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Effect of Pb(II) on phenanthrene degradation by new isolated *Bacillus* sp. P1

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Highlight

A new strain of bacteria *Bacillus sp.* P1 was isolated from compost to degrade PHE in the presence of Pb(II)

Abstract

A polycyclic aromatic hydrocarbons (PAHs)–degrading microbe was isolated and the effects of Pb(II) on the degradation of phenanthrene (PHE) by this microbe were studied in this research. The changes of metabolism, protein content, enzyme activities and PHE degradation induced by Pb(II) were investigated to elucidate the mechanism of Pb(II) affecting the degradation of PHE. In the system with Pb(II), protein content, catechol 2,3–dioxygenase activity and phenanthrene–degrading ability of enzymes were all enhanced at low concentration of Pb(II) (below 50 mg/L) but decreased at relatively higher concentrations (100 to 300 mg/L) of Pb(II). These results form the basis that the presence of Pb(II) is relevant to the enzyme changes of PAHs–biodegrading microbe and has helped in the process of biodegradation of PHE with low concentrations, but has not helped PHE biodegradation with excessive concentrations.

Keywords

Phenanthrene; Pb(II); Degradation; *Bacillus* sp.; Enzyme

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) can be appended into the environment through nature force or anthropogenic activities, ranging from industrial behaviors to

residential activities.[1] They can exert a detrimental effect on both the environment and human health because of their toxicity, mutagenicity and carcinogenicity. PAHs can be removed by adsorption, volatilization, photolysis, and degradation.[2, 3] Amongst these methods, biodegradation is becoming a popular process due to the advantages of low cost and non–secondary pollution.[4-6] Present studies indicate that the characteristic of composting system with large biomass of microorganisms is capable of decomposing organics such as pentachlorophenol and phenolic compounds.[7, 8] Low molecular weight PAHs such as naphthalene (NAP), anthracene (ANT) and phenanthrene (PHE) can also be degraded by microorganisms efficiently and effectively.[9, 10]

PAHs and heavy metals are prevalently occurred together in contaminated environment such as manufactured coking plant, railway yards and refinery sites, and the coexisting heavy metals may affect the PAHs biodegradation.[11] Niu et al.[12] pointed out that Pb(II) inhibited the biodegradation of PHE during composting the soil with combined pollutants of PHE and Pb(II). Yang et al.[13] demonstrated that Cd(II) significantly inhibited the activities of catalase, urease, phosphatase and invertase, thereby inhibiting the degradation of PAHs. Shen et al.[14] found that the activities of urease and dehydrogenase were far more inhibited by the synergistic inhibitory effect of Zn and PHE than the individual effect. Ke et al.[15] observed that metal ions posed a positive effect on the degradation of low molecular weight PAHs (fluorene (FLU) and PHE). Increasing studies have paid attention to the combined pollution of PAHs and

heavy metals,[16-18] mechanisms of heavy metals affecting PAHs degradation were still not deeply understood. Moreover, enzymes activities, which were involved in the main function of PAHs degradation, may change by heavy metals, thus affecting the PAHs degradation process. However, few studies combined analysis of enzyme changes resulted by PAHs and heavy metals.

In this work, a PAHs–degrading microorganism *Bacillus* sp. P1 was isolated. PHE is a three-ring PAH which is often considered as a model of PAHs because it is the smallest PAH to have a "K-region" and a "bay-region", which were basic structures shared with other carcinogenic PAHs. Lead is widely spread in the environment and can cause severe health effects to human beings with excessive concentrations. So PHE and lead were selected in this experiment as the typical and ubiquitous models of PAHs and heavy metal, respectively. The objectives of this research were to (1) investigate the effect of Pb(II) on PHE degradation by PAHs-degrading microbe; (2) discuss the variations of protein content, activities and phenanthrene–degrading ability of enzymes extracted from PAHs-degrading microbe in the presence of Pb(II), which may impact PHE degradation rate.

2. Materials and methods

2.1 Microorganism and medium

The bacterium was isolated with PAHs (containing fluoranthene (FLT), pyrene (PYR), PHE, and FLU) as sole carbon and energy source from the composting samples according to the previous studies.[12, 19] The 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） was used in this work.[20] The molecular identification was performed by TaKaRa Biotechnology (Dalian, China). The obtained 16S rDNA sequence and the similar sequences from National Center for Biotechnology Information (NCBI) were used to construct the phylogenetic tree. Branch support was assessed using 1000 bootstrap replicates.

2.2 PHE biodegradation by *Bacillus sp.* P1

Effect of Pb(II) on the biodegradation of PHE by *Bacillus sp.* P1 was assessed as follows. PHE stock solution was added to 100 mL mineral medium to obtain a final PHE concentration of 60 mg/L. The group without Pb(II) was served as control. Testing group was set with 60 mg/L PHE and 100 mg/L Pb(II).[12] Both groups were sterilized before inoculation. The isolated strain with an inoculum size (OD_{600}) of 0.8 was incubated at 30 ºC with 150 rpm. Residual PHE concentration was determined daily. PHE was extracted by n–hexane before being detected by a UV–visible spectrophotometer (SHIMADZU, UV 2550) at 254 nm.[21] All samples were analyzed in triplicate. The degradation rate of PHE calculated according to the equation $R = (C_0\text{-}C_t)/C_0$, where R is the degradation rate of PHE, C_0 is the initial concentration of PHE, and C_t is the final concentration of PHE.

99 2.3 Detection of PHE biodegradation intermediates

100 The effects of Pb(II) on metabolites of PHE biodegradation were analyzed by gas
101 chromatograph-mass spectrometer (GC–MS, QP2010, Shimadzu). The isolated strain
102 was cultured in mineral medium systems with PHE (60 mg/L) and PHE–Pb(II) (PHE,
103 60 mg/L; Pb(II), 100 mg/L) respectively for 2 days at 30 ºC with 150 rpm. The
104 supernatant after centrifugation (15 min, 9000 rpm) was extracted in dichloromethane
105 and then dried over by anhydrous sodium sulfate before used for the assessment of
106 metabolites. The injector temperature was programmed to 270 ºC. Helium was served
107 as the carrier gas at a rate of 3 mL/min. The column temperature program was set as
108 follows: 120 ºC for 1 min, and then 3 ºC /min up to 280 ºC.[22]

109 2.4 Extraction of extracellular enzymes and intracellular enzymes

110 The centrifuged supernatants (9000 rpm, 4 ºC) after culturing for 4 days in
111 mineral medium were extracellular proteins, which were approximately considered as
112 crude extracellular enzymes (EE). The residues were washed by phosphate buffer
113 (0.05 mM, pH 6.8) for three times and then sonicated (300 W, 3 s/8 s) for 10 min at 4
114 ºC with subsequent centrifugation (9000 rpm, 4 ºC). The supernatants were
115 intracellular proteins, which can similarly be seen as crude intracellular enzymes
116 (IE).[23]

117 2.5 Analysis of extracellular and intracellular protein content

118 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

fingerprint analysis was carried out to investigate the effects of Pb(II) on the protein composition extracted from the PAHs–degrading microbe. SDS–PAGE was performed according to the protocol of Laemmli [24, 25] with 12% polyacrylamide gel, using a mini–protein vertical electrophoresis system (Bio–Rad, USA). The samples were secretase extracted from the bacteria cultivated in PHE (60 mg/L) and PHE–Pb(II) (PHE, 60 mg/L; Pb(II), 50, 100, 150, 200, 300 mg/L) medium, respectively. They were salted and dialyzed before loading into the gel. SDS–PAGE gel was stained with Coomassie brilliant blue R–250 and the molecular mass of the samples were determined by the relative mobility of the stained SDS–PAGE marker running alongside.

2.6 Determination of catechol 2,3–dioxygenase activity

Catechol 2,3–dioxygenase is the key enzyme involved in the degradation of aromatic hydrocarbons.[26, 27] Crude extracellular and intracellular enzymes were extracted from PAHs–biodegrading microbe cultivated in PHE (60 mg/L) and PHE–Pb(II) (PHE, 60 mg/L; Pb(II), 50, 100, 150, 200, and 300 mg/L) medium for 2 days at 30 ºC with 150 rpm, respectively for the detection of catechol 2,3–dioxygenase. The mixture containing 2.4 mL phosphate buffers (0.05 mM), 0.4 mL catechol (20 μM) and 0.2 mL EE (or IE) were determined for the activity of catechol 2,3–dioxygenase at 375 nm by UV–visible spectrophotometer. One unit of its activity was defined as 1 μmol of substrate converted per minute. The soluble protein content was measured following the procedure described by Bradford.[28]

140 2.7 Assessment of Pb(II) on enzymes extracted from PAHs–biodegrading microbe

141 Conditions of pH and temperature were determined for a thorough understanding
142 of the effect of Pb(II) on PHE degradation. Experiments were conducted in five
143 Erlenmeyer flasks each containing 20 mL ultrapure water and 25 mg/L of PHE. The
144 pH value was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 with HCl or NaOH, respectively.
145 Enzymes (2 mL) were added into each flask and then it was shaken for 30 min at 30
146 ºC with 150 rpm. Moderate temperatures were set as 25, 30 and 37 ºC, respectively.
147 Group with enzymes, which were inactive because of the high temperature, was used
148 as a control for enzymatic biodegradation of PHE.

149 To investigate the degradation of PHE by enzymes, 2 mL enzymes were added
150 into 50 mL ultrapure water containing 5 mg/L PHE, followed by the addition of
151 various concentrations of Pb(II) (0, 50, 100, 150, 200, and 300 mg/L). Flasks were
152 incubated at 30 ºC and agitated at 150 rpm. PHE was extracted and determined after
153 30 min as the methods described in 2.2.

154 2.8 Statistical analysis

155 Statistically significant differences between the results were evaluated on the
156 basis of standard deviation determinations and the one–way analysis of variance
157 (ANOVA, 95% confidence intervals test) using the SPSS 19.0 software.

3. Results and Discussion

3.1 Effects of Pb(II) on PHE biodegradation by PAHs–biodegrading microbe

The bacteria isolated from compost belong to genus *Bacillus* and the highest homology obtained by this strain was 100% of similarity to *Bacillus malacitensis.* The 16S rDNA sequence was submitted to the GenBank and given the accession number KP888558. The phylogenetic tree based on a multiple sequence alignment of the 16S rDNA sequence is presented in Fig. 1. This strain was named as *Bacillus* sp. P1.

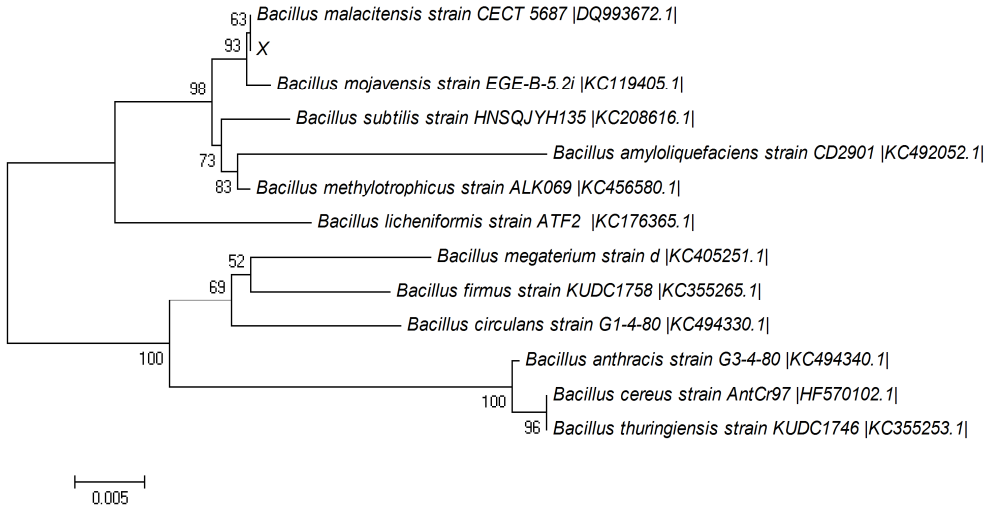
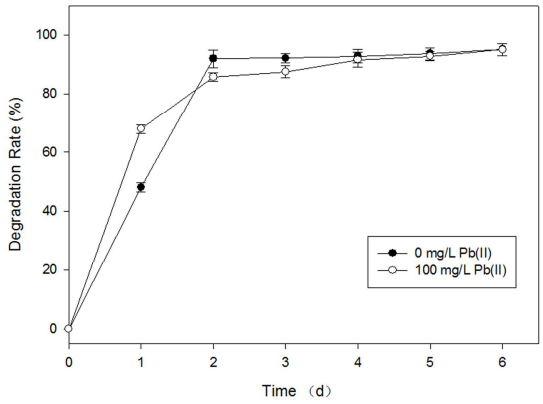


Fig. 1 Phylogenetic tree established by the neighbor-joining method based on the 16S rDNA sequence of Bacillus sp. P1 and the similar sequences obtained from NCBI.

Bacillus sp. P1 possessed satisfactory potential in degrading PHE according to Fig. 2. PHE in both media (with and without Pb(II)) were degraded significantly ($p < 0.05$) in the first two days. In this period, degradation rates in PHE (60 mg/L) and PHE–Pb(II) (PHE, 60 mg/L; Pb(II), 100 mg/L) media were rapidly increased to 91.9% and 85.7%, respectively. Degradation rate of PHE in the medium with Pb(II)

was higher than that in the medium without Pb(II) on the first day, mainly due to that Pb(II) increased PHE adsorption by *Bacillus* sp. P1, because Pb(II) might enhance the formation of *Bacillus* sp. P1–Pb(II)–PHE ternary surface complexes.[29, 30] *Bacillus* sp. P1 began to grow logarithmically on day 2 and promoted a higher rate of PHE degradation. During the 6-day incubation, PHE degradation reached a plateau in the two groups with the degradation rate of 95.1%. Degradation rates of PHE in the medium with Pb(II) were lower compared to medium without Pb(II) from day 2 to 6, because Pb(II) hindered the decay of PHE, which might be attributed to the impediment of Pb(II) to *Bacillus sp.* P1 and resulted in lower degradation rates of PHE. *Bacillus sp.* P1 could be impacted by Pb(II) for the following three reasons: (1) The motor function of the cytoskeletons was hindered by Pb(II), which might consequently retard cytokinesis of *Bacillus sp.* P1;[31] (2) Pb(II) could enhance the oxidation process produced by reactive oxygen species (ROS), thereby resulting in further damage to the cell organelles;[15, 32] (3) Pb(II) could penetrate through the cell wall of bacteria to oxidize the surface proteins on the plasma membrane directly and consequently disturb the cell homeostasis.[33, 34]



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190 **Fig. 2** Effects of Pb(II) on PHE biodegradation by Bacillus sp. P1 with respect to incubation time

191 at 30 ºC. The error bars represent ±1 standard deviation.

192 The intermediates of PHE were investigated in this study according to Fig. 3.

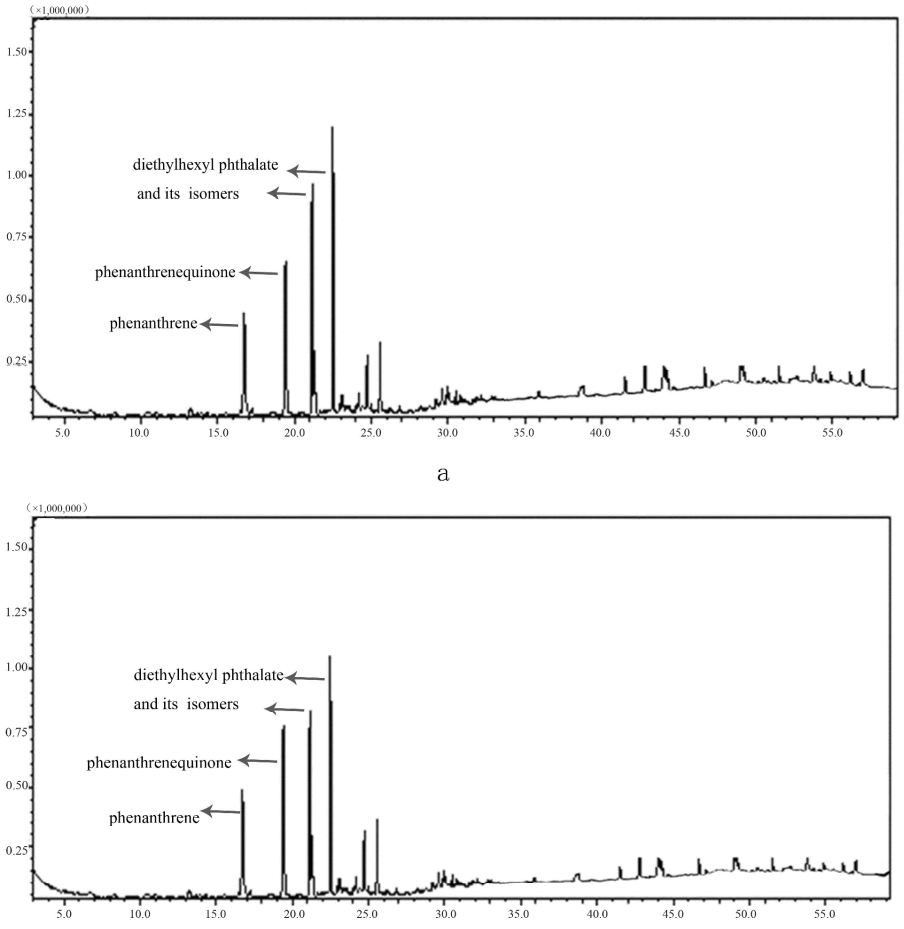
193 Qualitative analysis was based on retention indexes, mass spectra comparison with

194 data in the NIST08 library. The major metabolites detected in both PHE and

195 PHE–Pb(II) system were diethylhexyl phthalate (m/z 93, 104, 122, 149, 167, 205, 223)

196 and phenanthrenequinone (m/z 76, 152, 180, 208).[35] The result suggested that

197 *Bacillus* sp. P1 degraded PHE via protocatechuate pathway.



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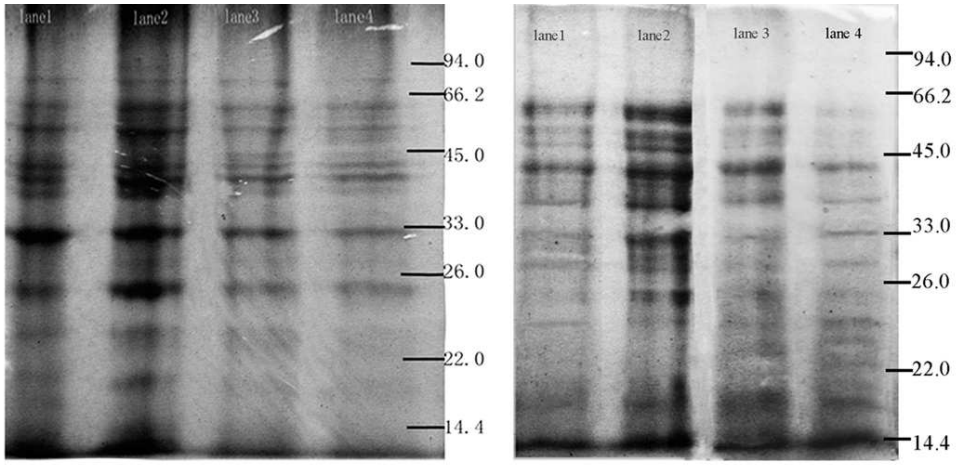
199 **Fig. 3** Effects of 0 mg/L (a) and 100 mg/L (b) Pb(II) on the intermediates of PHE.

200 3.2 Effects of Pb(II) on protein content of *Bacillus* sp.P1

201 The alterations of the extracellular protein (EP) and intracellular protein (IP)
202 concentrations were perceived through analyzing SDS–PAGE fingerprint by Gelpro
203 software (Fig. 4). With the increase of Pb(II) from 0 to 50 mg/L, the total contents
204 (absolute integrated optical density) of EP and IP increased from 5.8×10^5 to $6.4 \times$
205 10^5, and from 7.2×10^5 to 7.5×10^5, respectively, and then declined from 4.2×10^5 to
206 4.1×10^5, and from 7.1×10^5 to 5.3×10^5 respectively with the increase of Pb(II) from

100 to 150 mg/L. The change of expression of the total protein might be attributed to that gene clusters involved in the degradation of PHE were induced by Pb(II). Besides, hormesis induced by Pb(II) could cause the increase of proteins, which were planned to protect the cells from the toxic effect of Pb(II) by exporting the cation. The proteins finally could be brought down due to the toxic effect of Pb(II) on *Bacillus sp.* P1.[36] The protein bands with molecular mass of 33–45 kDa of EP (IP) increased from 8.6×10^4 to 1.3×10^5 (from 1.7×10^5 to 2.5×10^5), then decreased from 8.3×10^4 to 7.2×10^5 (from 1.4×10^5 to 1.2×10^5). It is probable that low concentration of Pb(II) could stimulate the release of the key ring–opening enzyme, thus promoting the degradation rate of PHE.



a b

Fig. 4 SDS-PAGE fingerprint analysis of extracellular proteins (a) and intracellular proteins (b). Lane 1 to 4 represents the proteins extracted from bacteria cultivated in the presence of 0, 50, 100, 150 mg/L Pb(II), respectively.

221 3.3 Effects of Pb(II) on catechol 2,3–dioxygenase activity

222 Table 1 lists the catechol 2,3–dioxygenase activity in EE and IE in the presence
223 of different concentrations of Pb(II) during the process of degrading PHE. Results
224 showed that remarkable variation ($p < 0.05$) in catechol 2,3–dioxygenase activities of
225 EE and IE could be observed in the presence of Pb(II) during the process of degrading
226 PHE. The activity of catechol 2,3–dioxygenase in EE showed a circuitous profile in
227 the presence of Pb(II). The activity increased in the presence of low concentration of
228 Pb(II) (below 50 mg/L), but conversely dropped down in the presence of high
229 concentration of Pb(II) (above 100 mg/L). For IE, the activity of catechol
230 2,3–dioxygenase increased with the increase of Pb(II) concentration and reached a
231 maximum in the system with 50 mg/L Pb(II), then declined as the Pb(II)
232 concentration increased. These results were consistent with the results demonstrated
233 in Fig. 4, indicating that catechol 2,3–dioxygenase was involved in degrading PHE
234 due to the positive correlation between PHE utilization and the activity of catechol
235 2,3–dioxygenase in the presence of low concentration of Pb(II). It might be possible
236 that enzyme activities were inhibited by Pb(II) by denaturing the protein structure or
237 masking catalytically active groups of enzymes.[37] The latter decreases in catechol
238 2,3–dioxygenase activities could be ascribed to the direct suppression of Pb(II) on
239 microbial growth.

240 **Table 1** Effects of different concentrations of Pb(II) on catechol 2,3-dioxygenase activity and total
241 protein of PHE-degrading bacteria.

Concentration of Pb(II) (mg/L)	Content of protein (μg)		Specific Activity of catechol 2,3-dioxygenase (U/mg)	
	Extracellular enzymes	Intracellular enzymes	Extracellular enzymes	Intracellular enzymes
0	132.5	117.5	274.6	890.1
50	186.7	133.3	296.1	1003.3
100	131.7	112.7	244.0	784.9
150	129.2	80.5	203.8	760.2
200	102.6	73.9	133.4	413.1
300	95.3	70.6	99.3	304.5

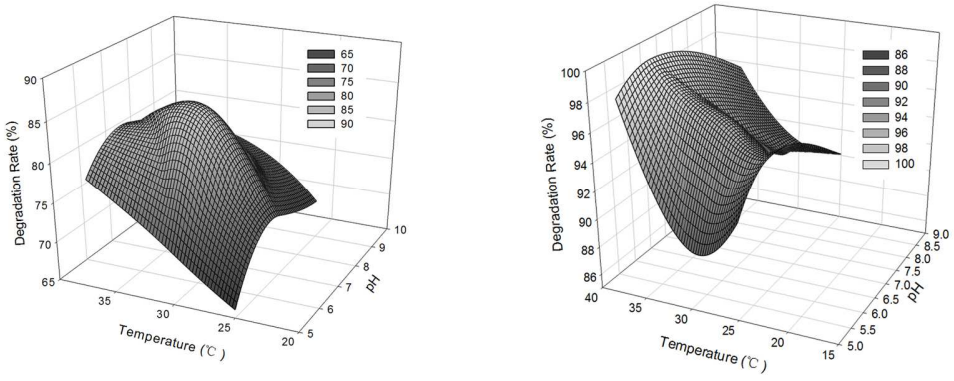
3.4 Effects of Pb(II) on PHE degradation by enzymes

3.4.1 Effects of pH and temperature on enzymatic degradation of PHE

PHE degradation by EE and IE as a function of pH and temperature is shown in Fig. 5. Both EE and IE could remove PHE from the medium. In EE medium, the highest removal rate of PHE was 88.1% at pH 7.0. Compared with EE, IE presented higher degradation ability of 97.2% under optimal pH of 6.0. The pH could affect the state of ionization of acidic or basic amino acids, thus altering the shape of the protein.[38] Extreme pH was considered as an inappropriate system for the degradation of PHE, which would cause an adverse effect on enzyme activities.

PHE degradation by EE and IE increased with the increase of temperature from 25 to 37 ºC, and degradation rates by EE and IE were 71.8% and 99.8% at 37 ºC,

253 respectively. Higher temperature could promote the enzymatic activities. Besides, the
254 solubility of PHE could increase with the increase of temperature, therefore leaded to
255 a higher bioavailability, which was governed by solubility.[39] IE displayed higher
256 degradation ability than EE, which could be attributed to the fact that more
257 dioxygenase enzymes involved in the steps of PHE catabolism such as Naphthalene
258 1,2–dioxygenase, were located in IE.[40]



259

260 **Fig. 5** Effects of pH and temperature on PHE degradation by extracellular
261 enzymes (a) and intracellular enzymes (b).

262 3.4.2 Effects of Pb(II) on enzymatic degradation of PHE

263 Dynamic patterns of PHE degradation by EE and IE under the exposure of
264 different concentrations of Pb(II) are illustrated in Fig. 6. For EE, experiment was
265 performed in a system at pH 7.0. The degradation rate increased initially from 68.4%
266 to 74.7% until the Pb(II) levels reached 50 mg/L, and then decreased gradually to
267 63.9% with the increase of Pb(II) levels. For IE, experiment was performed at pH 6.0.
268 The degradation rate increased from 85.2% to 87.0% as the ascending of Pb(II) from

0 to 50 mg/L, and then decreased from 84.9% to 80.6% as Pb(II) ascended from 100 to 300 mg/L. The degradation rate of PHE in control group was 2.8% (data are not shown). The results indicated that low concentration of Pb(II) (under 50 mg/L) had a stimulating effect on the degradation of PHE, while high concentration of Pb(II) (100-150 mg/L) had an inhibitory influence. PHE degradation under low concentration of Pb(II) stress was enhanced probably due to the formulation of enzyme–metal–substrate complexes. [41] Pb(II) might work as cofactors to bind a protein and be required for the protein's biological activity. Meanwhile, superabundant Pb(II) could compete with macronutrient (Mg^{2+}, Ca^{2+} et al.), which were commonly used as cofactors for the formulation of enzyme–metal–substrate complexes.[42] Additionally, they could also restrain the activity of enzymes by combining with sulfhydryl groups of proteins.[36]

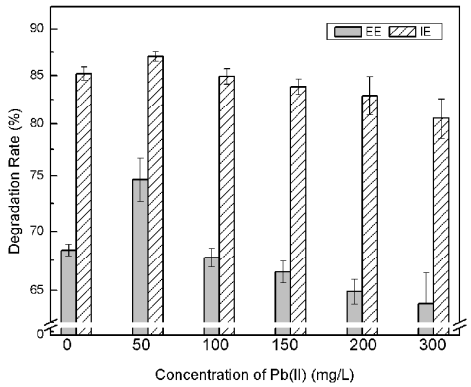


Fig. 6 Effects of Pb(II) on PHE degradation by extracellular enzymes and intracellular enzymes.

4. Conclusions

Low concentration of Pb(II) had a positive effect on PHE degradation, while

285 high concentration of Pb(II) had a side effect on degradation of PHE. Protein content,
286 activity of catechol 2,3–dioxygenase and PHE degradation by enzymes were all
287 gently enhanced under the exposure of relatively low concentration of Pb(II) due to
288 the formulation of enzyme–metal–substrate complexes, and the three indexes were all
289 inhibited by high concentration of Pb(II) because of its influence on enzymes of
290 PAHs–degrading microbe. The aforementioned results suggested that Pb(II) could
291 impact PHE biodegradation via changing protein content, enzyme activities and
292 phenanthrene–degrading ability of enzymes secreted by *Bacillus* sp. P1, which could
293 make contributions to the mechanism studies of combined pollution of heavy metals
294 and PAHs.

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