

Enhance Pyrene Degradation of Sodium Alginate Embedding Immobilization by Adding PAC

Zhaoyang You, Haiyang Xu, Kinjal Jainikkumar Shah*, Jiaqing Tao, Dongjian Cai

College of Urban Construction, Nanjing Tech University, Nanjing, 211800, China

kjshah@njtech.edu.cn

This study explored the development of immobilized microorganism carriers for pyrene degradation. Two bacterial strains *Klebsiella pneumoniae* (Kp) and *Pseudomonas aeruginosa* (Pa), along with bacterial consortium were cultured for 14 d. Powdered activated carbon (PAC), binder CaCl_2 , and sodium alginate (SA) were used as beads to immobilize the bacterial consortium for improving the degradation effects of the pyrene. Removal rate, mass transfer properties, embedding ratio, crosslinking time, pH, and temperature effects on Pyrene removal efficiency were studied for the immobilized particles. The obtained results indicate that the removal rate of pyrene was reached up to 91.6 %, which improved 89.26 % with compared to the control conditions. Scanning electron microscopy (SEM) characterization showed that the PAC has increased more porous structure in the immobilization SA beads, which was responsible for maintaining bacterial activity and improving mass transferability. PAC added SA immobilization can enhance the effect of pyrene degradation by improving the bacterial absorption ability and nutrient permeability.

1. Introduction

Pyrene is a toxic organic pollutant with a complex chemical structure and stable properties (Chirwa and Lutsinge, 2008). It accrues in large quantities in the environment (i.e. in soil) and poses a great threat to human health due to their low water solubility and difficult biodegradable in nature. Physical and chemical remediation for pyrene contaminated soil can effectively remove pollutants but will lead to many other problems such as high cost, soil disturbance, secondary pollution, and so on. Microbial immobilization technology is an important way of microbial reinforcement, which can effectively improve the quality of contaminated soil. It uses carrier materials to immobilize microorganisms to maintain high microbial biomass and oxidation activity, shield the competition of indigenous microorganisms and the invasion of adverse soil environment, and improve the degradation effect of microorganisms on environmental pollutants such as pyrene (Gan et al., 2009). It has the advantages of a large number of microorganisms in the carrier, high activity of microorganisms, good environmental tolerance, and high treatment efficiency (Ijoma et al., 2019). It has an important impact on bioremediation technology about temperature, pH, oxygen, nutrients, and other environmental conditions. Microbial immobilization technology can not only improve degradation efficiency of microorganisms, accelerate degradation reaction rate, but also enhance the stability of bacteria and the ability to resist harsh environment. For example, the immobilized SA beads with *Bacillus cereus* sp. for degrading the phenanthrene in Cd (II) wastewater increased the protective sites on the beads surface and the protective ability of beads, improving the degradation rate of phenanthrene (Liu et al., 2019). After immobilized *Bacillus cereus* sp. with SA, the degradation rate of petroleum hydrocarbon increased significantly, and the degradation rate of the immobilized strains increased to above 78 %, but degradation rate of free strains was 33 % (Bao et al., 2013). The choice of the immobilized carrier is one of the key techniques in microbial immobilization technology. The carrier material of immobilized should be characterized by good mass transfer, large surface area, high mechanical strength, stable performance, and decomposition resistance. Inorganic carrier materials such as coke, kaolin, and diatomite have the characteristics of high strength, resistance to decomposition, and low cost. On the other hand, agar, alginate, and other natural polymer materials have good mass transfer and low cost but are low strength and easy to anaerobic decomposition. Carriers of polyacrylamide, polyvinyl alcohol,

polystyrene, and other synthetic organic polymer have higher strength but poor mass transfer and cell inactivation (Tanaka H et al., 1984). In that case, PAC is the most appropriate material of choice considering higher strength and higher mass transfer. Similar to material choice, the selection of the immobilization methods is an important factor affecting the activity of microorganisms in immobilization beads. The crosslinking method, an adsorption method, embedding method, and composite fixation method are often used in immobilization. The adsorption method is simple and has little effect on the activity of microbial cells.

In recent years, the degradation rate of pyrene was increased from 19.7 % to 62.1 % while biochar immobilizing *Mycobacterium gilvum* and then degrading polycyclic aromatic hydrocarbons (PAHs) (Xiong et al., 2017). The immobilized biomass is greatly affected by the characteristics of carrier materials. The embedding method can immobilize the high concentration microorganisms, and improve crosslinking degree between microorganisms and the carrier but reduce the activation rate of microorganisms (Muyima and Cloete, 1995). Nanomaterials were also added to calcium alginate to enhance the degradation of anthracene with removal rate of 88.86 %. Although gel embedding method can effectively improve the microbial biomass of immobilization, the mass transfer between the embedded microorganisms and the extra bell pollutants is inhibited (Wang et al., 2014).

In this study, PAC was used to modify the structure of SA immobilization beads to improve the mass transfer and increase degradation effect of pyrene in soil. The ratio of PAC, gel, and crosslinking agent as well as the immobilized operating conditions are described in this work. The applicability of this research in practice has 3E benefits i.e. conservation of energy, sustainability to environment and economic aspects, which lead to be part of modern circular economy for bio materials to waste minimization and green chemistry aspects.

2. Materials and methods

2.1 Chemicals and culture medium

Luria-Broth or Bertani (LB) medium (g/L) was made by adding 5 g Beef extraction, 10 g peptone, and 5 g NaCl into 1 L deionized water with maintaining pH 7.2. The prepared medium was used for activation, enrichment, and preservation of the strains before degradation experiments. Mineral salt medium (MSN) (g/L) was prepared by adding 0.25 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, 2 g $NaNO_3$, 10 g $K_2HPO_4 \cdot 3H_2O$, 4 g KH_2PO_4 , and 5 g NaCl into 1 L of deionized water with maintaining pH 7.2 ± 0.2 . Pyrene-MSN (g/L) was prepared by dissolving 1 mL of the pyrene-acetone solution to a conical flask and volatilize by heat to remove acetone, then add 50 mL MSN medium at room temperature. It needs adding 15 ~ 20 g of agar in it to make a solid medium. All the organic solvents and inorganic chemicals used in this study were HPLC grade and analytical or higher grade.

2.2 Strains and culture

Two strains of pyrene degradation bacteria Kp and Pa were screened by our laboratory as per previously reported work (You et al., 2018). The strain was cultured in a conical flask with LB medium at 180 rpm and 30 °C on a shaking table until optical density (OD) OD_{600} (UV-5500 UV spectrophotometer, Shanghai) was 1.0 for later use.

2.3 Bacterial strain degradation and PAC adsorption

Bacterial strain degradation and PAC adsorption were determined by the OD values measured at wavelength 600 nm by UV spectrophotometer (You et al., 2018). The strain of Kp, Pa, and consortium Kp + Pa (1:1) of 1 ml 2 % to pyrene-MSN medium were centrifuged at 180 rpm, and 30 °C. Take 10 mL bacterial suspension, centrifuge it at 2000 rpm for 10 min, discard the supernatant, and use phosphate-buffered saline (PBS) buffer to adjust the substratum into bacterial solution $OD_{600} = 2.0$. Then, PAC of 0.1 g was added and adsorbed dynamically in a 25 °C shaker for 1 h. Calculate the adsorption amount of the bacterial suspension by measuring its OD_{600} values after 5 min of settling time. Repeat it for 3 times and get the average value.

2.4 PAC-SA immobilization

The PAC-SA immobilization beads were prepared by mixture of PAC (0.5 %, 1 %, and 1.5 %), SA (2 %, 2.5 %, and 3 %) and $CaCl_2$ (2 %, 3 %, 4 %), and the optimal ratio was obtained by comparing the degradation performance. During procedure, PAC was added to 10 ml 6 % bacterial suspension beaker (50 mL) and adsorbed for 3 h, then 2.5 % gel was added in and mixed with magnetic agitator. Transfer the mixture to a 10 mL syringe and let it drip freely into the 2 % $CaCl_2$ (pH 7.5) cross linker and stir it for 24 h to prevent the mixture from clumps. The beads were washed 3 times with 0.9 % normal saline and stored at 4 °C for later use. The effects of operating conditions of immobilization was investigated by changing crosslinking temperature (4, 10, 15, 20, 25, and 30 °C), crosslinking time (6, 12, 18, 24, and 30 h), pH (5, 6, 7, 7.5 and 8) and concentration of bacterial solution (0.2 %, 4 %, 6 %, 8 %, 10 %). The removal rate was determined by activating 5 g immobilization beads in LB medium for 8 h, and then placed in 50 mL pyrene-MSN medium at 30 °C and 180 rpm in a shaker

for 7 d. The mass transfer was determined by measuring luminosity of the solution at 665 nm for 50 beads in 20 mL methylene blue solution for every 10 min of measurement.

2.5 SEM

The SEM analysis was used to analyze the surface properties. The sample beads were pretreated by washing it in 0.9 % normal saline, and then soaked it in a 4 % formaldehyde solution at 4 °C for 12 h, then, rinsed it with normal saline for 3 times. The obtained rinsed samples were dehydrated in ethanol solutions with concentrations of 30 %, 50 %, 70 %, and 90 % for 30 min. The samples were then soaked in anhydrous ethanol for dehydration twice, each time for 15 min, and then soaked it in tert-butanol for 3 times for 15 min. The samples were then frozen at (-20 °C) and for 12 h. Finally, it was ground into powder after drying and sprayed with gold for 15 s (JMS-6510 scanning electron microscope, Japan electronics co., LTD.).

2.6 Statistical analysis

The difference in treatments was compared by one-way analysis of variance (ANOVA) with a significance level of $p < 0.05$ using the least significant difference (LSD) test in SPSS16 software. Origin 8.6 software was used in this work.

3. Results and discussion

3.1 Bacteria degrade pyrene and PAC adsorption

The degradation effect of pyrene (CK) was detected after 3 d, 7 d and 14 d, while Pa, Kp, and bacterial consortium (Pa:Kp =1:1) were added to pyrene-MSN medium (Figure 1a).

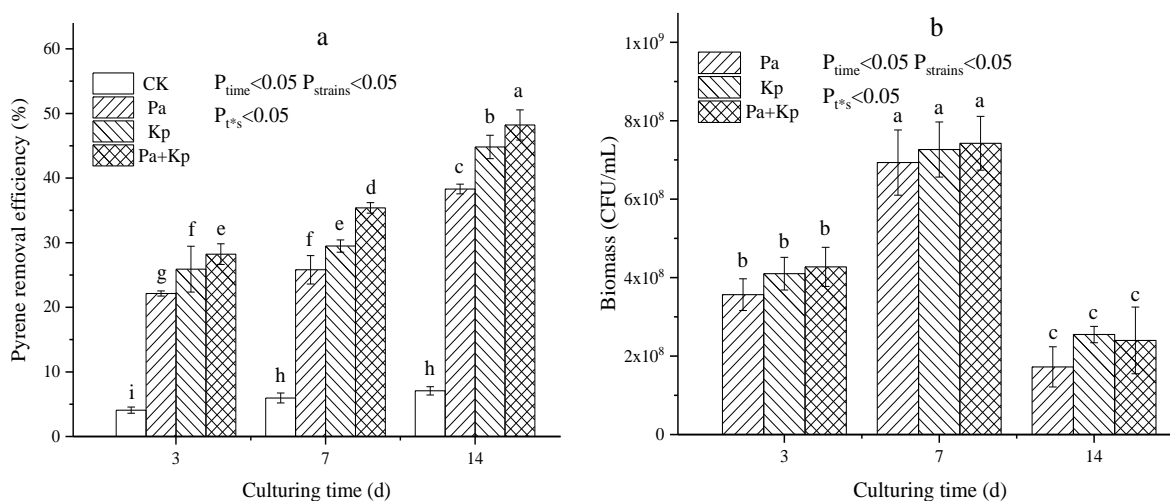


Figure 1: Degradation Efficiency of pyrene (a) and strain biomass (b)

In 14 d, Kp had higher degradation rates of pyrene than Pa, but bacterial consortium had the best degradation rates of 35.4 % and 48.2 % on 7 and 14 d. The number of strains increased and biomass of Pa increased from 3.6×10^8 CFU/mL to 6.9×10^8 CFU/mL on day 7 (See Figure 1b), but decreased to 1.7×10^8 CFU/mL on day 14. Although the biomass of Kp was higher than that of Pa, the trend of biomass was consistent with the degradation rate because of the limited inorganic nutrients in the medium. The nutrients are consumed and harmful metabolites accumulated by the time, which are more and more unfavorable to the growth of bacteria. Pyrene degradation efficiency was reached 41.2 %~44.3 % in the 14 d and indicated degradation effect of Kp, Pa strain is obvious. The degradation efficiency of the bacterial consortium was significantly improved. The mixed strains can use different substrates to improve the utilization range and degradation rate. Wang et al. (Wang et al., 2008) found that the degradation efficiency of bacterial consortium (*Aspergillus Niger* and *Fusarium sp*) for pyrene was up to 81 %, while the single *Fusarium sp* only was up to 69%. Single microorganism shows a certain degradation ability on PAHs, but the interspecific combination is better than that of single bacteria species. This indicates that when the two strains are mixed, they may have different enzyme systems or metabolic that work together to increase pyrene removal efficiency.

Table 1: Mass transfer properties of beads with different proportions after 60 min (SA %+ CaCl₂ %+ PAC %)

SA (%)	CaCl ₂ (%)	PAC (%)	Mass Transfer (%)	SA (%)	CaCl ₂ (%)	PAC (%)	Mass Transfer (%)	SA (%)	CaCl ₂ (%)	PAC (%)	Mass Transfer (%)
2	2	0.5	3.5	2.5	2	0.5	11.2	3	2	0.5	1.8
2	2	1.0	7.1	2.5	2	1.0	11.0	3	2	1.0	6.0
2	2	1.5	10.0	2.5	2	1.5	10.6	3	2	1.5	4.8
2	3	0.5	8.3	2.5	3	0.5	4.3	3	3	0.5	4.1
2	3	1.0	10.1	2.5	3	1.0	6.02	3	3	1.0	4.1
2	3	1.5	5.36	2.5	3	1.5	8.00	3	3	1.5	0.6
2	4	0.5	0.00	2.5	4	0.5	1.8	3	4	0.5	0.6
2	4	1.0	0.00	2.5	4	1.0	4.9	3	4	1.0	1.2
2	4	1.5	1.18	2.5	4	1.5	7.3	3	4	1.5	3.1

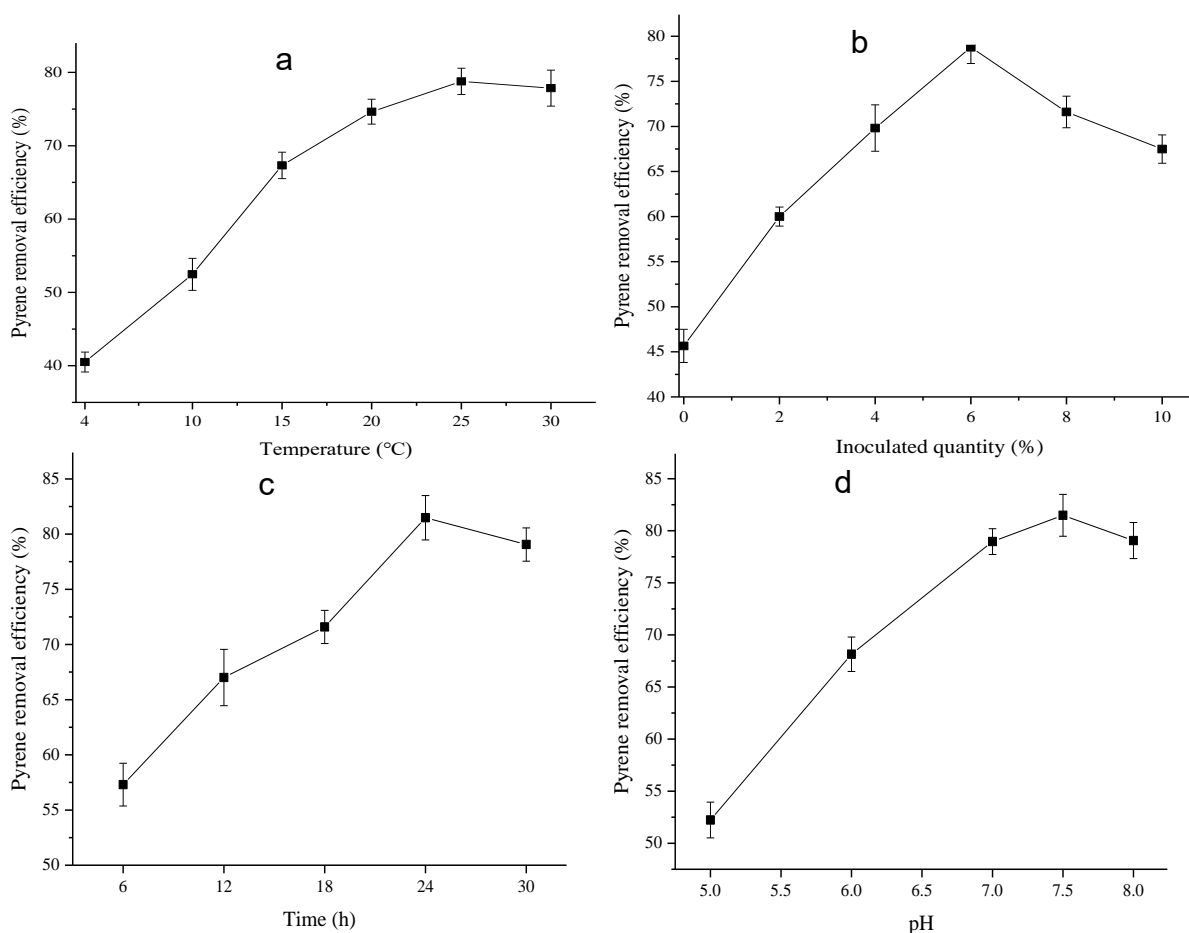


Figure 2: Effects of (a) Crosslinking temperature; (b) Inoculated quantity; (c) crosslinking time; (d) crosslinking pH on removal efficiency of pyrene

Mass transfer of 27 groups of immobilized beads with different proportions was tested within 60 min. The mass transfer of gel beads directly affects enzyme transfer and pyrene degradation of microorganisms. The mass transfer of gel beads was significantly improved after the addition of PAC, and 0.5 % PAC- beads were increased (Table 1). PAC has a strong adsorption capacity as a non-polar adsorbent. Materials with high adsorption properties can promote the migration of pyrene to the adsorption carrier, and enrich the high concentration of microorganisms, which will increase the contact between pyrene and microorganisms. The mass transfer was not improved significantly while increasing PAC proportion. Free bacteria cannot efficiently use pyrene in the soil because of the low solubility of pyrene. In SA-PAC beads, PAC plays a role of skeleton support, increase the porosity and permeability of the beads, reducing the internal diffusion resistance of organic matters. And it

can accelerate the adsorption of pyrene and dissolved oxygen and the migration to the interior of beads, also accelerate the exchange rate of substrates and oxygen of beads, which is conducive to keep the activity of strains. The mass transfer performance decreasing with the proportion of PAC increase, and beads might become an irregular shape, and might be swelling in the degradation process. This may be excessive PAC blocking the beads during crosslinking, resulting in incomplete cross-linking of SA and CaCl_2 . It was also found that 2.5 % ~ 3 % SA proportion in beads have the best effect. Similar findings were reported by Tao et al. in which, 3 % SA proportion in immobilization beads had better performance in terms of shape, strength, toughness, and permeability while using (Tao et al., 2009). A crosslinking agent of 2 % ~ 3 % proportion reached the best calcification effect, which improved the mass transfer of beads. It is concluded that the 2.5 % SA + 2 % CaCl_2 +0.5 % PAC is the best preparation proportion of beads.

Factors such as cross-linking temperature, cross-linking time, cross-linking pH and the number of embedded bacteria have important effects on pyrene removal efficiency of immobilized beads. With the increase of crosslinking temperature, removal efficiency improved continuously (Figure 2a). Temperature affects the density of cross-linking in the process of calcification of beads surface and then affects the efficiency of material exchange. The degradation rate of pyrene showed an increasing trend with increasing cross-linking temperature, and removal efficiency reached to its optimum at 25 °C. Figure 2b shows that the concentration of bacteria had a certain influence on pyrene degradation rate which increases continuously, and reaches to a maximum value of 78.8 ± 1.8 % while bacteria content 6 %. The degradation rate mainly depends on the number of bacteria adsorbed on the outside of the beads, because most of the bacteria inside are dormant or even dead. When the amount of bacteria embedded in beads is too large (this case above 6 %), a large number of bacteria will die because of the lack of space, nutrition, and dissolved oxygen. Also, the high concentration of bacteria will make the bead pores blocked, which affected the material transfer in and out. The removal efficiency of immobilization beads improved with the increase of crosslinking time (Figure 2c). The removal efficiency began to drop after crosslinking time 24 h. It may be that the immobilization beads become denser when the crosslinking time is too long. The SA gel might achieve complete crosslinking with too long crosslinking time. The removal efficiency of pyrene improved with the increase of cross-linking pH (figure 2d), reaching a maximum value of 81.5 ± 2.0 % while pH being 7.5. The effect of removal efficiency is mainly concentrated on the biological activity of bacteria because the optimal pH range of the two bacteria is between 7 and 8 (You et al., 2018). The immobilized strain grew best in the neutral and slightly alkaline environment, and the removal efficiency of beads reached the best. In summary, from the perspective of the pyrene removal rate, the best crosslinking temperature is 25 °C, the best bacterial content is 6 %, the best crosslinking time is 24 h, and the best-crosslinking pH is 7.5.

3.2 Properties of immobilized beads

To reveal the immobilization effect of beads, the internal structure and distribution characteristics of beads were observed and analysed by SEM. SA-PAC beads (2.5 % SA+2 % CaCl_2 +0.5 % AC, 6 % bacterial tolerance, 25 °C cross-linking for 24 h, pH 7.5) and no strains SA-PAC beads were selected for characterization.

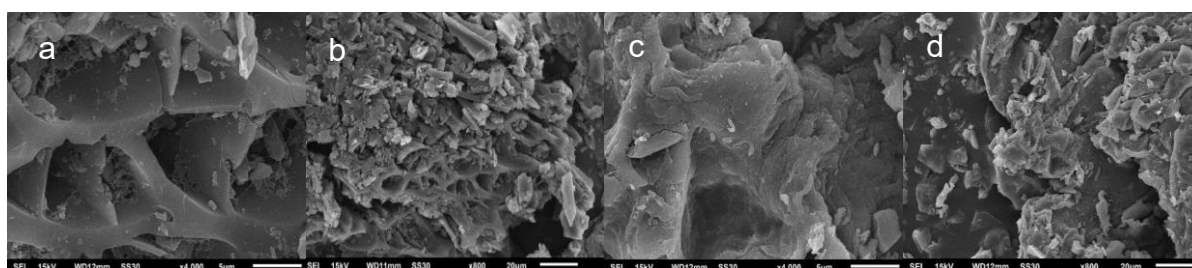


Figure 3: SEM of SA-AC beads in control at 4000X (a) and 800 X (b) and SA-AC immobilization beads at 4000 X (c) and 800 X (d)

Figure 3a and Figure 3b show the SEM image of SA-PAC after crushing and compaction. It can be seen that the bead has an obvious irregular network structure. It has more but small sparse pores on the surface of immobilization bead and dense and larger pore inside the bead. The mesh structure of the bead, which is sparsely outside and densely inside, is helpful for the internal strain to effectively obtain nutrients and dissolved oxygen and complete the material exchange of metabolites (Wang et al., 2014). At the same time, these rich mesh has a strong adsorption capacity of pyrene. This indicates that the most of the bacteria are embedded in the pores and adhere to grow after the microorganism is immobilized by the carrier. Figure 3c and Figure 3d are the SEM diagram of SA-PAC immobilization beads. There are many gaps in the cross-section of beads

because PAC can provide many tiny channels to the bead and act as the supporting framework of the beads which leads SA-PAC immobilization beads to have better mass transfer and strength (Wang et al., 2008). More bacteria were distributed on the surface of the beads. This is because PAC enhanced the permeability of the carrier, promote the exchange of nutrients and dissolved oxygen, more conducive to the bacteria activity. At the same time, PAC has a strong adsorption capacity, which can double adsorption of the bacteria and substrates, fixing more bacteria, and improving the utilization rate of the bacteria substrates. Bacterial strains are mainly adsorbed on the surface of the material, mainly through the adsorption of pyrene active contact with bacteria to play a role. The results of SEM showed that the internal space of the carrier was large, which provided a large space for microbial growth. The distribution of bacteria on the immobilization beads was not uniform, and the bacteria were concentrated on the outer surface of the beads while the interior was relatively sparse.

4. Conclusions

The choice of the immobilized carrier is one of the key techniques in microbial immobilization technology. PAC was used to immobilize the bacterial consortium Kp, Pa, and Kp+Pa with SA for improving the degradation effect of the pyrene. Pa, Kp, and the bacterial consortium were cultured in pyrene -MSN medium for 14 d, the bacterial consortium had better degradation effect than a single strain. The optimum proportion of SA, CaCl₂ and PAC were 2.5 %, 2 % and 0.5 % for immobilization SA beads. The removal rate of pyrene was observed to be 91.6 % with the condition of PAC adsorption time 3 h, bacterial inoculation load (v:v) 6 %, crosslinking temperature 25 °C, pH 7.5, crosslinking time 24 h. SEM characterization showed that immobilization beads had developed porous structure, which was beneficial to maintain bacterial activity and improve mass transfer. It could be concluded that PAC plays an essential role in increasing the porosity and permeability of the beads through which minimizing internal diffusion resistance and provides sites for pyrene adsorption.

References

- Bao M., Chen Q., Gong Y., Li Y., Wang H., Jiang G., 2013, Removal efficiency of heavy oil by free and immobilised microorganisms on laboratory-scale, *The Canadian Journal of Chemical Engineering*, 91, 1-8.
- Chirwa E.M. N., Lutsinge, T. B., 2008, Biological degradation of pyrene and fluoranthene in porous media and attached growth systems by biosurfactant producing bacteria, *Chemical Engineering Transactions*, 70, 1777-1782.
- Gan S., Lau E.V., Ng H.K., 2009, Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs), *Journal of Hazardous Materials*, 172, 532-549.
- Ijoma G.N., Selvarajan R., Tekere M., 2019, The potential of fungal co-cultures as biological inducers for increased ligninolytic enzymes on agricultural residues, *International Journal of Environmental Science and Technology*, 16, 305-324.
- Liu S. H., Zeng Z.T., Niu, Q.Y., Xiao, R., Zeng, G.M., Liu Y., Cheng M., Hu K., Jiang L.H., Tan X.F., Tao J.J., 2019, Influence of immobilization on phenanthrene degradation by *Bacillus* sp. P1 in the presence of Cd(II), *The Science of the total environment*, 655, 1279-1287.
- Muyima N.Y.O., Cloete T.E., 1995, Growth and phosphate uptake of immobilized *Acinetobacter* cells suspended in activated sludge mixed liquor, *Water Research*, 29, 2461-2466.
- Tanaka H., Matsumura M., Veliky I. A., 1984, Diffusion characteristics of substrates in Ca-alginate gel beads, *Biotechnology and Bioengineering*, 26(1), 53-58.
- Tao X., Lu G., Liu J., Li T., Yang L., 2009, Rapid degradation of Phenanthrene by using *Sphingomonas* sp. GY2B immobilized in calcium alginate gel beads, *International Journal of Environmental Research and Public Health*, 6, 2470-2480.
- Wang H.Q., Hua F., Zhao Y.C., E. Al., 2014, Immobilization of *Pseudomonas* sp. DG17 onto sodium alginate – attapulgite – calcium carbonate, *Biotechnology & Biotechnological Equipment*, 28, 834-842.
- Wang X., Gong Z., Li P., Zhang L., Hu X., 2008, Degradation of Pyrene and Benzo(a)pyrene in contaminated soil by immobilized fungi, *Environmental Engineering Science*, 25, 677-684.
- Xiong B., Zhang Y., Yanwei Houc., Arp H.P.H., Reid B.J., Cai C., 2017, Enhanced biodegradation of PAHs in historically contaminated soil by *M. gilvum* inoculated biochar, *Chemosphere*, 182, 316-324.
- You Z., Xu H., Zhang S., Kim H., Chiang P., Yun W., Zhang L., He M., 2018, Comparison of Petroleum hydrocarbons degradation by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, *Applied Sciences*, 8, 2551 (1-19).