PAHs in contaminated soil, and better results can be obtained with a microbial consortium with different metabolic properties [4]. Active microbial consortia can be used to effectively degrade mixed PAHs [18]. Additional carbon sources in complex media can assist in enhancing the degradative activity of a microbial consortium. BES/NB system indicates that the composition of NB regulates the hydrocarbon utilization by microorganisms in a two-liquid phase system.

The organic phase should generally meet several criteria such as being biocompatible, resistant to biodegradation, inexpensive, high affinity for the target compound, and low emulsification. Also, various organic phases used in this field were mentioned in the literature [23]. Wang et al. [24] reported that the naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, and pyrene at low concentrations were completely biodegraded by two-liquid phase systems using silicone oil in 4-50 days. Mahanty et al. [25] used a two-phase partitioning bioreactor using silicone oil for the degradation of pyrene by Mycobacterium frederiksbergense, and they observed the effective degradation at high concentrations. Also, it was shown that a high concentration of pentachlorophenol (PCP) was effectively removed by *Sphingobium chlorophenolicum* DSM 8671 using Dioctyl sebacate (synonym BES) [26]. MacLeod and Daugulis [27] stated that BES has superior chemical properties and low cost compared to other solvents examined for the study. They reported that using a two-liquid phase system with BES, 1 g of phenanthrene and 1 g of pyrene were completely degraded by *Mycobacterium* PYR-1a within 4 days at rates of 168 mg/L and 138 mg/L per day, respectively.

It was mentioned in a study that two-liquid phase systems are a promising approach for the removal of phenolic compounds and other xenobiotics, and they mainly facilitate substrate biodegradation by reducing substrate toxicity [28]. The data from this study show that the organic phase can reduce the long-term toxic effects of high hydrocarbon concentrations, and support the maintenance of the degradation process. On the other hand, it was mostly studied with high agitation values in the literature. Results indicate that the reason for prolonged degradation time may be the application of lower agitation rates and high levels of mixed PAHs.

In conclusion, metabolic interactions among consortium members may result in further degradation. Besides, an additional organic phase can boost the degradation rates. Nutrient contents of fermentation liquid may affect the stability of hydrocarbon solubility by regulating the behavior of microorganisms in harsh environments. From the findings of this study, it can be stated that two-liquid phase systems are promising approaches for the efficient removal of toxic chemicals. Also, these systems can be developed with diverse chemical and bioreactor technologies.

Acknowledgment

I would like to thank Tuğba Topbaş who helped with the study at some stages, and Prof. Dr. Güven Özdemir for providing the laboratory facilities.

Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

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