Effect of heavy-metal on synthesis of siderophores by *Pseudomonas aeruginosa* ZGKD3

Peili Shi[1], Zhukang Xing[2], Yuxiu Zhang[1, 4], Tuanyao Chai[3]

[1] Department of Environmental & Biological Engineering, School of Chemical & Environmental Engineering, China University of Mining & Technology (Beijing), D11 Xueyuan Road, Beijing 100083;
[2] Huozhou Secondary School of Shanxi, Huozhou 031400, China;
[3] College of Life Science, University of Chinese Academy of Sciences, A19 Yuquan Road, Beijing 100049, PR China.

E-mail: zhangyuxiu@cumtb.edu.cn

Abstract. Most siderophore-producing bacteria could improve the plant growth. Here, the effect of heavy-metal on the growth, total siderophore and pyoverdine production of the Cd tolerance *Pseudomonas aeruginosa* ZGKD3 were investigated. The results showed that ZGKD3 exhibited tolerance to heavy metals, and the metal tolerance decreased in the order $Mn^{2+}>Pb^{2+}>Ni^{2+}>Cu^{2+}>Zn^{2+}>Cd^{2+}$. The total siderophore and pyoverdine production of ZGKD3 induced by metals of Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+} and Mn^{2+} were different, the total siderophore and pyoverdine production reduced in the order $Cd^{2+}>Pb^{2+}>Mn^{2+}>Ni^{2+}>Zn^{2+}>Cu^{2+}$ and $Zn^{2+}>Cd^{2+}>Mn^{2+}>Pb^{2+}>Ni^{2+}>Cu^{2+}$, respectively. These results suggested that ZGKD3 could grow in heavy-metal contaminated soil and had the potential of improving phytoremediation efficiency in Cd and Zn contaminated soils.

1. Introduction

Soil contamination with heavy metals has become increasingly prominent with the rapid development of human industry and agriculture which causes great hazard to natural environment [1]. Heavy metal ions could be absorbed by plant root and then transport from roots to shoots and cause a considerable threat to human health through food chain due to its high toxicity [2]. Nowadays, in comparison with conventional methods such as physical separation, washing and stabilization, more attention has been paid to plant-microorganism combined bioremediation phytoremediation [3-4]. Various studies have reported that heavy-metal tolerant microbes can directly improve the efficiency of phytoremediation, the possible strategies including metal mobilization, metal chelation and oxidation/reduction reactions [5-7]. In particular, many metal resistant bacteria are capable of synthesize siderophores which provides benefits to plants [8-9]. Siderophores play an important role in phytoremediation due to its strong affinity for Fe^{3+}, Cu^{2+}, Zn^{2+}, $Ni^{2+}$and Cd^{2+}. Siderophore-producing *Pseudomonas aeruginosa* increased the concentrations of Cr and Pb in maize by mobilizing Cd and Pb in soils [10]. Sharma reported that siderophore-producing *P. aeruginosa* GRP_3 alleviated the chorotic symptoms and significantly enhanced chlorophyll content and biomass of *Vigna radiate* L [11]. Ni tolerant endophytic bacteria isolated from *Alyssum bertolonii* were capable of producing siderophores, enhanced the biomass and Ni accumulation of inoculated plants [12]. Similarly, Dimkpa found that siderophores produced by *Streptomyces acidiscabies* E13 alleviated oxidative stress induced by heavy metal of cowpea [13]. A large amount of siderophore-priducing bacterial were screened and applied to improve phytoremediation efficiency, and it has been evidenced that siderophores produced by bacteria can protect microbes against the toxicity of heavy metals [14-15], whereas the mechanism of heavy-metal tolerance of siderophore-producing bacteria is still unknown.

In the present study, *P. aeruginosa* ZGKD3 isolated from soil contaminated by gangue pile of coal area in our laboratory exhibited high tolerance to Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+} and Mn^{2+}. The objectives of this study were to investigate the effect of different concentrations of multiple heavy metals on synthesis of siderophores of *P. aeruginosa* ZGKD3. Furthermore, determine the potential of ZGKD3 in improving phytoremediation efficiency in heavy-metal contaminated soils and these results may provide a base for revealing the heavy metal tolerance mechanism of bacteria.

2. Materials and Methods

2.1. Bacteria and media.

The heavy-metal resistant *P. aeruginosa* ZGKD3 were isolated from soil contaminated by gangue pile of coal area of Shanxi province in our laboratory. ZGKD3 were cultivated in broth medium which contained 3.0 g beef extract, 10.0 g peptone, and 5.0 g sodium chloride per liter with an initial pH of 7.2 and MSA (sugar-Asp) medium which contained 20.0 g sucrose, 2.0 g aspartic acid, 1.0 g K_2HPO_4 and 0.5 g $MgSO_4$ per liter with an initial pH of 7.2 [16].

2.2. The growth of ZGKD3 under heavy-metal stress.

The growth of *P. aeruginosa* ZGKD3 under different concentrations of heavy-metal stress was assayed in MSA. ZGKD3 were grown on nutrient broth medium for 16 h, 2 ml cells of ZGKD3 were inoculated into 100 ml Erlenmeyer flasks containing 50 mL of sterile MSA medium with $CdCl_2$, $CuCl_2$, $ZnCl_2$, $NiCl_2$, $Pb(NO_3)_2$ and $MnCl_2$ (0, 200, 400 and 1000 μM), respectively, and incubated in an rotary shaker (150 rpm) at 37 °C for 24 h, the effect of heavy-metal on growth and the ability of alkaline production of ZGKD3 were investigated at 16 h. The biomass of bacterial cell was determined by a UV-Visible spectrophotometer, the absorbance was measured at 600 nm (OD_{600}). All of the chemical reagent were analytical reagent.

2.3. Quantitative analysis siderophore synthesis by ZGKD3 under heavy-metal stress.

The effects of different concentrations of heavy-metal on siderophore synthesis of ZGKD3 were test. Bacterial samples were obtained from MSA medium contained $CdCl_2$ (0, 200, 400, 1000 and 3000 μM), $CuCl_2$, $ZnCl_2$, $NiCl_2$, $Pb(NO_3)_2$ and $MnCl_2$ (0, 200, 400 and 1000 μM), respectively. Detection of total siderophores and pyoverdine production by ZGKD3 were carried out, the total siderophore production was assayed by chromo azurol S (CAS) plate assay [17]. For quantification of siderophore and pyoverdine production were investigated at 12, 24 and 48 h by a UV-Visible spectrophotometer at the absorbance of 630 and 400 nm, respectively.

2.4. Statistical analysis.

All data were analyzed by SPSS 16.0 for significant differences ($P < 0.05$). Statistical analyses were performed by one-way ANOVA.

3. Results

3.1. The growth of ZGKD3 in MSA medium under heavy metal stress

The effect of six heavy metals on growth of ZGKD3 was assayed. As shown in Figure 1, the concentrations of Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+} and Mn^{2+} (200, 400, 1000 μM) inhibited the growth of ZGKD3 and as a consequence of the increasing dose of heavy meal, the stronger inhibitive effects on growth of bacterial were observed, suggested that there is a significant negative correlation between the heavy-metal concentration and the growth of ZGKD3. Moreover, ZGKD3 could tolerate Ni^{2+}, Pb^{2+}

and Mn^{2+} at the concentrations of 200, 400 and 1000 μM, and the growth of ZGKD3 was significantly inhibited by Cd^{2+}, Zn^{2+} and Cu^{2+} at the concentration of 200 μM. The strongest inhibitory effect on the growth of ZGKD3 was observed at the high concentrations of Cd^{2+}, the biomass (OD_{600}) of ZGKD3 dreased from 0.792 to 0.098 with the increasing dose of Cd^{2+}. Thus, the effects of various heavy metals on growth of ZGKD3 were different.

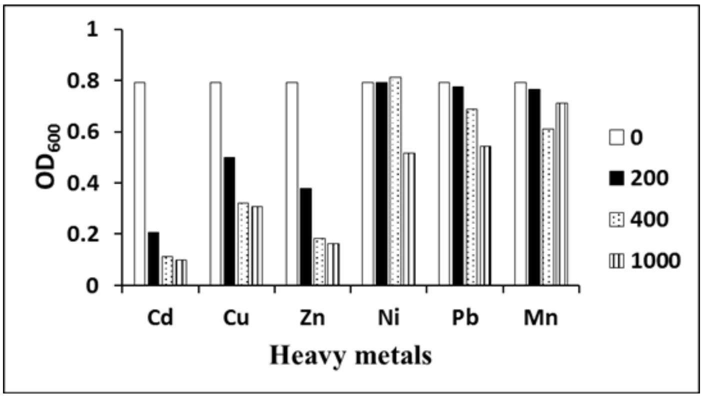

Figure 1. Effect of heavy metals on the growth of strain ZGKD3 in MSA medium contained heavy metals at the concentration of 0, 200, 400 and 1000 μM at 16 h

3.2. The total siderophore and pyoverdine production of ZGKD3 in MSA medium under heavy metal stress

Siderophore production of ZGKD3 was confirmed by adding the culture supernatant into the holes on CAS agar plate and orange haloes were observed. Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+} and Mn^{2+} exhibited different effects on siderophore production of bacteria (Figure 2). The largest orange haloes was found in the Zn^{2+} group, next is Cd^{2+}. Significant increase in the total siderophore and poverdine production of strain ZGKD3 was found, with the increase of 8-90% for 200-1000 μM of Cd^{2+} and 35-242% for 200-1000 μM Zn^{2+}. However, Cu^{2+} significantly inhibited the total siderophore and poverdine production of strain ZGKD3, and the reduction was range from 26-88%. In comparison with control, Ni^{2+}, Pb^{2+} and Mn^{2+} had no markedly difference in total siderophore and poverdine production (Figure 3 and 4), the total siderophore and pyoverdine production reduced in the order $Cd^{2+}>Pb^{2+}>Mn^{2+}>Ni^{2+}>Zn^{2+}>Cu^{2+}$ and $Zn^{2+}>Cd^{2+}>Mn^{2+}>Pb^{2+}>Ni^{2+}>Cu^{2+}$, respectively.

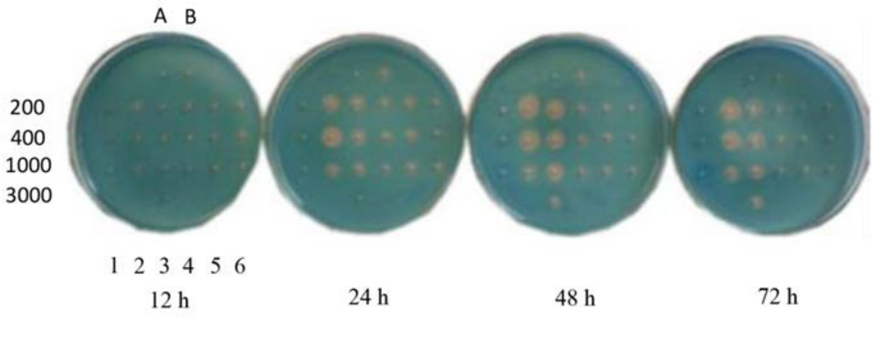

Figure 2. Pyoverdine production by ZGKD3 exposed to heavy metals at the concentrations of 200, 400, 1000 and 3000 μM. The orange haloes on each plate are: A and B in No.1 row indicate negative control and positive control, respectively. The numbers from 1to 6 indicate Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+} and Mn^{2+}, respectively.

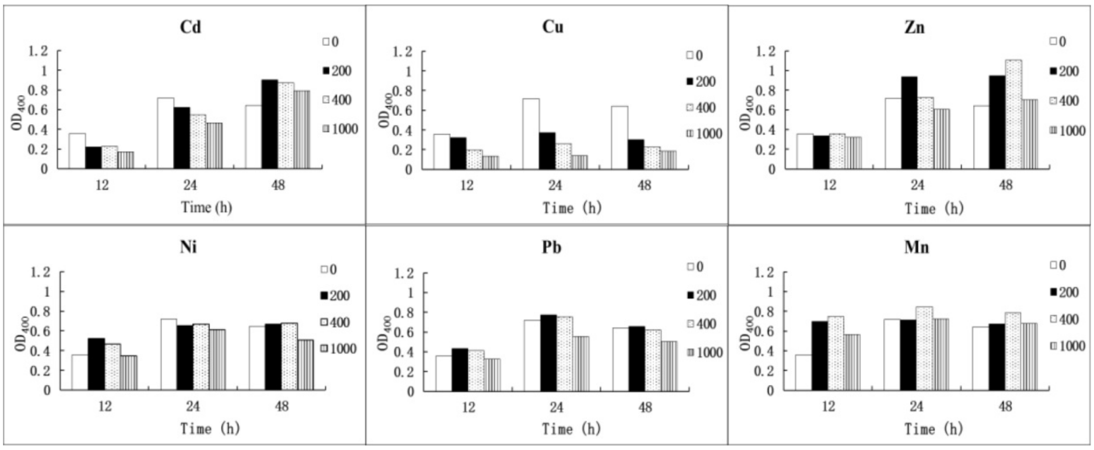

Figure 3. Effects of heavy metals on pyoverdine production by strain ZGKD3 in MSA medium contained heavy metals at the concentration of 0, 200, 400 and 1000 μM.

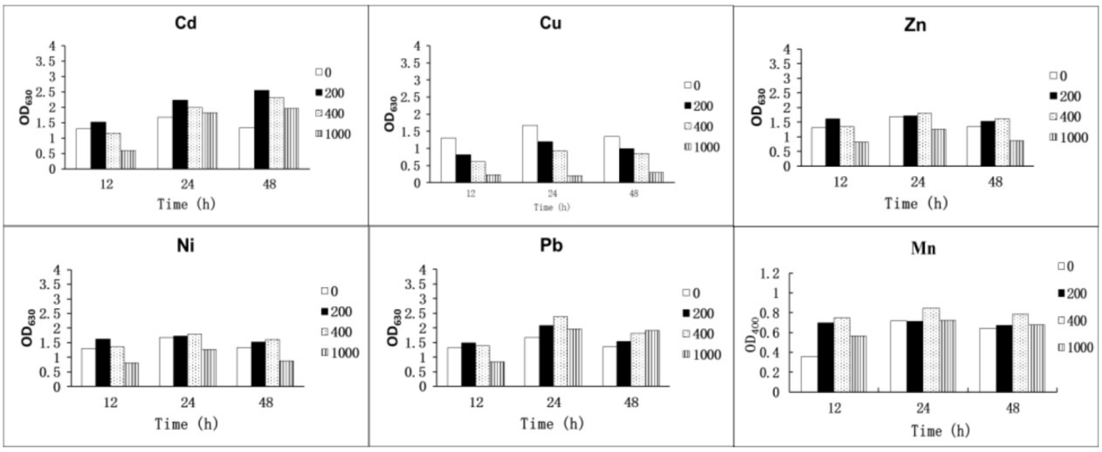

Figure 4. Effects of heavy metals on total siderophore production by strain ZGKD3 in MSA medium contained heavy metals at the concentration of 0, 200, 400 and 1000 μM.

4. Discussion

4.1. The heavy metals inhibited the growth of ZGKD3

Various studies have evidenced that heavy metals could induce inhibitory effects on growth of bacteria. For instance, Zhang found that the biomass of *Bacillus subtillus* decreased by 96.1% at 0.2 mM of Cd [18]. Jiang reported that the growth of *Bacillus subtillus* was inhibited and decreased by 95% at the concentration of 0.25 mM Cd compared to control [19]. In this paper, a experiment was used to determine the growth of ZGKD3 in response to different concentrations of heavy metals. According to the growth curves, ZGKD3 present a great variance in tolerance towards different heavy metals. In this study, an increase in bacteria biomass of ZGKD3 was observed under Ni^{2+} stress of 200 and 400 μM, the result was similar with the reports previously [20]. Thus, low concentration of heavy metal might promote the growth of bacteria, in the contrary, high concentrations of Ni inhibited the growth of ZGKD3. For the Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+} and Mn^{2+}, all the concentrations of heavy metals exhibited inhibitory effect on bacteria. Therefore, the role of heavy metals on bacteria was dependent on their respective concentration and the kind of heavy metals, suggested that ZGKD3 had different tolerance to multiple heavy metals, ZGKD3 is more sensitive to Cd^{2+} than other matals and the metal tolerance of ZGKD3 decreased in the order $Mn^{2+}>Pb^{2+}>Ni^{2+}>Cu^{2+}>Zn^{2+}>Cd^{2+}$.

4.2. The heavy metals induced the siderophore production of ZGKD3

In the present study, Cd^{2+} and Zn^{2+} were the heavy metal that showed the greater toxicity than that of other heavy metals. In this sense, most of bacteria couldn't grow at high concentrations of heavy metals. Furthermore, bacteria could remove heavy metals from their growth environment with intracellular and surface accumulation of Cu, Zn, Pb and Cd [21]. A large number of bacteria can produce diffusible light-green pigment on a CAS agar plate, and the light-green pigment has been characterized previously to be siderophores [22]. Most of *P. aeruginosa* could produce siderophores, including pyoverdine and pyochelin. It has been suggested that Al, Cu, Mn, Ga and Ni could induce synthesis of siderophores [10]. Similarly, Sinha demonstrated that Cd-resistant strain KUCd1 induced siderophore production maximally at 1.75 mM of Cd concentration [23]. Dao found that the presence of 0.125-1 mM of Cd could stimulate pyoverdine production of *P. aeruginosa* strain PAO1 [24]. Dimlpa observed that Al, Cd, Cu and Ni induced three hydroxamate siderophores by *Streptomyces* sp. Strains [25]. Furthermore, it has been confirmed that siderophores produced by bacteria could chelate many heavy metals, such as Al, Cd, Zn, Cu and Pb, and it was different in chelate ability of bacteria for every heavy metal [26]. Some studies demonstrated that siderophore could increase or decrease the toxicity of heavy metals in bacteria. Pyochelin produced by *P. aeruginosa* increased the toxicity of vanadium to bacteria [27]. However, Braud found synthesis of siderophores decreased the toxicity of multiple heavy metals to *P. aeruginosa* [28]. According to our results, the effects of heavy metals on growth of ZGKD3 were different, the ability of producing siderophore of ZGKD3 varied with different heavy metals might be one of the reasons. The toxicity of heavy metals on bacteria dependent on the amount of heavy metal accumulation in bacteria cells. Hence, the more siderophore produced, the more heavy metals were absorbed by ZGKD3 through the chelation of siderophres for heavy metals, which found to be high toxic to bacteria. In the present study, Cd^{2+} and Zn^{2+} significantly induced the synthesis of pyoverdine, and remarkably inhibited the growth of ZGKD3, indicated that the absorption of ZGKD3 for Cd^{2+} and Zn^{2+} was more than that for other metals. Therefore, *P. aeruginosa* ZGKD3 might have the potential of improving the phytoextration efficiency in Cd and Zn contaminated soils.

5. Conclusions

P. aeruginosa ZGKD3 exhibited different tolerance to multiple heavy metals. In comparison with Cd^{2+}, Zn^{2+}, Ni^{2+}, Pb^{2+} and Mn^{2+}, and the metal tolerance decreased in the order $Mn^{2+} > Pb^{2+} > Ni^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+}$. Moreover, Cd^{2+} and Zn^{2+} significantly stimulated the total siderophore and poverdine production of strain ZGKD3. Therefore, *P. aeruginosa* ZGKD3 can act as siderophore-producing bacteria and applied to microbe and plants combined remediation in Cd and Zn contaminated soils.

Acknowledgements

This work was supported by the National Natural Science Foundation of China under Grant No. 31370281 and Fundamental Research Funds for the Central Universities under Grant No. 2010YH05.

References

[1] Kermani J N, Ghasemi F, Khosravan, A. Farahmand, et al. 2010 *Iran. J. Environ. Health. Sci. Eng.* **7** 279-286
[2] Nawrot T S, Staessen J A, Roels H A, Munters E, Cuypers A, Richart T, Ruttens A, Smeets K, Clijsters H, Vangronsveld J 2010 *Biometals* **23** 769-782
[3] Chehregani A, Noori M, Yazdi H L 2009 *Ecotoxicol Environ Saf* **72** 1349–53
[4] Haque N, Peralta-Videa J R, Jones G L, Gill T E, Gardea-Torresdey J L 2008 *Environ Pollut* **153** 362-368
[5] Ma Y, Prasad M N V, Rajkumar M, Freitas H 2011 *Biotechnol Adv* **29** 248–258
[6] Rajkumar M, Ae N, Prasad MNV, Freitas H 2010*Trends Biotechnol* **28** 142–149
[7] Kidd P, Barcelo J, Bernal M P, Navari-Izzo F, Poschenrieder C, Shilev S,Clemente R, Monterroso C 2009 *Environ Exp Bot* **67** 243–59

[8] Kuffner M, Puschenreiter M, Wieshammer G, Gorfer M, Sessitsch A 2008 *Plant Soil* **304** 35-44
[9] Rajkumar M, Ae N, Prasad M N V, Freitas H 2010 *Trends Biotechnol* **28** 142-149
[10] Braud A, Jézéquel K, Bazot S, Lebeau T 2009 *Chemosphere* **74** 280–286
[11] Sharma A, Johri B N, Sharma A K, Glick B R 2003 *Soil Biol. Biochem* **35** 887–894
[12] Barzanti R, Ozino F, Bazzicalupo M, Gabbrielli R, Galardi F, Gonnnelli C, Mengoni A 2007 *Microb. Ecol* **53** 306-316
[13] Dimkpa C O, Merten D, Svatoš A, Büchel G, Kothe E 2009 *Soil Biol Biochem* **41** 154–162
[14] Cortese M S, Paszczynski A, Lewis T A, Sebat J L, Borek V, Crawford I 2002 *Biometals* **15** 103-120
[15] Fiedler H P, Krastel P, Müller J, Gebhardt K, Zeeck A 2001 FEMS *Microbiol. Lett* **196** 147-151
[16] Shin S H, Lim Y, Lee S E, Yang N W, Rhee J H. 2001 *Journal of Microbiological Methods* **44** 89-95
[17] Schwyn B, Neiland J B 1987 *Anal Biochem* **160** 47–56
[18] Zhang W, Chen G H, Guo J, Zhao Y J, Gao Y C, Wang J N 2013 *Industrial safety and environmental protection* **39** 42-45
[19] Ma G F, Wang J, Zhang C B 2009 *Microbiology* **35** 882-887
[20] Cabrero A, Fernandez S, Fernando M, Julian G 1998 *Water research* **32** 1355-62
[21] María V C, Antonio C H, Antonio F V, María V M T 2014 *Advances in Microbiology* **4** 644-655
[22] Meyer J M, Abdallah M A 1978 *J Gen Microbiol* **107** 319–328
[23] Sinha S, Mukherjee S K 2008 *Curr Microbiol* **56** 55-60
[24] Dao K H T, Hamer K E, Clark C L, Harshman L G 2001 *Ecol Appl* **9** 441–448
[25] Dimkpa C O, Svatos A, Dabrowska P, Schmidt A, Boland W, Kothe E 2008 *Chemosphere* **74** 19-25
[26] Schalk I J, Hannauer M, Braud A 2011 *Microbial* **13** 2844-54
[27] Baysse C, Vos D D, Naudet Y, Vandermonde A, Ochsner U, Meyer J M 2000 *Microbiol-SGM* **146** 2425-34
[28] Braud A, Geoffroy V, Hoegy F, Mislin G L A, Schalk I J 2010 *Environ Microbiol Report* **2** 419-425