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, P.R. China*

[b]*College of Chemistry and Material Engineering, Hunan University of Arts and Science, Changde 415000, Hunan, P.R.China*

[c]*Department of Dermatology, Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, P.R.China*

[d]*School of Minerals Processing and Bioengineering and Key Laboratory of Biometallurgy of Ministry of Education, Central South University, Changsha 410083, P.R. China*

[1]*These authors contributed equally to this article.*

** Corresponding author address: College of Environmental Science and Engineering, Hunan University, Changsha 410082, P.R. China. Tel.: +86 0731 88822754; Fax: +86 0731 88823701.*

E–mail addresses: zgming@hnu.edu.cn (G.M. Zeng)

*Corresponding author address: College of Environmental Science and Engineering,Hunan University, Changsha 410082, P.R. China. Tel.: +86 0731 88822754; Fax:+86 0731 88823701.

E-mail address: zgming@hnu.edu.cn (G.-M. Zeng)

[1] These authors contributed equally to this article.

Abbreviations and explanations

PAHs—polycyclic aromatic hydrocarbons

SOD—superoxide dismutase

CAT—catalase

GSH—glutathione

PVA—polyvinyl alcohol

SA—sodium alginate

Abstract

Suspended microbes gradually lost advantages in practical applications of PAHs and heavy metals bioremediadion. 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.

Keywords

Phenanthrene; Cd(II); Immobilization; *Bacillus* sp.; Detoxification

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; Cheng et al., 2016b; Oleszczuk et al., 2014). Amongst 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 (Zhang et al., 2017a; 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 studyare 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 degradationin 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 ºC 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 ºC for 24 h to reach equilibrium. The bacteria were inactivated in an autoclave at 121 ºC for 30 min. Since the bacteria were inactive and

133 metabolism-independent, the consumption rate of phenanthrene can be treated as the
134 adsorption rate. The cells concentration was maintained at 2×10^6 CFU/mL. For the
135 determination of the residual phenanthrene concentrations, the whole bacterial culture
136 were mixed with an equal volume of n-hexane in a 100 mL separatory funnel and
137 extracted (200 rpm) on an extraction shaker (JTLDZ-8) for 10 min. Extraction was
138 repeated three times and n-hexane phase were separated. All the experiments were
139 performed in triplicate.

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

151 To investigate the influence of immobilization on phenanthrene degradation in
152 the presence of different Cd(II) concentrations, a set of experiments with a
153 phenanthrene content of 50 mg/L and various Cd(II) contents (0, 50, 100, 200 and 300
154 mg/L) were prepared. The immobilized bacteria and non-immobilized *Bacillus sp*. P1
155 (2×10^6 CFU/mL) was inoculated in the systems and shaken for 48 h at 30 ºC and 150
156 rpm. Phenanthrene was extracted by n-hexane before being analyzed by a UV-visible
157 spectrophotometer at 254 nm (Giger and Blumer, 1974; Marsh et al., 2000). To test
158 the possibility of reusing the immobilized cells, the beads were washed with 0.5%
159 NaCl and distilled water before performing another phenanthrene degradation test in

160 the presence of 50 mg/L Cd(II) for five cycles, using the same conditions as in the
161 first test. Phenanthrene degradation properties at different pH (4.0, 5.0, 6.0 7.0, 8.0 and
162 9.0) and temperature (20, 30, 40 and 50 ºC) were studied to determine the advantages
163 of using immobilized cells.

164 2.4 Scanning electron microscopy (SEM)

165 SEM (FEI QUANTA 200, Czech) was performed to investigate the modification
166 of *Bacillus sp*. P1 by immobilization with or without Cd(II). The immobilized cells
167 and non-immobilized bacteria were dehydrated with a series of ethanol concentrations
168 (50%, 60%, 80% and 100% ethanol in distilled water). They were air dried before
169 being coated with gold and observed at 20 kV.

170 2.5 Detoxification indices

171 Enrichment of phenanthrene and Cd(II) can increase the level of reactive oxygen
172 species ($O_2^{\bullet-}$, $^{\bullet}OH$ and H_2O_2), thus providing a detoxification mechanism, and
173 expressing a high antioxidant capacity. Antioxidant enzymes, such as superoxide
174 dismutase (SOD), catalase (CAT) and low molecular weight antioxidant components
175 such as glutathione (GSH), are able of removing reactive oxygen radicals such as $O_2^{\bullet-}$,
176 $^{\bullet}OH$ and H_2O_2. Therefore, SOD activities, CAT activities and GSH contents were
177 determined in this study. Bacteria were sonicated (300 W, 3 s/8 s) for 10 min at 4 ºC
178 with subsequent centrifugation (9000 rpm, 4 ºC). The supernatants were used for the
179 determination of detoxification indices. Assay kits of SOD activities, CAT activities
180 and GSH contents (Jiancheng bioengineering institute, Nanjing, China) were utilized.
181 The total protein content of the bacteria was also analyzed by enzyme-linked
182 immunosorbent assay kits based on BCA methods to determine the SOD, CAT and
183 GSH values (Zhang et al., 2017). SOD activity was measured by a modified nitrite
184 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 miligram 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:

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

(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 transferand 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.

Please insert Figure 1

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 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 the growth, motion and other physiological processes caused by the microbes. Therefore the adsorption capacity of the immobilized cells was improved.

Please insert Table 1 and Figure 2

Please insert Table 2 and Figure 3

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 (Xu et al., 2016a; 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).

Please insert Table 3

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(II) on the cells are illustrated in Fig. 4. These micrographs confirm that a more porous structures is present on the immobilized gel beads (Fig. 4a and 4c) compared to non-immobilized cells (Fig. 4b and 4d), 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.

Please insert Figure 4

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.

Please insert Figure 5

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

The effect of pH and temperatureon the immobilized and suspended cells in phenanthrene degradation in the presence of Cd(II) is reported in Fig. 6a and 6b, 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 occured 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 ºC, 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.

Please insert Figure 6

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; Zhang et al., 2017b; 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. 8a 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 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. 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

346 stresses related to Cd(II) increased (Garg and Chandel, 2015). However, when an
347 excessive SOD consumption occurs, the SOD synthesis capacity drop, thus limiting
348 the SOD production and activity (Pramanik et al., 2017; Cao et al., 2012). This could
349 explain that without the gel protection effect, the SOD activity decreased at the
350 highest Cd(II) content (300 mg/L).

351 The variation of CAT activity for the immobilized and non-immobilized *Bacillus*
352 *sp.* P1 are shown in Fig. 7b. By increasing the Cd(II) concentration from 0 to 300
353 mg/L, the CAT activity increased from 1.46 and 12.74 to 23.63 and 30.09 U/mgprotin
354 immobilized and non-immobilized cells, respectively. The PVA-SA carrier helped the
355 bacteria to survive to the environmental conditions, especially being in contact with
356 phenanthrene and Cd(II). Therefore, the oxidative damage was minimized (Chen et al.,
357 2007; Haghighi et al., 2017). The scavenging capacity was higher than the ROS
358 production rate based on CAT activitysince CAT activity kept increasing instead of
359 decreasing with increasing Cd(II) concentration from 0 to 300 mg/L (Tang et al.,
360 2014).

361 GSH, especially reduced GSH, was very significant in keeping the ROS balance
362 related to PAHs and heavy metals. Fig. 7c reports on the total GSH and reduced GSH
363 content in immobilized and non-immobilized cells exposed to different Cd(II) content
364 when degrading 100 mg/L of PHE. For the immobilized system, reduced GSH content
365 increased from 13.11 to 46.62 µmol/L with increasing the Cd(II) concentration from 0
366 to 200 mg/L, and then dropped to 34.96 µmol/L when exposed to 300 mg/L Cd(II).
367 Likewise, a maximum value (466.28 µmol/L) of total GSH was found at 200 mg/L
368 Cd(II) before decreasing when exposed to higher Cd(II) concentration. This behavior
369 can be attributed to the fact that GSH act as a reduction agent or a substrate for ROS
370 scavenging at high Cd(II) concentration (> 200 mg/L) (Xu et al., 2016b; Xu et al.,
371 2012b). Immobilization did not change this trend. Similarly, the reduced GSH and
372 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.

Please insert Figure 7

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 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 reseaches 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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