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Influence of immobilization on phenanthrene degradation by *Bacillus* sp. P1 in the presence of Cd(II)

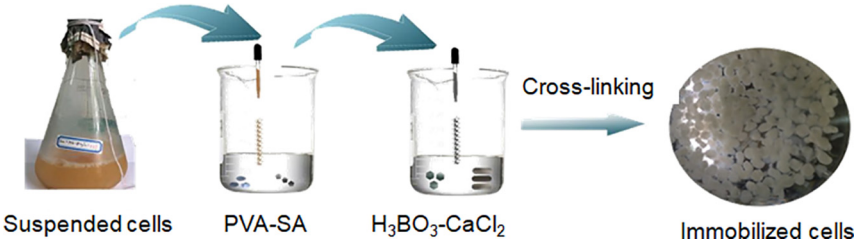
Shao-Heng Liu [a,b,1], Zhuo-Tong Zeng [c,1], Qiu-Ya Niu [a,1], Rong Xiao [c,1], Guang-Ming Zeng [a,*], Yang Liu [a], Min Cheng [a], Kai Hu [a], Lu-Huang Jiang [d], Xiao-Fei Tan [a], Jian-Jun Tao [b]

[a] College of Environmental Science and Engineering, Hunan University and Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, PR China
[b] College of Chemistry and Material Engineering, Hunan University of Arts and Science, Changde 415000, Hunan, PR China
[c] Department of Dermatology, Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, PR China
[d] School of Minerals Processing and Bioengineering and Key Laboratory of Biometallurgy of Ministry of Education, Central South University, Changsha 410083, PR China

HIGHLIGHTS

- Immobilization effect on phenanthrene biodegradation was investigated.
- Gel beads increased adsorption sites thus accelerating phenanthrene degradation.
- Detoxification indices showed the protection mechanism of immobilization on cells.

GRAPHICAL ABSTRACT



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ABSTRACT

Suspended microbes gradually lost advantages in practical applications of PAHs and heavy metals bioremediation. Therefore this study investigated the effect of immobilization on phenanthrene degradation by *Bacillus* sp. P1 in the presence of different Cd(II) concentrations. Condensed *Bacillus* sp. P1 was immobilized with polyvinyl alcohol and sodium alginate and PVA-SA-cell cryogel beads were prepared. The results indicated that the use of gel beads increased the number of adsorption sites thus accelerating phenanthrene degradation. In addition, changes in detoxification indices, including superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH), were determined to elucidate the immobilization mechanisms related to cells protection from Cd(II) when degrading phenanthrene. By protecting the gel membrane, oxidative damage was minimized, while SOD activity increased from 55.72 to 81.33 U/mgprot as Cd(II) increased from 0 to 200 mg/L but later dropped to 44.29 U/mgprot as Cd(II) increased to 300 mg/L for the non-immobilized system. On the other hand, the SOD activity kept increasing from 52.23 to 473.35 U/mgprot for the immobilized system exposed to Cd(II) concentration between 0 and 300 mg/L. For CAT and GSH, immobilization only slowed down the depletion process without any change on the variation trends. The changes in surface properties and physiological responses of microbes caused the differences of immobilization effect on phenanthrene biodegradation in the presence of Cd(II), which is a novel finding.

Abbreviations: PAHs, polycyclic aromatic hydrocarbons; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; PVA, polyvinyl alcohol; SA, sodium alginate.

* Corresponding author at: College of Environmental Science and Engineering, Hunan University, Changsha 410082, PR China.
E-mail address: zgming@hnu.edu.cn (G.-M. Zeng).

[1] These authors contributed equally to this article.

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1. Introduction

Widely distributed contamination of polycyclic aromatic hydrocarbons (PAHs) has aroused the attention of people around the world because of their potential detrimental effect on human and ecosystem (Lamichhane et al., 2016; Chen et al., 2015, 2016b; Oleszczuk et al., 2014). Among PAHs, phenanthrene is often studied by researchers because it both contains K regions and bay regions, which are considered as the basic carcinogenic and mutagenic molecular structure in most of the high molecular weight PAHs (Rodriguez et al., 2017). With respect to its relatively low hydrophobicity and high solubility, the concentration of phenanthrene can be easily detected in the aqueous phase (C. Zhang et al., 2017; Tan et al., 2015). Therefore, phenanthrene is selected as an optimum PAHs model for laboratory studies.

Among all the PAHs removing techniques, biodegradation is considered as an ecological and economical method where the bacteria play an important role in the process (Liu et al., 2017; Zhang et al., 2016; Gong et al., 2009). In our previous studies, freely suspended bacteria were used in degrading PAHs in the presence of heavy metals, but freely suspended bacteria tend to be involved in competitions with indigenous microbes, thus losing the advantages of the dominant bacteria (Ye et al., 2014; Long et al., 2011). Besides, some cultured exogenous microbes are screened in soft conditions and might be difficult to adapt to natural environmental conditions (Lang et al., 2016; Zhou et al., 2018). Immobilization is a process fixing the bacteria in polymeric matrices, improving the bioremediation efficiency of PAHs via higher microbe density, making them more resistant towards the environmental conditions and other microbes (Moritz and Geszke-Moritz, 2013; Xu et al., 2012a; Jézéquel and Lebeau, 2008). A variety of carrier materials have been used for immobilization including inorganic, polymeric and composite materials (Dong et al., 2014). Both natural and synthetic materials can be used for micro-organisms immobilization. For example, Garcia-Delgado et al. (2015) reported that *Pleurotus ostreatus* immobilized on sterilized wheat straw achieved the best PAH degradation rate mainly because of increased ligninolytic enzymes activity. Ali and Naeimpoor (2013) concluded that immobilized cells were able of degrading higher phenanthrene concentrations because the carrier protected the cells from soluble toxic intermediates produced in pollutant consumption. Other studies have investigated the application of immobilized microorganisms for PAHs degradation. The mechanisms of PAHs treatment by immobilized microorganisms are still not completely understood, especially in the presence of heavy metals as they are very common in natural conditions. Heavy metals usually occur together with PAHs in places like refinery sites (Ren et al., 2018; Zhang et al., 2015). In general, toxic heavy metals or high concentrations of metal ions have a detrimental effect on the microbes by suppressing the oxidative stress, breaking DNA and deactivating zymoprotein (Liang et al., 2017). Among heavy metals, Cd(II) is widely investigated because it is widespread and inevitably released into environments. The pollution of Cd (II) is very severe and has aroused attentions all over the world. Cd(II) is considered as a highly toxic metal and can be easily transported in biologic chain. Heavy metals can influence bacteria even if they were protected by gel membranes. Actually, the detoxification mechanisms of immobilized cells could be affected by inducing extracellular cells secretions, therefore making it different in systems without heavy metals. Moreover, detoxification of immobilized bacteria reveals the process and response of toxicological effect, as well as oxidative damage that PAHs and heavy metals impose on micro-organisms. Better detoxification effect helps keeping the microbes vigorous, thus improving PAHs degradation (Cheng et al., 2016a). However, few studies comprehensively analyzed the detoxification step in PAHs degradation by immobilized bacteria.

In this work, polyvinyl alcohol (PVA) and sodium alginate (SA) were chose as carrier materials because they are low costs and easily available porous hydrophilic gels. Today, several types of immobilization materials have been reported in the literature including agar, glutin, polyacrylamide, PVA and SA. But materials properties must be considered in choosing the carrier materials. Compared with other porous materials, PVA-SA have high mechanical strength, low microbial toxicity, high mass transfer efficiency, easy to decompose by microbes and low cost. Therefore, PVA and SA were selected in this work, while phenanthrene and Cd(II) were selected as models of PAHs and heavy metals, respectively. The objectives of this study are to: (i) investigate the effect of immobilization on phenanthrene degradation in the presence of Cd(II); (ii) explore the detoxification differences between immobilized bacteria and free suspended bacteria on phenanthrene degradation in the present of Cd(II), which should improve our understanding of the immobilization effect on bacteria.

2. Materials and methods

2.1. Micro-organism and medium

The strain of bacteria was isolated from activated sludge in a sewage treatment of a coking plant of Hualing iron steel Co., Ltd. in Lianyuan City (Hunan, China). The strain was then domesticated with PAHs (containing 16 kinds of priority pollutants) as the sole carbon and energy source and identified as *Bacillus* sp. P1 based on a 16S rDNA gene sequence analysis. This strain is able of degrading high PAHs concentrations in a very short time, especially in the presence of heavy metals. According to our previous work (Liu et al., 2015), 60 mg/L of phenanthrene can be degraded with almost 90% degradation rate in two days with or without heavy metals. The highly efficient PAHs degrading bacteria were cultured in a beef extract peptone medium at 30 °C and 150 rpm in a rotary shaker for 2 days. Then they were sub-cultured in a mineral medium ($MnSO_4$ 0.0447 mg/L, $ZnSO_4$ 0.0686 mg/L, $(NH_4)_6MO_7O_{24}\cdot 4H_2O$ 0.0347 mg/L, K_2HPO_4 129.15 mg/L, Na_2HPO_4 167 mg/L, KH_2PO_4 43.5 mg/L, NH_4Cl 25 mg/L, $MgSO_4$ 13.8 mg/L, $CaCl_2$ 36.4 mg/L, $FeCl_3$ 0.42 mg/L) before use.

2.2. Immobilization

PVA (Guaranteed Reagent) and SA (Guaranteed Reagent) were used as the bacteria gel carriers. A solution of 12% (w/v) PVA and 0.3% SA was first prepared and sterilized (Lin et al., 2014). Then, 20 mL of highly condensed *Bacillus* sp. P1 (2×10^6 CFU/mL) was injected through an injection needle into the prepared 200 mL PVA-SA solutions at room temperature to obtain PVA-SA-cell suspensions. Subsequently, the suspension was inoculated into 5 L H_3BO_3-$CaCl_2$ solutions and then shaken for 8 h at 150 rpm to form immobilized PVA-SA-cell cryogel beads. Every gel bead was about 3 mm in diameter. The immobilized cell beads were collected by filteration through filter paper and washed with sterilized water. Then, they were re-cultured in a beef-protein medium for 2 days and stored in a 0.5% NaCl solution before use.

2.3. Adsorption studies and the effect of Cd(II)

A series of flasks containing different phenanthrene concentrations (5, 20, 50 mg/L) with 0 and 50 mg/L of Cd(II) were prepared. Phenanthrene was dissolved to 1% (w/v) in acetone. The mineral medium was added to each flask after complete evaporation of the solvent. Appropriate amount of soybean lecithin were added under sterile conditions to ensure complete solubilization of phenanthrene (Fava et al., 2004; Soeder et al., 1996). The adsorbents (immobilization materials, immobilized inactive cells and suspended inactive cells) were agitated in 20 mL solutions in a rotary shaker at 150 rpm and 30 °C for 24 h to reach equilibrium. The bacteria were inactivated in an autoclave at 121 °C for 30 min. Since the bacteria were inactive and metabolism-independent, the consumption rate of phenanthrene can be treated as the adsorption rate. The cells concentration was maintained at 2×10^6 CFU/mL. For the determination of the residual phenanthrene concentrations, the whole bacterial culture were mixed with an equal

volume of *n* hexane in a 100 mL separatory funnel and extracted (200 rpm) on an extraction shaker (JTLDZ-8) for 10 min. Extraction was repeated three times and n-hexane phase were separated. All the experiments were performed in triplicate.

The efficiency of mass transform by the immobilized cells and immobilization materials were compared by dying with an inert red ink. A total of 14 beads of immobilized cells and materials (d = 3 mm) were added into flasks containing 50 mL of sterilized water. Three drops of inert red ink were then injected into each flask. At specific time intervals (0, 5, 10, 20, 30, 60 and 120 min), two beads were taken out and washed three times with distilled water. Each bead was cut apart to observe the erosion extent. The slicing positions are presented in Fig. 2. BET specific surface area, pore volume and pore distribution of active immobilized cells and immobilization materials were characterized by an automatic surface analyzer (Micromeritics Tristar II 3020, USA) after dehydration. The analysis bath temperature was 77.3 K and the sample density was 1 g/cm^3.

To investigate the influence of immobilization on phenanthrene degradation in the presence of different Cd(II) concentrations, a set of experiments with a phenanthrene content of 50 mg/L and various Cd(II) contents (0, 50, 100, 200 and 300 mg/L) were prepared. The immobilized bacteria and non-immobilized *Bacillus* sp. P1 (2×10^6 CFU/mL) was inoculated in the systems and shaken for 48 h at 30 °C and 150 rpm. Phenanthrene was extracted by *n* hexane before being analyzed by a UV–visible spectrophotometer at 254 nm (Giger and Blumer, 1974; Marsh et al., 2000). To test the possibility of reusing the immobilized cells, the beads were washed with 0.5% NaCl and distilled water before performing another phenanthrene degradation test in the presence of 50 mg/L Cd(II) for five cycles, using the same conditions as in the first test. Phenanthrene degradation properties at different pH (4.0, 5.0, 6.0 7.0, 8.0 and 9.0) and temperature (20, 30, 40 and 50 °C) were studied to determine the advantages of using immobilized cells.

2.4. Scanning electron microscopy (SEM)

SEM (FEI QUANTA 200, Czech) was performed to investigate the modification of *Bacillus* sp. P1 by immobilization with or without Cd (II). The immobilized cells and non-immobilized bacteria were dehydrated with a series of ethanol concentrations (50%, 60%, 80% and 100% ethanol in distilled water). They were air dried before being coated with gold and observed at 20 kV.

2.5. Detoxification indices

Enrichment of phenanthrene and Cd(II) can increase the level of reactive oxygen species (O_2•$^-$, •OH and H_2O_2), thus providing a detoxification mechanism, and expressing a high antioxidant capacity. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and low molecular weight antioxidant components such as glutathione (GSH), are able of removing reactive oxygen radicals such as O_2•$^-$, •OH and H_2O_2. Therefore, SOD activities, CAT activities and GSH contents were determined in this study. Bacteria were sonicated (300 W, 3 s/8 s) for 10 min at 4 °C with subsequent centrifugation (9000 rpm, 4 °C). The supernatants were used for the determination of detoxification indices. Assay kits of SOD activities, CAT activities and GSH contents (Jiancheng Bioengineering Institute, Nanjing, China) were utilized. The total protein content of the bacteria was also analyzed by enzyme-linked immunosorbent assay kits based on BCA methods to determine the SOD, CAT and GSH values (Zhang et al., 2017). SOD activity was measured by a modified nitrite method (Oyanagui, 1984). Superoxide generated by hypoxanthine and xanthine oxidase was changed to nitrite ion by hydroxylamine. Nitrite ion was measured spectrophotometrically at 550 nm by the use of a chromogen (Luca et al., 2007). One unit of SOD activity was defined as 50% of SOD inhibition rate caused by enzymes. CAT can decompose H_2O_2, therefore the concentration of H_2O_2 can indirectly indicate CAT activities. The excess H_2O_2 can form complexation with ammonium molybdate to produce light yellow solution, which can be measured by spectrophotometer at 450 nm (Zhang et al., 2017). One unit of CAT activity was defined as 1 μmol of H_2O_2 decomposed by protein per milligram per second. GSH determination method is based on the reaction of GSH with the thiol reagent DTNB (5,5 dithiobis(2 nitrobenzoic acid)) to form GSSG and TNB (5 thionitrobenzoic acid), which is detected spectrophotometrically at 412 nm (Salbitani et al., 2017).

2.6. Data analysis

The degradation rate of phenanthrene (*R*) was calculated as:

$$R = \frac{C_0 - C_t}{C_0} \quad (1)$$

where C_0 is the initial phenanthrene content (mg/L), and C_t is the phenanthrene concentration at a specific time *t* (mg/L).

The amount of sorbed phenanthrene (*Q*) (mg/g) within 12 h onto the immobilized or non-immobilized bacteria was determined by:

$$Q = \frac{V(C_0 - C_t)}{M} \quad (2)$$

where *V* is the initial volume of phenanthrene in the solution (L), while *M* is the mass of bacteria immobilized or non-immobilized. A pseudo first order kinetics equation was used to plot the results as:

$$\log(Q_e - Q_t) = \log Q_e - \frac{K_1}{2.303} t \quad (3)$$

where *t* is the adsorption time (min), Q_e is the amount of phenanthrene adsorbed at time t (mg/g), Q_t is the amount of phenanthrene adsorbed at equilibrium (mg/g) and K_1 is the pseudo first order reaction rate constant (g/mg·min^{-1}).

A pseudo second order kinetics equation was also investigated as:

$$\frac{t}{Q_t} = \frac{1}{K_2 \times Qe^2} + \frac{t}{Q_e} \quad (4)$$

where K_2 is the pseudo second order reaction rate constant (g/mg·min^{-1}).

Statistically significant differences ($p < 0.05$) among various treatments were evaluated by using a Duncan's Multiple Range Test via the SPSS 19.0 software.

3. Results and discussion

3.1. Effects of immobilization on phenanthrene consumption

Phenanthrene consumption as a function of time was determined by taking samples at 10, 30, 60, 120, 240, 720 and 1440 min. Phenanthrene consumption rate can be approximated by the adsorption rate since the bacteria were inactive. The results in Fig. 1 show that the immobilized *Bacillus* sp. P1 have a satisfactory potential in phenanthrene adsorption compared to suspended cells. This can be attributed to improved mass transfer and number of adsorption sites of the carrier materials (Lin et al., 2014; Deng et al., 2013) and less Cd(II) inhibited phenanthrene adsorption especially in PVA-SA system since Cd(II) would compete with phenanthrene for these adsorption sites. The adsorption reached equilibrium within 24 h and the absorbents, immobilized cells and suspended cells results were all better fitted by a pseudo second order model than a pseudo first order one.

Mass transfer performances of the immobilized cells and immobilization materials are compared in Table 1. The results showed that both groups of beads were completely dyed in 120 min. The cells were more promoted by PVA-SA mass transfer mainly because of

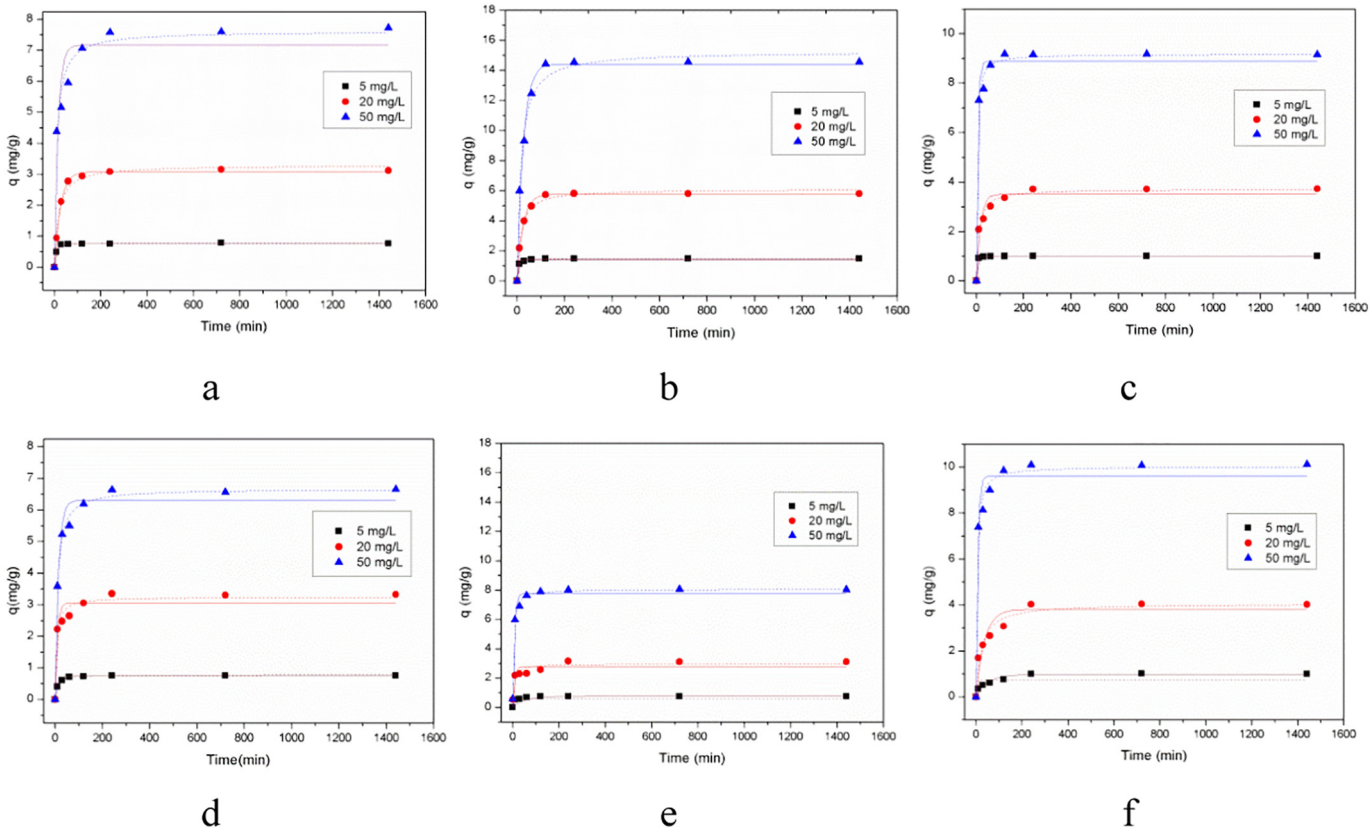


Fig. 1. Time profiles of phenanthrene adsorption with inactive immobilized/suspended bacteria with/without Cd(II). (a, suspended cells without Cd(II). b, PVA-SA without Cd(II). c, PVA-SA-Cell without Cd(II). d, suspended cells with Cd(II). e, PVA-SA with Cd(II). f, PVA-SA-Cell with Cd(II)).The solid lines represent pseudo-first-order kinetics model and dash lines represent pseudo-second-order kinetics model. The error bars represent ±1 standard deviation.

increased porosity. The results of Table 2 show that the BET specific surface area and pore volume of the immobilized cells were all increased compared to the immobilization materials. The volumes of micropores, mesopores and macropores were all increased in PVA-SA-cells compared to that in PVA-SA materials (Fig. 3), which may because of the growth, motion and other physiological processes caused by the microbes. Therefore the adsorption capacity of the immobilized cells was improved.

3.2. Effects of immobilization on phenanthrene degradation at different Cd (II) contents

The effect of immobilization on phenanthrene degradation with different Cd(II) concentrations (0, 50, 100, 200 and 300 mg/L) is presented in Table 3. The phenanthrene degradation rate by immobilized cells gradually decreased from 98.62% to 79.64% with increasing Cd(II) concentration (0 to 300 mg/L) in 48 h, while for the non-immobilized system, the phenanthrene degradation rate decreased from 92.05% to 66.28%. Compared to the literature, most of the phenanthrene degradation rates reported were up to 60–80%. For example, Xiong et al. (2017) reported that *Mycobacterium gilvum* immobilized with rice straw biochar led to 62.6% phenanthrene degradation. Alessandrello et al. (2017) used a composite material to fix *Pseudomonas monteilii* P26-*Gordonia sp.* H19 in polyurethane foams and reported that 78% of phenanthrene was degraded. Here, this strain of *Bacillus* sp. P1 immobilized on PVA-SA was very effective with up to 98% phenanthrene degradation. As expected, Cd(II) inhibited degradation on both immobilized and non-immobilized *Bacillus* sp. P1, mainly because it restrained the enzymatic production process, as well as modified the enzymes composition, concentration and activity (Liu et al., 2015). Therefore, phenanthrene degradation rate by immobilized and non-immobilized cells decreased. The phenanthrene degradation rate for the immobilized system was nevertheless higher than for the non-immobilized system. This result is associated to a "protective effect" of the gels from exposure to toxic Cd(II) and toxic intermediates of the phenanthrene consumption (H. Xu et al., 2016; Wan et al., 2018). Furthermore, immobilization not only enabled more phenanthrene to be fixed to the bacteria, but also to immobilize more extracellular secretion on the carrier such as polysaccharides which could improve the contact efficiency, thus increasing the phenanthrene degradation rate (Szczesna et al., 2001).

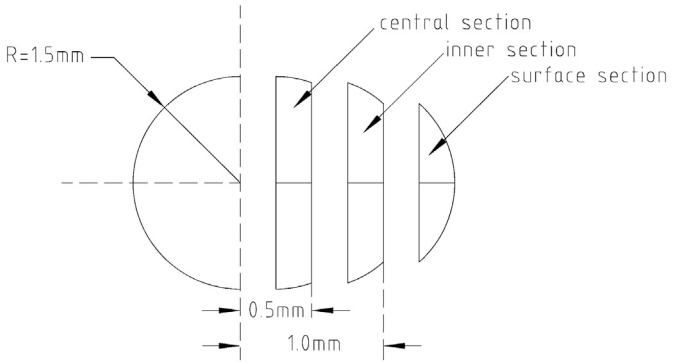


Fig. 2. Slicing positions of immobilized cells and immobilization materials.

3.3. Immobilization effect on physical characteristics

In order to investigate the effects of immobilization on cells surface characteristics, SEM was performed and typical micro-structures of the immobilized *Bacillus* sp. P1 and suspended cells with/without Cd

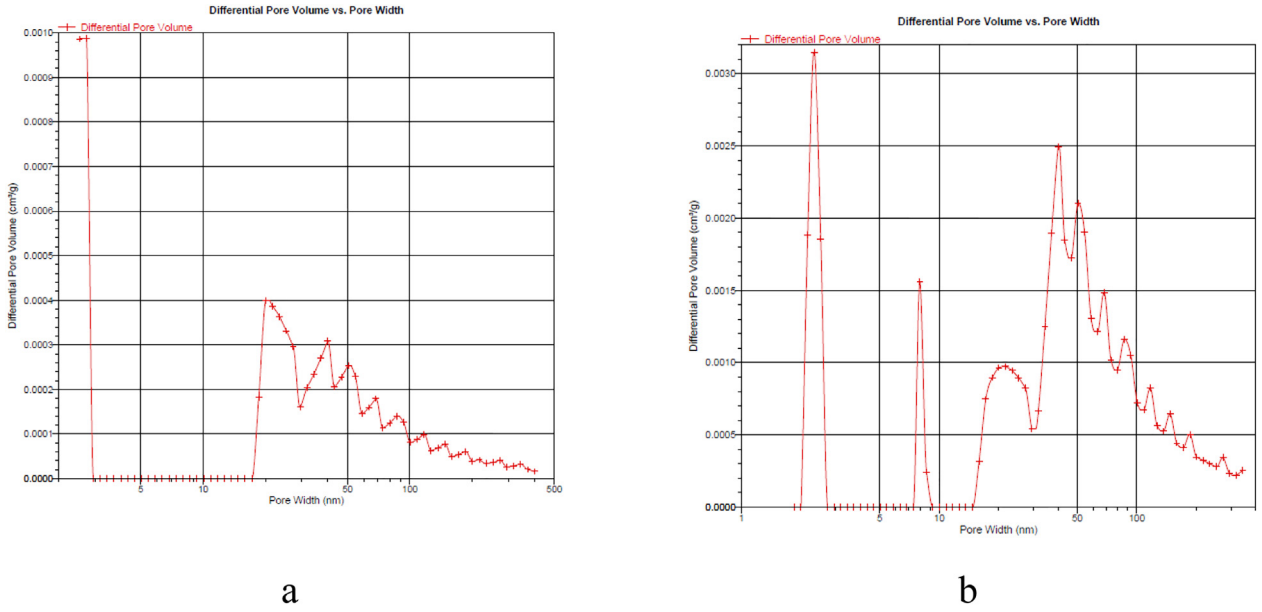


Fig. 3. Porous distribution of immobilization material (a) and immobilized cells (b).

(II) on the cells are illustrated in Fig. 4. These micrographs confirm that a more porous structure is present on the immobilized gel beads (Fig. 4a and c) compared to non-immobilized cells (Fig. 4b and d), which increased the number of adsorption sites for the contaminants and the bioavailability, therefore accelerating the phenanthrene removal rate. The porous structures also improved the micro density and fixed more extracellular secretion on the carrier, thus increasing the contact efficiency with phenanthrene.

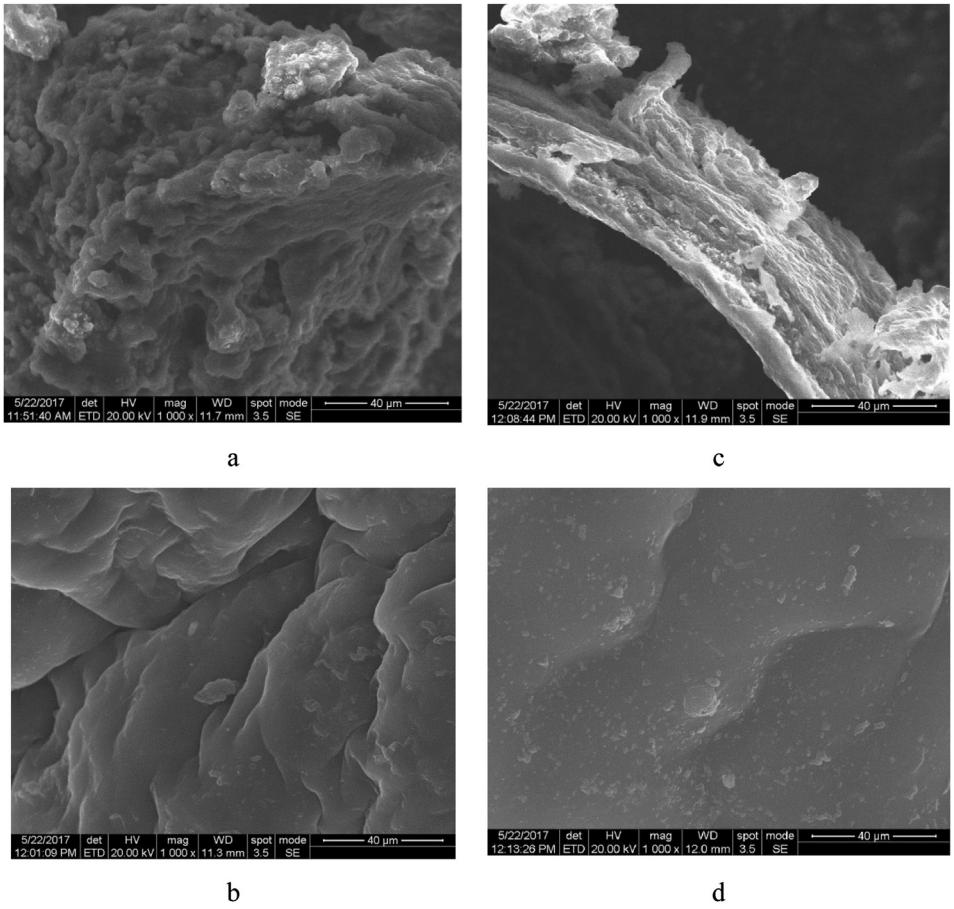


Fig. 4. Scanning electron microscope micrograph of immobilized bacteria and suspended bacteria with/without Cd(II) (a, immobilized cells without Cd(II). b, suspended cells without Cd(II). c, immobilized cells with 50 mg/L Cd(II). d, suspended cells with 50 mg/L Cd(II)).

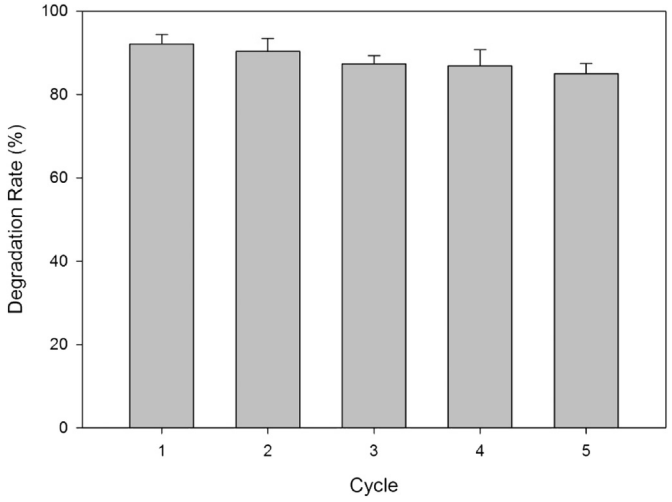


Fig. 5. Repeated phenanthrene degradation by reusing immobilized cells.

3.4. Possibility to reuse the immobilized Bacillus sp. P1

The possibility of reusing the immobilized bacteria was examined via batch experiments. As presented in Fig. 5, after five consecutive degradation experiments, up to 85% of the immobilized cell beads of phenanthrene degradation in the presence of Cd(II) can be observed in each cycle test. The results indicate that the gel membranes provided high mechanical strength for *Bacillus* sp. P1, therefore reducing the operational costs.

3.5. Effect of pH and temperature on the immobilized and suspended Bacillus sp. P1

The effect of pH and temperature on the immobilized and suspended cells in phenanthrene degradation in the presence of Cd(II) is reported in Fig. 6a and b, respectively. In the suspended systems, the highest phenanthrene removal rate was 88.06% at pH 7.0, while the lowest phenanthrene removal rate was 69.81% at pH 4.0. Acid or alkaline conditions could both reduce enzymes activity by affecting the state of ionization of acidic or amino acids, thus affecting the degradation of PAHs. Besides, pH has impacts on the solubility and redox of heavy metals, which occurred together with PAHs. Discrepancy valence states of heavy metals could pose different effects on bacteria, which in turn influenced PAHs degradation (Liu et al., 2017). In immobilized systems, the highest phenanthrene removal rate was 88.33% at pH 7.0, while the lowest phenanthrene removal rate was 82.07% at pH 4.0. Immobilization protected the cells from the adverse pH condition and improved the tolerance to pH variations, thus improving phenanthrene degradation over a wide range of conditions.

Phenanthrene degradation by suspended and immobilized cells increased from 62.79% and 80.51% to 77.29% and 86.71% respectively, as the temperature increased from 20 to 30 °C, but then declined to 67.89% and 84.25%, respectively. The solubility of phenanthrene increased with the increase of temperature, which improved the bioavailability of phenanthrene. Besides, the activities of microbes increased with the increase of temperature in the appropriate range, because it enhanced the bacterial metabolism, which accelerated the bioremediation process of phenanthrene. When the temperature was too high for the microbe, the enzymes activity could be inhibited, therefore, phenanthrene degradation decreased. Immobilization gave the cells some protection against increased temperature, making them again more tolerant towards temperature variations, thus increasing the conditions range for possible phenanthrene degradation.

3.6. Antioxidant responses of immobilized and non-immobilized bacteria

Both PAHs and heavy metals can impose oxidative stress on *Bacillus* sp. P1. When degrading phenanthrene with or without Cd(II), the cells attempt to fight with an oxidant effect and adjust the redox balance by producing antioxidant enzymes such as SOD, CAT and low molecular weight antioxidant components such as GSH (Khan et al., 2017; X. Zhang et al., 2017; Khan et al., 2015; Bianucci et al., 2013). Fig. 7a shows the variations of SOD activity and protein content in immobilized and non-immobilized bacteria exposed to different Cd(II) contents when degrading 100 mg/L of phenanthrene. The protein contents were analyzed for the calculation of SOD, CAT and GSH. The curves in Fig. 7a show that the protein contents continuously decreased by increasing the Cd(II) concentration from 0 to 300 mg/L in both immobilized and non-immobilized systems. This inhibition effect on protein content became more important with increasing Cd(II) content. The protein contents are generally higher in immobilized cells compared with non-immobilized bacteria, which can be ascribed to adsorption and aggregation effects caused by immobilization.

In non-immobilized systems, the SOD activity increased from 55.72 to 81.33 U/mgprot as Cd(II) increased from 0 to 200 mg/L, and then

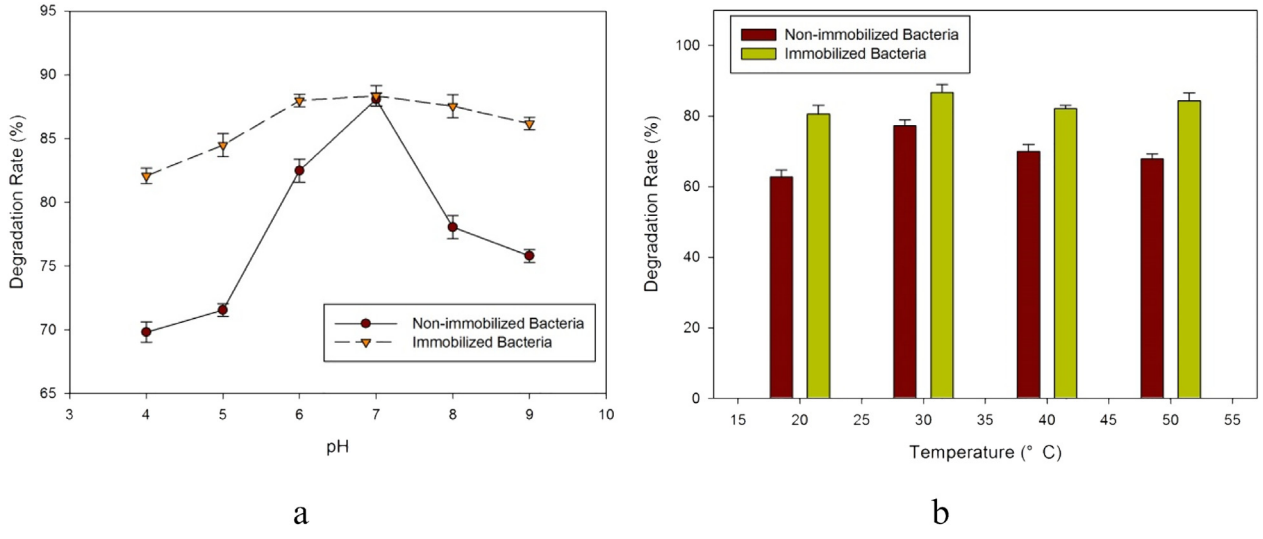
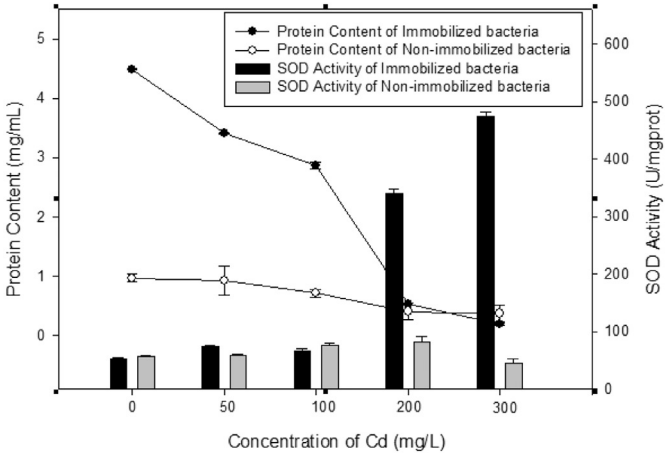
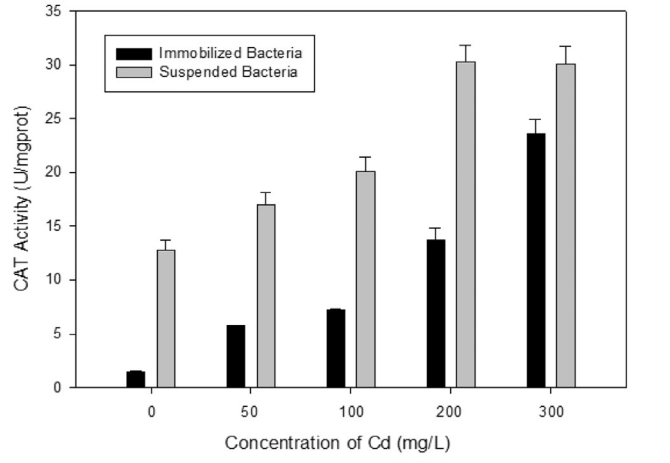


Fig. 6. pH (a) and temperature (b) tolerance variations of immobilized and free suspended *Bacillus* sp. P1 in phenanthrene degradation in the presence of Cd(II).



a



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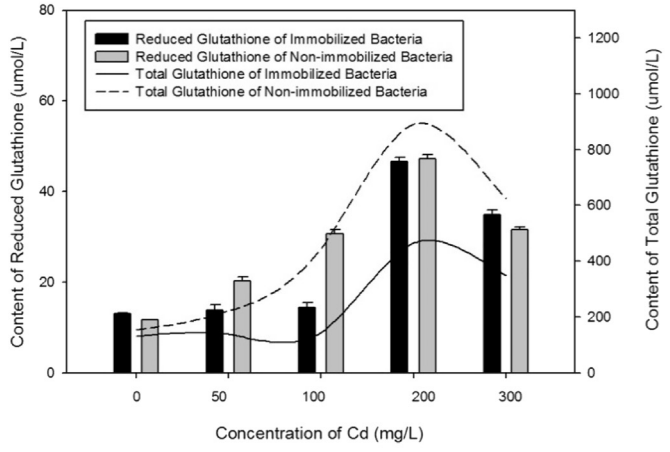

c

Fig. 7. Variations of detoxification indexes of immobilized bacteria and non-immobilized bacteria in the presence of Cd(II). (a) represents changes of SOD activities and protein contents, (b) represnts changes of CAT activities, (c) represents changes in total glutathione content and reduced glutathione content. The error bars represent ±1 standard deviation.

dropped to 44.29 U/mgprot as Cd(II)increased to 300 mg/L. For the immobilized system, the SOD activity kept increasing from 52.23 to 473.35 U/mgprot with increasing Cd(II)content from 0 to 300 mg/L.

Table 1
Mass transfer performance of immobilized cells and immobilization materials.

Time/min	0	5	10	20	30	60	120
PVA-SA	−	+	+	+	+	++	+++
PVA-SA-Cells	−	+	+	+	++	+++	+++

− beads were not dyed. + the surface section of bead was dyed. ++ the inner section of bead was dyed. +++ the central section of bead was dyed.

This behavior can be attributed to higher Cd(II)-concentrations inducing more oxidative stresses and phenanthrene-Cd(II) synergetic effect on cells associated with the protection effect of the gel membrane (Tao et al., 2015). Much higher SOD activity in both immobilized and non-immobilized systems was observed when exposed to higher Cd (II) concentrations (200 and 300 mg/L), which is related to some stress responses to oxidative damage. The reactive oxygen species (ROS) production rates caused by these oxidative stress and the cells scavenging capacity usually kept this balance in steady state conditions, so SOD activity increased as the stresses related to Cd(II) increased (Garg and Chandel, 2015). However, when an excessive SOD consumption occurs, the SOD synthesis capacity drop, thus limiting the SOD production and activity (Pramanik et al., 2017; Cao et al., 2012). This could explain that without the gel protection effect, the SOD activity decreased at the highest Cd(II) content (300 mg/L).

The variation of CAT activity for the immobilized and non-immobilized *Bacillus* sp. P1 is shown in Fig. 7b. By increasing the Cd (II) concentration from 0 to 300 mg/L, the CAT activity increased from 1.46 and 12.74 to 23.63 and 30.09 U/mgprot in immobilized and non-immobilized cells, respectively. The PVA-SA carrier helped the bacteria to survive to the environmental conditions, especially being in contact with phenanthrene and Cd(II). Therefore, the oxidative damage was minimized (Chen et al., 2007; Haghighi et al., 2017). The scavenging capacity was higher than the ROS production rate based on CAT activity since CAT activity kept increasing instead of decreasing with increasing Cd(II) concentration from 0 to 300 mg/L (Tang et al., 2014).

GSH, especially reduced GSH, was very significant in keeping the ROS balance related to PAHs and heavy metals. Fig. 7c reports on the total GSH and reduced GSH content in immobilized and non-immobilized cells exposed to different Cd(II) content when degrading 100 mg/L of phenanthrene. For the immobilized system, reduced GSH content increased from 13.11 to 46.62 μmol/L with increasing the Cd (II) concentration from 0 to 200 mg/L, and then dropped to 34.96 μmol/L when exposed to 300 mg/L Cd(II). Likewise, a maximum value (466.28 μmol/L) of total GSH was found at 200 mg/L Cd(II) before decreasing when exposed to higher Cd(II) concentration. This behavior can be attributed to the fact that GSH act as a reduction agent or a substrate for ROS scavenging at high Cd(II) concentration (>200 mg/L) (P. Xu et al., 2016, 2012b). Immobilization did not change this trend. Similarly, the reduced GSH and total GSH content increased with increasing Cd(II) concentration from 0 to 200 mg/L, and then decreased at 300 mg/L of Cd(II) in the non-immobilized system. GSH played roles in the protective mechanism against the contaminants including phenanthrene and Cd(II), but becomes depleted when the contaminants concentrations were too high (Corticeiro et al., 2006). In this case, the immobilization can only slow down the depletion process.

Overall, the three indices played an essential role in detoxification. SOD can directly convert O_2 to $O_2^{\bullet -}$, which was one of the principal toxicants induced by Cd(II). Therefore, heavy metals enrichment in bacteria increased the SOD activity. CAT played a role in eliminating H_2O_2, which

Table 2
Surface area and pore volume of immobilized cells and immobilization materials.

	PVA-SA	PVA-SA-cells
Surface area	0.0513 m^2/g	2.9763 m^2/g
Pore volume	0.000316 cm^3/g	0.001554 cm^3/g

Table 3
Effect of Cd(II) on PHE degradation by suspended and immobilized *Bacillus* sp. P1.

Concentration of Cd(II) (mg/L)	PHE degradation rate by suspended *Bacillus* sp. P1 (%)	PHE degradation rate by immobilized *Bacillus* sp. P1 (%)
0	92.05	98.62
50	86.52	94.91
100	78.49	90.70
200	70.73	85.36
300	66.28	79.64

was generated when $O_2^{\bullet -}$ was eliminated by SOD. So the CAT response rate to Cd(II) was slower than SOD. The small molecule antioxidant GSH could form a complex with Cd(II), which entered into the cells via active transport, Therefore, GSH depletion was observed. However, this consumption generated much more GSH to balance this depletion trend. The antioxidants would change in a dynamic process of continuous synthesis and consumption as the suppression of Cd(II).

Some researches reported that the PVA-SA improved the heavy metals adsorption by forming covalent bonds (Liu et al., 2012; Liao et al., 2018). Therefore, more heavy metals were released. The carriers would also concentrate pollutants increasing the effective contents of contaminants around micro-organisms. Also, PVA-SA was able to immobilize the extracellular secretions on the carrier, thus improving the contact efficiency between the pollutants and the degrading microbes (Chen et al., 2012). Moreover, PVA-SA with a large specific areas and a porous structure provided some protection for the bacteria as well, therefore increasing the amount of micro-organisms biomass. These effects of the immobilized materials on contaminants or micro-organisms were all related to the detoxification mechanisms of the microbes.

4. Conclusions

In this work, the results showed that cell immobilization made the system more effective to degrade phenanthrene in the presence of Cd (II) by altering the physical and chemical characteristics. Because of the aggregation effect and increased adsorption sites, phenanthrene removal rates by the immobilized cells were accelerated. In principle, immobilization protected *Bacilllus* sp. P1 from Cd(II) when degrading phenanthrene, thus delaying the oxidative stresses by altering the antioxidant enzymes activities (SOD and CAT) or by changing the antioxidant component contents (GSH). The SOD activity kept increasing from 52.23 to 473.35 U/mgprot for the immobilized system exposed to Cd(II) concentration between 0 and 300 mg/L. For CAT and GSH, immobilization only slowed down the depletion process without any change on the variation trends. The changes in surface properties and physiological responses of microbes caused the differences of immobilization effect on phenanthrene biodegradation in the presence of Cd(II), which is a novel finding.

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