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Application of cold-adaptive Pseudomonas sp. SDR4 and Mortierella alpina JDR7 co-immobilized on maize cob in remediating PAH-contaminated freeze-thawed soil

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Abbreviations, PAHs, Polycyclic aromatic hydrocarbons
Phe, Phenanthrene, Pyr, Pyrene
BaP, Benzo[a]pyrene, SDR4, Pseudomonas sp., JDR7, Mortierella alpine
SEM7, 7Scanning electronic microscope
TPH, Total petroleum hydrocarbons
TOC, Total organic carbon
Total nitrogen
TN, Total phosphorus
TP, Maximum water holding capacity, WHC, Maize cob
MC, High-performance liquid chromatography
HPLC, HMW, High molecular weight
LMW, Low molecular weight

ABSTRACT

There are large areas of contaminated soils with low- and medium-concentration of polycyclic aromatic hydrocarbons (PAHs) in the coldest regions of the earth, potentially threatening ecological safety and human health. Using maize cobs (MC) as the carrier, two cold-adaptive PAHs-degrading microorganisms, bacterial (*Pseudomonas* sp., SDR4) and fungal (*Mortierella alpine*, JDR7) strains were co-immobilized. The degradation characteristics of phenanthrene (Phe), pyrene (Pyr) and benzo[a]pyrene (BaP) in freeze-thawed soil by both co-immobilized and free microorganism were studied. The removal rate of Phe, Pyr and BaP using the co-immobilized fungal-bacterial consortium within 60 d was 59.2±3.7%, 46.6±3.3%, and 36.8±2.7%, respectively, which was obviously higher than that of free fungal-bacterial consortium in the same time. Ten percent (w/w) was the optimal amount of inoculum for PAH degradation in the co-immobilized fungal-bacterial consortium. Under low-temperature conditions, when the initial concentration of PAH was between 10-100 mg•kg^{-1}, the immobilized cold-adaptive fungal-bacterial consortium displayed the desired degradation of PAHs. The scanning electronic microscope (SEM) observation and mass transmission showed that the microstructure of co-immobilized mixed system was beneficial to the growth of SDR4 and JDR7 at low temperature. These results imply that the cold-adaptive fungal-bacterial consortium, co-immobilized on MC, has the potential for application in remediating PAH contaminated soil under the freeze-thawing environment.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a class of chemical compounds with two or more benzene rings that are common organic pollutants and ubiquitous in the environments. The detection rate has increased from less than 20% to more than 80% and the magnitude of pollution levels has risen to mg•kg^{-1} from μg•kg^{-1} in the last two decades (Jennifer et al., 2019). Approximately 20% of primary agricultural products were detected to contain PAHs above the critical levels for food safety (Bouchard et al., 1988; Bakker et al., 2000). Due to human activities PAHs compounds have been detected around the world, even in the Polar and tropics far from industrial sites (Wilcke, W., 2007). High bioaccumulation potential and phytotoxic effects of PAHs pose a threat to ecosystem stability and human health (Wu et al., 2017). Research had showed that human exposure to PAHs was greater through soil than water or atmosphere (Fismes et al., 2002). Therefore, remediation of PAH-contaminated soil is critical to ensure food safety and human health.

As one of the bioremediation technologies, the microbial remediation method has attracted continuous interests for its high efficiency, low cost, and environmental friendliness for remediating PAH-contaminated soil (Singh et al., 2017). Organisms with desired PAH degradation performance include *Mycobacterium, Haematococcus, Pseudomonas, Bacillus* and *Mortierella* (Li et al., 2005; Su, et al., 2006). Currently, microbial degradation with monomeric strains at ambient temperatures is the primary method of PAH removal. However, the diversity

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and uneven distribution of PAHs in contaminated soils, as well as low winter temperatures in the coldest regions of the earth, often lead to low efficiency, which has become a significant hurdle to the remediation of PAH-contaminated soils in cold areas. Freeze-thawing significantly affects the activities of fungi, bacteria and other microorganisms in the soil (Bölter et al., 2005; Juan et al., 2018). The microbial cell structure is changed at a relatively low temperature, resulting in low metabolism in most microorganisms (Zhang et al., 2008). How to improve the remediation efficiency of PAH-contaminated soils in cold environments has become an essential scientific issue to be addressed.

Most of the biosphere on the earth is cold (<5°C), it triggers physiological reaction(s) to ensure microorganisms survive in frozen and cold ecosystems, thereby sustaining a wide diversity of microbial life. By synthesizing more short-chain fatty acids and unsaturated fatty acids at low temperatures, cold-adaptive microorganisms (psychrophiles and psychrotolerant bacteria) can increase the fluidity of their membranes (Lipson et al., 2004). These microorganisms have cold-adaptive proteins and enzymes, which can adapt to freezing environments, decrease the freezing point of water and exhibit more excellent resistance to freezing and thawing (Hamdan, 2018). At -20°C, cold-adaptive bacteria take advantage of water molecules on the soil particle surface to remain in a solution state (Freppaz et al., 2007). Therefore, under low-temperature conditions, cold-adaptive microorganisms can still degrade PAHs in freeze-thawed soils. During the last fifteen years, a growing number of cold-adaptive microorganism with biodegradation potential for many kinds of contaminants, such as crude oil (Pham et al., 2018), TPH (Camenzuli and Freidman, 2015), petroleum oil (Wang et al., 2016), PAHs (Ruberto et al., 2005; Chronopoulou et al., 2015; Su, et al., 2017), have been isolated and have been used in the bioremediation of contaminated sites. The cold-adaptive microorganisms with potential application for bioremediation mostly include *Roseovarium, Halomoans, Glaciecola, Phodococcus, Rhodococcus, Arthrobacter, Pimelobacter*, etc. (Aislabie et al., 2006; Chaudhary and Kim, 2019). However, compared with the use of mesophilic microorganisms, this approach of introducing potential cold-adaptive microorganisms into the contaminated areas is still in its infancy.

Studies showed that fungal-bacterial mixed cultures could improve the degradation efficiency of PAHs in contaminated soils (Hays et al., 2015). Mixed cultures had a more remarkable degrading ability than single strains of bacteria, while fungi are generally more effective in PAH degradation than bacteria (Qian et al., 2012). When high molecular weight (HMW) PAHs like Pyr and BaP, is present as a sole source of carbon and energy, the effect of mixed culture is higher than that of a single strain, and the total biomass of the bacteria is also larger (Stanley et al., 2000). Furthermore, extracellular enzymes secreted by fungi, such as catalase, can hydrolyze a variety of complex PAHs and exert a ring-opening effect on the benzene ring (Juhasz et al., 2000). Bacteria can decompose these compounds into CO_2 and water (Singh and Chaudhary, 2015). Therefore, the use of high efficient cold-adaptive PAH degrading fungal-bacterial co-cultures that are resistant to freezing and thawing is a promising approach for improving the bioremediation efficiency of contaminated soils in the cold regions.

In cold environments, biotic factors (interspecies competition between exogenous microorganisms and indigenous) and abiotic factors (contaminant characteristics, moisture, pH, temperature, nutrient contents, organic matter and aeration) should be considered in selecting suitable cold-adaptive microbial strains (Vogel, 1996). Free microorganisms have a disadvantage over indigenous bacteria in adapting to the new environment. Arulazhangan (2011) and Debajyoti (2016) reported that the use of immobilization technology to remediate PAH-contaminated soils could enhance the competition of introduced microorganisms with indigenous species, provide protection from toxic compounds, extremes of pH and predation in the contaminated soils. According to Su et al. (2008), the *Mucor* sp. immobilized on maize cobs (MC) had a faster start-up speed of reaction and greater environmental resilience, thus performed a higher degradation rate of BaP from contaminated soils (Su et al., 2008). Studies have shown that the MC as a carrier have a very high affinity and capacity for sorbing organic contaminants and encapsulating the strains since it has a large surface area and high microporosity, which is advantageous in shielding against the interference of external indigenous bacteria, making it fully exposed to pollutants and promoting the degradation of PAHs (Song et al., 2013). Studies have also reported that microbial activity could be stimulated by the elemental nutrients in MC, thereby enhancing the biodegradation of pollutants such as PAHs in soil (Reid et al., 2000).

In this study, two cold-adaptive PAHs-degrading microorganisms, *Pseudomonas* sp. SDR4 and *Mortierella alpine* JDR7 were co-immobilized on MC with the objective to determine the optimal condition parameters (i.e., inoculum amount, temperature, initial PAH concentration) of bacterial-fungal symbiotic system for enhancing the remediation of freeze-thawed soil contaminated with PAHs. We also evaluated the performance of the co-immobilized cold-adaptive fungal-bacterial consortium when compared with that of free counterparts. Besides, the mass transmission processes and morphology of the co-immobilized fungal-bacterial consortium were examined. The results can provide theoretical reference for in-situ microbial remediation of PAH-contaminated freeze-thawed soils in low-temperature environments.

1. Materials and methods

1.1. Materials

MC was collected from the National Field Research Station of Shenyang Agroecosystem. PAHs-degrading fungal-bacterial were consisted of two cold-adaptive indigenous strains, a bacterial *Pseudomonas* sp. SDR4 and a fungal *Mortierella alpine* JDR7. SDR4 were isolated from freeze-thawed soil of heavily contaminated farmland in upstream of Shenfu Irrigated Areas (SIA, southeast of Shenyang, Liaoning Province, China), JDR7 were isolated from freeze-thawed soil of the Jixi Coking Plant (JCP, Northeast of Jixi, Heilongjiang Province, China) according to the method of enrichment and isolation of degrading strains by a modification of Rajendran (2017) and Zeng (2010). The degrading capability of SDR4 and JDR7 were maintained in a mineral plate where phenanthrene (Phe), pyrene (Pyr) and benzo[a]pyrene (BaP) were added as sole carbon and energy source, and they were cultured according to Hu et al (2011).

The soil was collected from the topsoil (0-10 cm) of Shenyang Ecological Station, with the following characteristics: pH 6.72, silt 50.8%, and 29.0%, 20.2% clay, total organic carbon (TOC) 17.8%, total phosphorus (TP) 0.35 g•kg^{-1}, total nitrogen (TN) 1.1 g•kg^{-1} and cation exchange capacity (CEC) 45.04 g•kg^{-1}. No PAHs detected in the soil. Before use, the soil was air-dried and sieved (2-mm), while stored a portion of fresh soil at 4°C.

Phenanthrene (Phe) with a purity of 95%, Pyrene (Pyr) with a purity of 98% and Benzo[a]pyrene (BaP) with a purity of 96% were bought from Beijing BL Technology Co., Ltd. Acetone and N-hexane (AR) were purchased from Shenyang Economic and Technological Development Zone Reagent Factory. Dichloromethane (AR) (Tianjin Fine Chemical Co., Ltd), and 60~100-mesh silica gel (Sinopharm Chemical Reagent Co., Ltd) were all used in the present study.

1.2. Experimental methods

1.2.1. Microorganism cultivation

Inorganic salt selection medium used for isolation of PAH-bacteria contained K_2HPO_4 1.0 g, $(NH_4)_2SO_4$ 5 g and $MgSO_4$•$7H_2O$ 0.5 g, and distilled water 1000 mL. The pH of the medium was adjusted to 7.0-7.2 and the medium was serialized at 121°C for 25min before use. The isolation of PAH-degrading bacteria was conducted in inorganic salt selection liquid medium with a mixture of Phe (10-30 mg•L^{-1}), Pyr (10-30 mg•L^{-1}) and BaP (10-30 mg•L^{-1}), Utilization of PAH as a sole source

of carbon and energy was performed by adding PAH in acetone solution to empty sterile 250 ml flasks, followed by evaporation of acetone with gentle shaking. Each flask was added with 25 ml of inorganic salt selection medium to give a concentration of 10 mg•L^{-1} of PAH (2.5 mg PAH/0.25 mL acetone solution for 10 mg•L^{-1}, 5.0 mg PAH/0.50 ml acetone solution for 20 mg•L^{-1} and 7.5 mg PAH/0.75 ml acetone solution for 30 mg•L^{-1}), and placed in an ultra-clean table for ultraviolet sterilization, and then used for screening and enrichment of strains.

The seed medium which was used to culture the mixed consortium of bacteria and fungi contained beef extract 5 g, peptone 10 g glucose 10 g, yeast powder 5 g, NaCl 5 g, agar 20 g, and distilled water 1000 mL, with pH 7.1-7.2, sterilized at 121°C for 20 min.

The proliferation medium which was used for multiplication of co-immobilized consortium contained sugar 4 g, yeast extract 3 g, KH_2PO_4 0.5 g, $(NH_4)_2HPO_4$ 2 g, $MgSO_4•H_2O$ 0.25 g, and distilled water 1000 mL, with pH 6.0-6.5, sterilized at 121°C for 20 min.

1.2.2. MC pretreatment and microbial immobilization

The dried MC were crushed to particles with diameter 1.5-2.0 cm and were chosen as the main microorganism co-immobilizing carrier mixed with other accessories. The carrier components include (wt, %): wheat bran 20.0%, MC 80.0%, $(NH_4)_2HPO_4$ 1.0%, $CaSO_4$ 1.0%, $MgSO_4•7H_2O$ 0.1%, 45% water.

SDR4 and JDR7 were separately inoculated to the nutrient medium and cultured on a shaker at 110 rpm and 15°C for 30-40 h. The prepared suspensions had a strain density of $6×10^8$ cfu•g^{-1}. SDR4 and JDR7 were inoculated in a nutrient medium at the volume ratio of 1:1. They were mixed and cultured for three days under the same conditions to obtain the mixing microbial agent.

Portions of 10 g MC carrier were weighed and infiltrated with immobilizing proliferation medium for 12 h. Composited bacterial agent was inoculated at 10% (w/w) dose and placed in an incubator at 15°C, supplemented with an appropriate amount of proliferation medium, and cultured in dark for 7 days. After culture, the immobilized microorganisms were rinsed with deionized water several times to obtain co-immobilized fungal-bacterial consortium.

1.2.3. Experimental design

The target PAHs, Phe, Pyr, and BaP were formulated by acetone and kept at -20°C until use. The prepared contaminated soil was frozen at -20°C for 24 h and then defrosted at 5°C for 24 h, with each freeze-thaw cycle for 48 h. After 15 freeze-thaw cycles, an aged 30-day freeze-thaw contaminated soil was prepared. Each bottle was filled with 4 g of sieved (2 mm) sterilized or non-sterilized freeze-thaw diluted soil samples, which were spiked with Phe, Pyr, and BaP. Co-immobilized SDR4 and JDR7 were then added to the soil samples. The ratio of bacteria to fungi was 1:1 in the fungal-bacterial consortium. The bacterial density was $6×10^8$ mL^{-1}. The experimental fungal agent was a spore suspension with a spore density of $6×10^8$ $mL^{-}1$.

In order to obtain the optimal condition parameters for the immobilized fungal-bacterial symbiotic system, co-immobilized cells were used to remove Phe, Pyr, and BaP in sterilized soil samples under different environmental conditions (i.e., inoculum amount, temperature, initial PAH concentration). The impact factor design groups included the following: (a) varied inoculum amounts (0%, 5%, 10%, 15% and 20%, w/w), with an initial PAH concentration of 30 mg•kg^{-1}, at 20% (w/w) of maximum water holding capacity (WHC), and a freeze-thaw processing mode of 10°C (thaw at 10°C for 2 h, frozen at -20°C for 12 h); (b) different freeze-thaw processing modes -20°C (thawed at 5°C for 12 h, frozen at -20°C for 12 h), -10°C (thawed at 5°C for 12 h, frozen at -10°C for 12 h), 0°C (thaw at 5°C for 12 h, frozen at 0°C for 12 h), 10°C (thaw at 10°C for 12 h, frozen at -20°C for 12 h), and 25°C (thaw at 25°C for 12 h, frozen at 10°C for 12 h), with inoculum amount of 10% (w/w), with an initial PAH concentration of 30 mg•kg^{-1}, with 20% of WHC; and (c) varied initial PAH concentrations (10, 20, 30, 50, and 100 mg•kg^{-1}), at 20% (w/w) of WHC, inoculum amount of 10% (w/w)and a freeze-thaw processing mode of 10°C (thaw at 10°C for 12 h, frozen at -20°C for 12 h). The experiment for each treatment was designed with three replications.

Then the non-sterilized soil samples with an initial PAH concentration of 30 mg•kg^{-1}, inoculum amount of 10% (w/w), and a 20% (w/w) of WHC, a temperature of 10°C was used to investigate the degradation performance of introduced fungal-bacterial consortium in the presence of indigenous species.. The stability of PAHs spiked in the soil without MC was monitored (designated as control). Another control medium without microorganisms (sterilized MC only) was also prepared and used to account for any change of the compound concentration caused by adsorption rather than biodegradation by the fungal-bacterial consortium being studied (designated as MC).

Samples were incubated in a darkened freeze-thaw incubator for the duration of the experiment. Based on weight loss, added sterilized water to maintain the WHC every three days. Subsamples were taken at intervals of 0, 15, 30, 45, and 60 d after incubation. The collected samples before analysis for the residual concentration and removal rate of PAHs were stored at 4°C.

1.3. Determination method

The extraction and measurement of PAHs is based on the method of Song et al. (1995). The WHC in soil was determined based on the method of Veihmeyer and Hendrickson (1949) with some modifications following Peake et al. (2014). The functional groups of MC were identified using an FTIR analysis (Guibal et al., 1995). To determine the mass transmission processes and morphology of the co-immobilized fungal-bacterial consortium, SDR4 and JDR7 co-immobilized on MC were fixed in 2.5% glutaraldehyde in 0.1 mol•L^{-1} phosphate buffer for 3 h, rinsed twice for 10-15 min each in phosphate buffer and post-fixed in 1% osmium tetroxide buffer for 1-2 h at 4°C. The MC was then washed twice in phosphate buffer for 10-15 min each at room temperature and dehydrated with a series of ethanol (30%, 50%, 70%, 80%, 90% and 100%) at room temperature. The samples were dried using the CO_2 critical-point technique (Hitachi Critical Point Dryer-HCP2, Japan) and sputter-coated with gold (Eiko IB•5 Ion Coater, Japan). Observations were performed using a SEM (Hitachi SDR4800) operated at 5kV (Jastrzebska et al., 2016).

1.4. Statistical analysis

The degradation rate of PAHs by microorganisms was calculated by the following equation:

Degradation rate=(total amount-natural loss–residual amount of PAHs in soil degraded by free or immobilized mixed strains)/total amount×100%.

All the data obtained in the study were subjected to statistical analysis of two ways ANOVA, and post hoc Turkey test with SPSS Version 18.0. Statistical charts were conducted using the software of Origin Pro 8.0. The error bars represent standard deviation, calculated from triplicate data.

2. Results and discussion

2.1. Effect of initial inoculum amounts

The initial amount effect of co-immobilized fungal-bacterial consortium on degradation of Phe, Pyr, and BaP is shown in Figure 1. When the inoculation amount was 10%, the removal efficiency of Phe, Pyr and BaP reached the highest which were 59.5±3.3%, 51.9±4.1%, and 37.3±3.9%, respectively ($P<0.05$). Above 10% (w/w) inoculation the removal efficiency of PAHs did not significantly improve and below 10% (w/w) the removal efficiency dropped markedly (Figure 1). As for Phe and BaP, in Pyr 15% (w/w) inoculation was no better than 10%, and

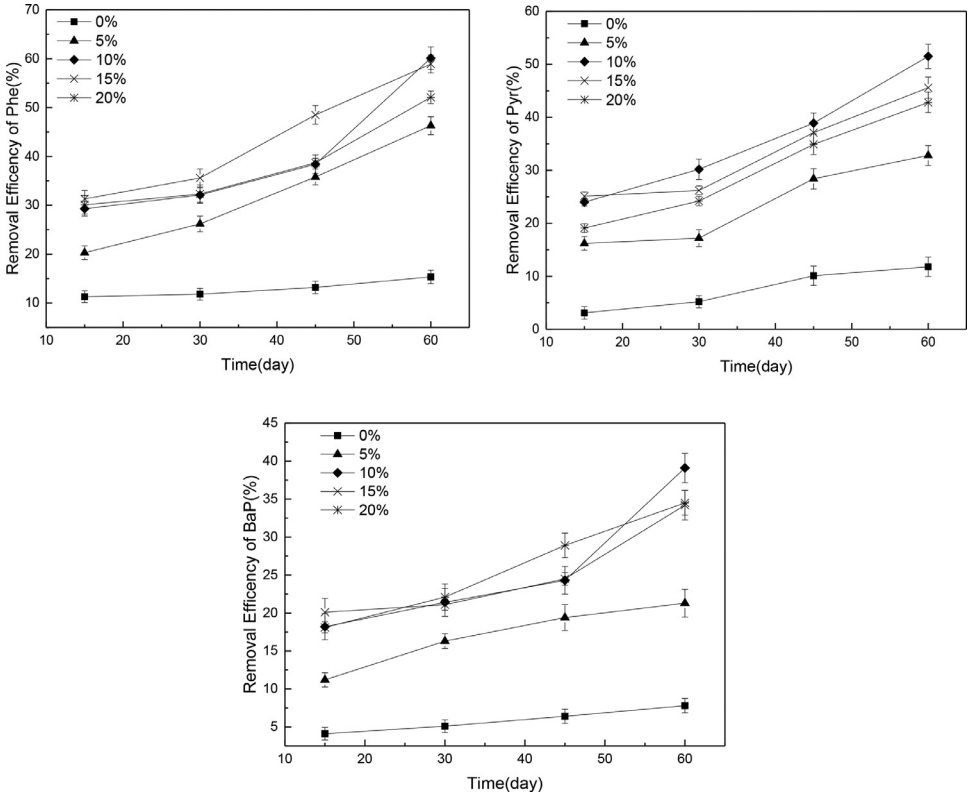

Fig. 1. Removal efficiency of Phe, Pyr and BaP by various amounts of inoculated co-immobilized SDR4 and JDR7 within 60 day. The concentrations of co-immobilized microbes are given in weight percent (wt %). Values represent the mean of three replicates; bars indicate standard deviation.

therefore the latter was considered optimal in terms of cost. At 20% (w/w) inoculum, the removal efficiency of Phe, Pyr, and BaP decreased significantly ($P<0.05$). Thus, the optimal inoculum amount of the co-immobilized fungal-bacterial consortium was 10% (w/w).

When the inoculation was too low, the immobilized cells had no obvious effect on the degradation of Phe, Pyr, and BaP, which may be due to the toxic effect of PAH on both fungal and bacterial strains. In this case, the cell survival rate is low, and the small number of surviving microbes might also die from the depletion of nutrients (Genisheva et al., 2011). Consequently, the tolerance of the low-temperature Nthe cells to degrade PAHs were significantly increased (Singh et al., 2015). With further increase in inoculum size, the relative ratio between PAHs and degrading strains were not proportional, and the chance of strains in contact with PAHs was reduced. Excessive organism inoculation also resulted in an insufficient supply of soil nutrients and the competition among the PAH-degrading strains due to limited living space and nutrients (Meena et al., 2017; Ajent et al., 2020). This competition ultimately caused removal rate of PAHs to decrease (Magesin et al., 2001). Besides, some more toxic intermediate products generated during the degradation process may hinder the growth of the degraders, thus resulting in a decrease in the removal rate (Sivaram et al., 2018; Harmsen et al., 2019).

2.2. *Effect of freeze-thaw processing temperature on biodegradation*

Temperature is a very important factor to maintain microbial activity in the soil, which affects the cell membrane and intracellular enzymatic activities of microorganisms (Mikael et al., 2001; Akbari and Ghoshal, 2015). Temperature also causes changes in the characteristics of PAHs and oxygen content, indirectly affecting the degradation of PAHs in the soil (Margesin, 2000; Liu et al., 2016). These changes lead to significant differences in the degradation of organic pollutants by fungi and bacteria under different temperature conditions (Zheng and Liu, 2007).

The biodegradation performance of BaP, Pyr, and Phe by co-immobilized cold-adaptive SDR4 and JDR7 at varied freeze-thaw processing temperatures are summarized in Fig. 2. At approximately 10°C the removal of PAHs was enhanced by the co-immobilized system, with the degradation rate of 58.9±3.1%, 48.9±2.4%, and 37.1±2.9%, respectively for Phe, Pyr, and BaP ($P<0.05$) (Fig. 2). The degradation rates were reduced at higher temperatures because the optimum temperature range of the cold-adaptive microorganisms is 10-20°C. Margesin (2004) investigated that for mesophilic microorganisms, the toxicity of high concentrations of PAHs at 25°C showed lower than their at 10°C, while for cold-adapted microorganisms, the toxicity of PAHs at 10°C was significantly lower than their toxicity at 25°C. This may reflect the difference in adaptability between mesophilic and cold-adapted microorganisms.

Fig. 2 also showed that the co-immobilized system displayed remarkable degradation of LMW PAHs (Phe) at 10°C, compared with the degradation rate of the HMW PAH (Pyr and BaP) ($P < 0.05$). However, there was no significant difference in the degradation of HMW PAHs between Pyr and BaP ($P> 0.05$). At low temperatures, the degradation of Pyr and BaP were 49.5±2.4% and 37.1±2.9%, respectively, in the freeze-thawed soil (Fig. 2).

In winter, because of the low temperature, the activity of microorganisms decreased, and the efficiency of PAH degradation was reduced, especially in the colder regions of the earth where the degradation efficiency was particularly low. To cope with these challenges, the utilization of cold-adaptive PAH degraders are beneficial. There is minimal information available on the cold-adapted PAH degraders at freeze-thaw temperatures. Aislabie et al. (2000) screened old-tolerant isolates (*Pseudomonas* spp. or *Sphingomonas*) from oil-contaminated soils in cold regions, Margesin et al. (2003) isolated cold-adaptive yeast and bacterial strains from alpine habitats at 10°C, and Sartoros et al. (2005) described a strain belonging to the genus *Rhodococcus* that was able to degrade recalcitrant hydrocarbons a mixed microorganisms degrading pyrene and anthracene at 10°C and 25°C. Ruberto et al. (2005) reported the degradation of hydrocarbon by *Psychrotolerant Phodococcus* strains in

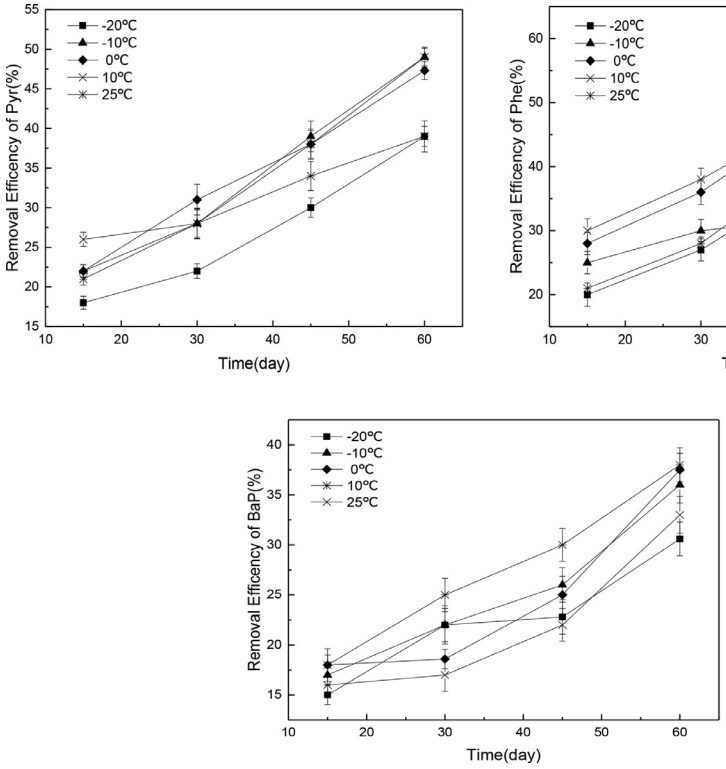

Fig. 2. Removal efficiency of Phe, Pyr and BaP by the co-immobilized SDR4 and JDR7 in different freeze-thawing processing models within 60 days. (Initial PAH concentration was 30 mg•kg^{-1}, with 20% (w/w) of WHC, inoculum amount of co-immobilized microbes was 10% (w/w), the inoculation and freeze-thaw processing modes are described in the text. Values represent the mean of three replicates, bars indicate standard deviation.)

Antarctica soil, and showed that when the temperature ranged between -2 to +10°C, the degradation of hydrocarbon in microcosms was 81.1% higher than that of in the control, suggesting that *Phodococcus* strains may play an essential role in bioremediation of organic pollutants in cold environments. Lee et al. (2010) also investigated that *Rhodococcus* was able to degrade Pyr at low temperatures. In the present study, the results showed that the co-immobilized SDR4 and JDR7 cells could function at varied freeze-thaw processing temperatures, and they could be potentially applied to the degradation of PAHs in the contaminated soils of cold northern regions.

2.3. Degradation of PAHs at different concentrations

The biodegradation performance of Phe, Pyr, and BaP by co-immobilized cold-adaptive fungal-bacterial consortium was investigated with varied initial PAH concentrations over 60 days and summarized in Fig. 3. The co-immobilized microorganisms can degrade Phe, Pyr, and BaP with an initial concentration of 10-100 mg•kg^{-1} (Fig. 3). However, at higher initial concentrations of Phe, Pyr, and BaP, the co-immobilized fungal-bacterial consortium showed a longer adaptation period before the degradation for the high concentrations of PAHs. The results in Fig. 3 also showed that when the initial PAH concentration were 30 mg•kg^{-1}, the removal rates of Phe, Pyr, and BaP were 59.9±2.6%, 51.9±3.2% and 37.5±3.9%, respectively (P<0.05) (Fig. 3).

In order to remove PAHs, degraders should be able to get enough biomass and ideally mineralize and grow on PAHs as carbon and energy source in contaminated soil (Noordman et al., 2000; Alexander, 1999). When the initial PAH concentration was 10 mg•kg^{-1}, the availability of PAH is poor, and the degradation is not obvious. The lack of PAH may have inhibited the growth of the strains, and the amount of PAH-induced degradation enzymes also decreased.

When the concentration increased from 10 mg•kg^{-1} to 30 mg•kg^{-1}, the PAHs were more likely to come into contact with the strains due to a concentration gradient, which increased the chance of contact between the low-temperature mixed cell consortium and PAHs. The proportion of degradative enzymes produced by strains with a relatively abundant carbon source is correspondingly increased, thereby increasing the removal efficiency of PAHs (Wang et al., 2011).

However, when the concentration of PAHs reached a certain level (100 mg•kg^{-1}), the relative proportions of PAHs and strains become unbalanced. PAHs are toxic to the immobilized cells, and their excessive concentrations may hurt cell growth. Based on the previous studies, the growth and metabolism of SDR4 and JDR7 were the main reasons of PAHs degradation by the immobilized SDR4, JDR7 mixed strains (Wang et al., 2020), the strong inhibition of PAHs on microbial activity reduced the amount of strains, thereby reduced the activity of degrading enzymes produced by PAH-induced strains (Waigi et al., 2015), then resulting in an overall reduction in PAHs degradation (Lu et al., 2019).

Little is known about the fate of PAHs in cold habitats. Margesin et al. (2013) reported that R. *erythreus* BZ4 could efficiently degrade high concentrations (50 mg•L^{-1}) of pyrene and anthracene at 15°C. The anthracene could be completely degraded at 15°C at the initial concentration of 20 mg•L^{-1} within 28 days and with the residual concentration of 13% at 15°C at the initial concentration of 50 mg•L^{-1} within 48 days.

2.4. Comparison of Phe, Pyr and BaP degradation in soil by immobilized and free cells

The degradation of Phe, Pyr, and BaP in non-sterilized soils (with indigenous microorganisms) by co-immobilized or free fungal-bacterial consortium was studied under similar conditions (Fig. 4). Fig. 4A showed the final removal efficiency of Phe, Pyr, and BaP with different treatments at 60 d. Fig. 4B shows the removal efficiency change over time.

The degradation of PAHs in treatments incubated with the co-immobilized fungal-bacterial consortium was 36.8-59.2%, which was 6.9-23.2% higher than the free mixed microorganisms (25.2-36.3%). Significant differences were observed in PAHs degradation among the four-ring PAHs, the three-ring and five-ring PAHs (P < 0.05), and the degradation of Phe, Pyr, and BaP by the co-immobilized fungal-bacterial

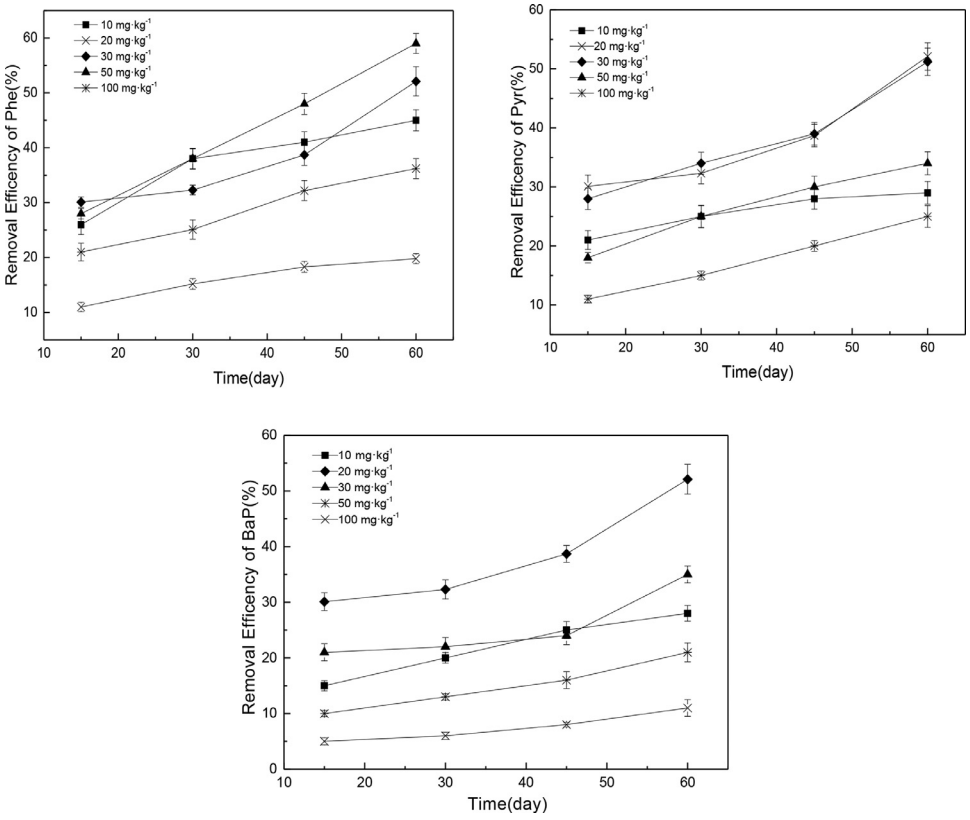

Fig. 3. Removal efficiency of Phe, Pyr and BaP by co-immobilized SDR4 and JDR7 at different initial PAH concentrations within 60 days. (Freeze-thaw processing mode was frozen at -20°C for 12 h and thaw at 10°C for 12 h, with 20% (w/w) of WHC, inoculum amount of co-immobilized microbes was 10% (w/w) and inoculation and growth conditions are described in the text. Values represent the mean of three replicates, bars indicate standard deviation.)

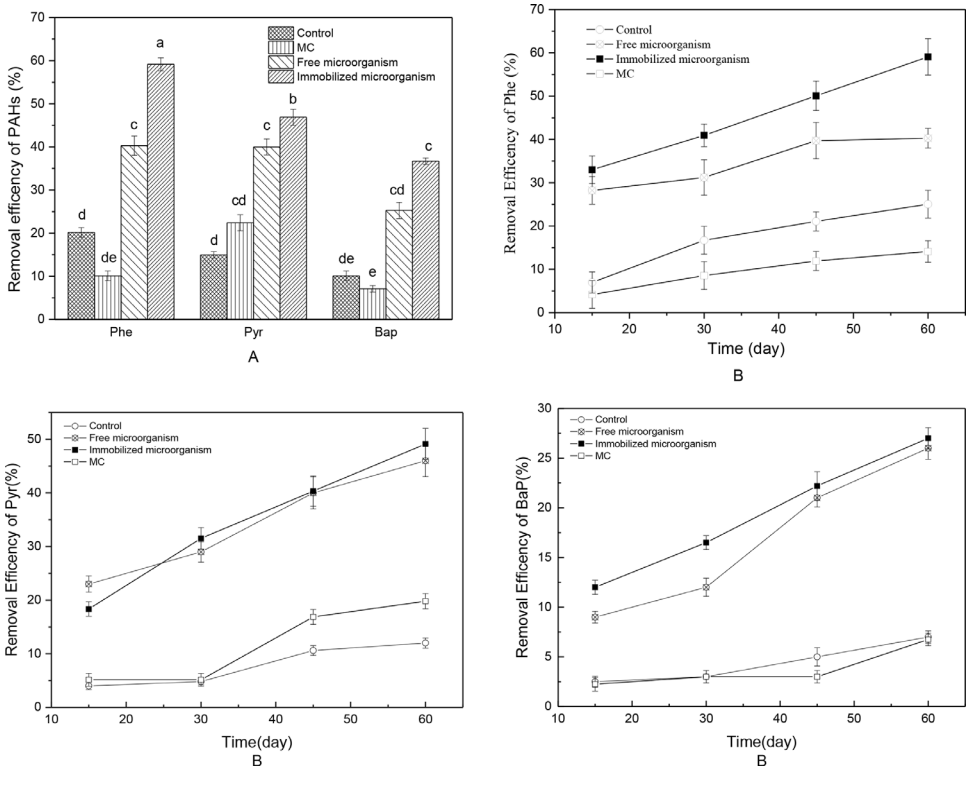

Fig. 4. (A) Final removal efficiency of Phe, Pyr and BaP for different treatments at 60 d; (B) Removal efficiency of Phe, Pyr and BaP as a function of incubation time for different treatments. (Inoculum amount co-immobilized microbes was 10% (w/w); values represent the mean of three replicates; bars indicate standard deviation)

Table 1
Dynamics equations and parameters.

PAHs	Treatments	Reaction kinetic equation, $C=C_0e^{-kt}$, mg•L^{-1}	Correlation coefficient (R^2)	Half-life period, $T_{1/2}$= ln2/k, d
Phe	Control	C =29.231$e^{-0.003\ t}$	0.9221	231.05
	MC	C =28.944$e^{-0.003\ t}$	0.8779	231.05
	Free microorganism	C =27.679$e^{-0.007\ t}$	0.8600	99.02
	Immobilized microorganisms	C =28.445$e^{-0.014\ t}$	0.9705	49.51
Pyr	Control	C =30.133$e^{-0.002\ t}$	0.9815	346.57
	MC	C =30.054$e^{-0.002\ t}$	0.9963	346.57
	Free microorganism	C =29.069$e^{-0.007\ t}$	0.9764	99.02
	Immobilized microorganisms	C =28.363$e^{-0.01\ t}$	0.9614	69.31
BaP	Control	C =15.001$e^{-0.001\ t}$	0.9871	693.15
	MC	C =14.958$e^{-0.001\ t}$	0.9838	693.15
	Free microorganism	C =15.026$e^{-0.005\ t}$	0.9721	138.63
	Immobilized microorganisms	C =14.423$e^{-0.007\ t}$	0.9630	99.02

Note: the concentration versus time (t) curve was calculated to fit the data in Figure 4. k is the constant, C is the concentration of PAH.

consortium was 59.2±3.7%, 46.6±3.3% and 36.8±2.7%, respectively (Fig. 4).

There is comparatively little information on cold-adapted PAHs degraders. Margesin et al. (2013) reported that R. *erythreus* BZ4 could efficiently degrade high concentrations (up to 50 mg•L^{-1}) of pyrene and anthracene, which only 8±2% and 13±6 % were detected after 48 days, respectively, at 15°C. A mixed enrichment culture degraded 99.3% for anthracene and 97.2% for pyrene at 2 mg•L^{-1} PAHs, at the higher concentration of 20 mg•L^{-1}, 98.6% anthracene and 98.7% pyrene were degraded at 10°C (Sartoros et al., 2005).

For a given PAH substrate, the rate of change in concentration as a result of biodegradation is modelled as $C=C_0e^{-kt}$ (El-Mansi et al., 2007; Bezza and Chirwa, 2016), where C is the concentration of the PAH substrate (mg•L^{-1}), k is constant, and time t (d). According to kinetic analysis, the concentration (C) verses time (t) (C-t) curve and the ln (C-t) curve were calculated to fit the data of the biodegradation of Phe, Pyr, and BaP by cold-adaptive SDR4 and JDR7. The fitting results and the parameters obtained from the fitting for Phe, Pyr, and BaP are shown in Table 1. The kinetic results indicated that Phe, Pyr, and BaP treated with co-immobilized microorganisms had the shortest half-lives of 49.5, 69.3 and 99.0 d, respectively (see Table 1).

Michael et al. (1997) found that the half-life of 3-ring PAHs is less than 100 days, while the half-life of most 4-ring and 5-ring PAHs is generally more than 100 days. In comparison, in the soil treated with mixed strains, the half-life of PAHs was significantly shortened (P<0.05). The degradation effect of mixed microbial population on PAHs appears to be better than that of single strain inoculation. During the complex biodegradation process, the catabolic activity of inoculums might improve due to the co-metabolism (Hadibarata et al. 2012; Hays et al., 2015; Sun et al., 2020). Kanaly et al. (1999) pointed out that only under the joint action of soil microorganisms, the degradation of BaP is the most, which may explain why the treatment of mixed SDR4 and JDR7 resulted in the best degradation rate of PAH.

The indigenous microorganisms play a significant role in accelerating PAH biodegradation in the low temperature environments or in the cold regions. Not only temperature can affect the number of microorganisms present in cold environment, but factors such as soil properties, nutrient availability and contaminant concentration can also affect the number of microorganisms (Chaudhary and Kim, 2019). Microbial immobilization technique, as one of the bio-augmentation techniques, could be the best approach for increasing microbial population size in contaminated sites. When the strains are co-immobilized on the MC carrier, the carrier can act as a shield and a buffer when the concentration of toxic and harmful pollutants in the soil is high in cold areas. The environment of the carrier itself provides a site for the proliferation of the strains and more favorable conditions for the proliferation of microorganisms. The MC structures have many cavities, which provided a natural entrapment of the cells. The surface of the MC itself was loosely packed, rough and porous structures (Fig. 5A and B), permitted to obtain bigger surfaces for the cells attachment. It was also found that the MC as the support materials released nutrients to the medium, which favored the cell development (Genisheva et al., 2011). As the immobilized strains cannot be readily reproduced to replenish any loss, it is necessary to accumulate the strains at a high initial concentration; and the activity of the immobilized microorganisms towards the degradation of toxic substances in the soil showed that the degradation rate was fast and that the degradation efficiency was high (Martienssen and Gründl, 2000; Thion et al., 2012). At low temperatures, the immobilized cells also have great advantages, and this result provides a certain theoretical basis for the practical application of this system for remediating contaminated soils. The application of co-immobilized cold-adaptive microorganisms has the potential to remediate PAH-contaminated soil in cold environments.

2.5. Mass transmission and microenvironment of co-immobilized fungal-bacterial mixed system

The surface of immobilized MC carriers was loosely packed and have many cavities (Fig. 5A and B), which provided a natural entrapment of the cells. The internal tissues were uniform and well-developed, which increased their contact with contaminants. Fig. 5C and 5D showed the adsorption of the fungal spores on some of the SDR4 cells, and the SDR4 cells and JDR7 fungal spore were also adsorbed by the MC surface (Fig. 5E and 5F). With the growth of fungal mycelium, the SDR4 adsorbed on JDR7 mycelium can move freely in the carrier medium and soil medium (Fig. 5). The SEM microstructure analysis demonstrated that SDR4 and JDR7 were readily co-immobilized on the MC by entrapment and adsorption permitting excellent oxygen and mass diffusion of substrate.

Numbers of microorganisms adsorbed and fixed into the MC carriers, which makes the continuous growth of fungi and bacteria can be sustained subsequently. More bacteria and fungi were released into the surrounding environment, accompanied by the replication and growth in the MC carriers. Therefore, it has a two-fold effect on the degradation of PAHs by microorganisms: on the one hand, the degradation of PAHs within the impacted area is enhanced due to the slow release of fungal and bacterial; on the other hand, the degradation rate of PAHs within the protected MC environment is increased. Therefore, the MC as a carrier not only have a high microbial count but also adsorbs and encapsulates the strains, which is advantageous in shielding against the interference of external indigenous strains, making it fully exposed to pollutants and promoting the degradation of PAHs. During the co-cultivation process of fungi and bacteria, the cooperative growth of the *Pseudomonas* sp. and *Mortierella alpine* promote the degradation of PAHs in the cold environment.

3. Conclusions

This study presented a practical bio-augmentation approach in remediating PAH-contaminated soil in cold environment. Co-immobilization

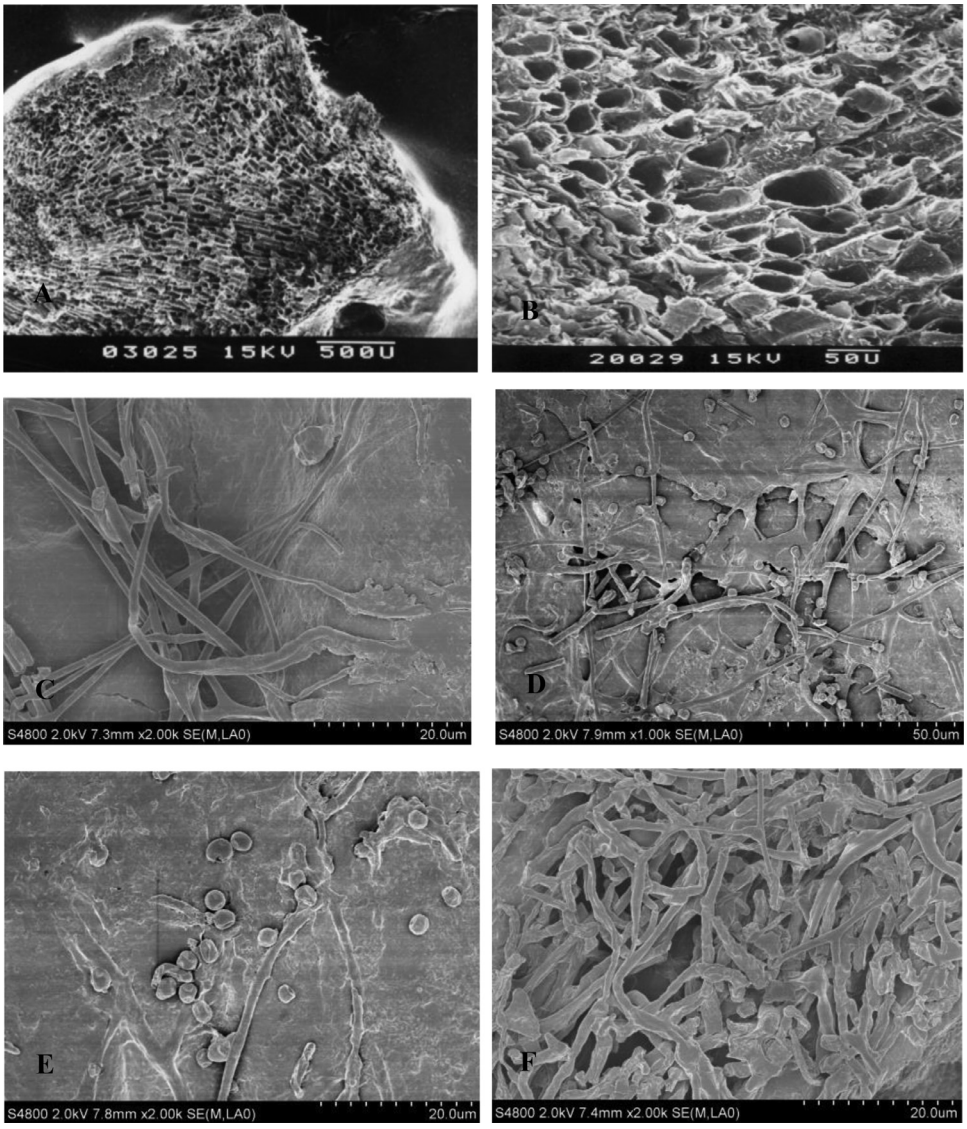

Fig. 5. Immobilized mixed consortium observed by SEM. (A) ×30; (B) ×200; (C) ×2000; (D) ×1000; (E) ×2000; (F) ×2000.

of indigenous cold-adaptive PAH-degrading SDR4 and JDR7 strains on MC as a carrier showed obvious advantages, compared with free strains. With the co-immobilized system, the PAH degradation rate in the contaminated freeze-thawed soil after 60 days incubation reached 36.8–59.2%, which was 6.9-23.2% higher than the free mixed microorganisms (25.2-36.3%). Ten percent (w/w) was the optimal inoculum for the co-immobilized fungal-bacterial mixed microorganisms. Significant differences were observed in PAHs degradation among the four-ring PAHs, the three-ring and five-ring PAHs ($P<0.05$). When the initial concentrations of PAH were 30 mg•kg^{-1}, the highest degradation of Phe, Pyr, and BaP by the co-immobilized fungal-bacterial consortium was 59.2±3.7%, 46.6±3.3% and 36.8±2.7%, respectively. The SEM analysis demonstrated the advantages of the process of mass transmission and the immobilized microstructure. In the cold regions of the earth, the use of co-immobilized cold-adaptive fungal-bacterial consortium to repair soil contaminated by PAHs has great application prospects.

Declaration of compting interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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