How *Serratia marcescens* HB-4 absorbs Cadmium and its implication on phytoremediation

Yakui Chen^{a,b}, Qifa Zhu^c, Xiangzhou Dong^c, Weiwei Huang^c, Chenyu Du^{d**}, Diannan Lu^{a,b*}

^a Key Lab of Industrial Biocatalysis, Ministry of Education, China

^b Department of Chemical Engineering, Tsinghua University, Beijing, 100084, China

^c Anhui Southern Tobacco Co., Ltd. Xuancheng, Anhui Province, 242000, China

^d School of Applied Science, The University of Huddersfield, Queensgate, Huddersfield, HD1 3DH,

UK

* To whom it corresponds: ludiannan@tsinghua.edu.cn; c.du@hud.ac.uk.

Graphical abstract

14 **Abstract:** A novel strain *Serratia marcescens* HB-4 with high Cadmium adsorption capacity was
15 isolated from heavy metal contaminated soil in Hunan province, China. *S. marcescens* HB-4 reduced
16 the concentration of Cd present in wastewater to less than 0.1 mg/L when the inlet stream contained
17 no higher than 5.0 mg/L Cd. After treatment, wastewater meets Integrated Wastewater Discharge
18 Standard of China (GB8978-1996). The naturally dead *S. marcescens* HB-4 still maintained over
19 80% of its Cd adsorption capacity. Scanning electron microscope (SEM), transmission electron
20 microscope (TEM) and energy dispersive spectroscopy (EDS) results suggested that the mechanism
21 of Cd adsorption can be explained as the synergy of extracellular adsorption, periplasm accumulation
22 and intracellular absorption. The size of the accumulated Cd particular is at the nanometer scale,
23 which can be washed out by EDTA without damaging cell integrity. SDS-polyacrylamide gel
24 electrophoresis experiment showed that the heavy metal binding protein (especially Fe binding
25 protein), transporter, amino acid and histidine periplasmic binding proteins and oxidoreductases were
26 responsible for Cd removal. The pot experiment of *S. marcescens* HB-4 combined with *Houttuynia*
27 *cordata* to detoxify Cd contaminated soil showed that the cadmium content in the aboveground and
28 underground parts of *Houttuynia cordata* increased by 34.48% and 59.13% (w/w), respectively. The
29 cadmium accumulation in *Houttuynia cordata* increased by 44.27% compared with the blank group
30 which was not combined with *S. marcescens* HB-4. This work demonstrates that microbial
31 synergistic phytoremediation has a significant potential to treat heavy metal contaminated soil.

32

33 **Key words:** microorganism, extracellular adsorption, periplasm accumulation, intracellular
34 absorption, binding protein, bioremediation

35

	Abbreviation
<i>Serratia marcescens</i>	<i>S. marcescens</i>
Scanning electron microscope	SEM
Transmission electron microscope	TEM
Energy dispersive spectroscopy	EDS
Ethylenediaminetetraacetic acid	EDTA
Luria-Bertani	LB
Inductively Coupled Plasma – Mass Spectrometry	ICP-MS
Polyacrylamide gel electrophoresis	PAGE
Phosphate buffer saline	PBS
Cadmium	Cd
Arbuscular mycorrhizal fungi	AMF
Polycyclic aromatic hydrocarbons	PAHs

37

38 **1 ▪ Introduction**

39 Cadmium (Cd) is a heavy metal which is toxic to humans. It is widely present in waste battery,
40 glazes, plastics, phosphate fertilizers and household waste [Adamis et al.,2003; Angela et al.,2009;
41 Al-Khaldi et al,2015]. If the irrigating water contains over 0.04 mg/L Cd, both soil and grown crops
42 are deemed as contaminated [Alkorta et al.,2004]. If the concentration of Cd exceeded 0.1 mg/L, the
43 surface water self-cleaning function is suppressed (GB5084-1992). Heavy metal pollution in the soil
44 not only adversely affects the quality of soil and agricultural products, but also directly impacts
45 human health via transference through the food chain [Yang and Wu.,2016; Salem et al.,2000;
46 El-Sayed et al,2014;Saha et al,2011], or indirectly threatens human health by polluting water and air
47 [Lin et al.,2007; Ozbolat and Tuli.,2016; Varol and Sen.,2012; Li et al.,2014; Al-Musharafi et
48 al.,2013]. With the increasing awareness of heavy metal pollution, there are increasing research
49 focus on the remediation of heavy metal contaminated soil.

50 The phytoremediation of heavy metal contaminated soil was firstly proposed by Chaney et al.
51 [Parr et al.,1983]. Compared with chemical immobilization [Crannell et al.,2000; Hafsteinsdóttir et
52 al.,2014; Zhang et al.,1999] and electrochemical removal methods [Pedersen et al.,2015],
53 phytoremediation has advantages of low cost, high economic benefit, easy control of secondary
54 pollution, and reducing soil erosion after vegetation formation [Zhang et al.,2014]. Sunflower was
55 the first plant to be discovered to have Cd enrichment ability. When it was planted on
56 cadmium-contaminated soil for one month; the average Cd content in plants reached 50 mg/kg
57 [Turgut et al.,2004 and 2005]. Rosa et al reported that the accumulation of Cd in roots, stems and
58 leaves of *Salsola kali* plants were 2696, 2075 and 2016 mg/Kg, respectively [Rosa et al.,2004].
59 However, phytoremediation is limited by slow plant growth rate, low biomass amount, the variation
60 of soil physical and chemical properties, and the variation of pollutant distribution in the soil
61 [Cameselle et al.,2013].

62 Alternatively, microbial remediation has the advantages of simple implementation, low cost,
63 little damage to the environment, and high specificity [Ghosh et al.,2016; Espinosaortiz et al.,2016].
64 Microorganisms not only accumulate heavy metals by self-adsorption, but also facilitate heavy
65 metals reduction in the soil by secreting ironophore, functional enzymes, organic acids (heavy metal
66 binding chemicals) and biosurfactants to promote the absorption of heavy metals by plants. Yang et
67 al [Yang et al.,2009] treated heavy metal Cd, Pb and Zn-contaminated soil using *Bacillus*
68 *megaterium* and *Bacillus mucilaginosus* together with plant *Brassica juncea*. The results showed that
69 the microbial mixture can promote plant growth, enhancing the absorption of heavy metals by
70 *Brassica juncea*. The extraction amount of Cd was increased by 1.18 times. Ma et al [Ma et al.,2013]
71 reported that *Phyllobacterium myrisacearum* produced ACC deaminase and indole acetic acid, which

72 promoted the growth of *Sedum plumbizincicola*, and increased Cd and Zn adsorption in plants
73 through Fe carrier. Promoting the effect of microorganisms on phytoremediation technology of
74 heavy metal contaminated soil is one of the recent hotspots [Ma et al.,2016; Ullah et al.,2015;
75 Rajkumar et al.,2012].

76 In this study, a Cd high adsorption strain *Serratia marcescens* HB-4 was isolated from the
77 heavy metal contaminated paddy soil in Chenzhou, Hunan Province, China. This strain was cultured
78 with the Cd super-accumulating plant *Houttuynia cordata* for Cd removal, with a hypothesis that
79 co-cultivation of microorganism may improve plant absorption of Cd [Baker et al.,1989; Han et
80 al.,2018; Park et al.,2018]. *S. marcescens* HB-4 can also secrete heavy metal binding protein, Fe
81 binding protein, amino acid, histidine periplasmic binding proteins and protocatechuic acid to
82 promote the Cd recovery efficiency of *Houttuynia cordata*. To our knowledge, this is the first report
83 that investigated the synergetic effect of *Serratia marcescens* and *Houttuynia cordata* on
84 phytoremediation.

85

86 2 ▪ Materials and Methods

87 2.1 Materials

88 2.1.1 Microorganisms

89 Strain *Serratia marcescens* HB-4 was isolated in heavy metal contaminated soil in Hunan
90 province, China, following the method described by Chen et al., 2017 [Chen et al.,2017]. The strain
91 was stored in a –20°C freezer until use. Luria-Bertani (LB) medium was used for the cultivation of *S.*
92 *marcescens* HB-4 unless specified otherwise. It contains 5 g/L yeast extract, 10 g/L tryptone and 10
93 g/L NaCl. The cultivation of *S. marcescens* HB-4 was carried out in an air bath shaking incubator at

94 30°C, 170 rpm to the required period.

95 2.1.2 *Houttuynia cordata*

96 *Houttuynia cordata* was provided by a local farmer in Sichuan Province, China. *Houttuynia*
97 *cordata* that had two or three leaves after around 15 days of cultivation was used in the synergized
98 Cd removal experiments.

99 2.1.3 Soil

100 The soil used for strain isolation was collected from a heavy metal contaminated field near an
101 industrial estate, Chenzhou, Hunan province, China, on September 2015. Around 1,000 g of surface
102 soil sample was collected using a sterilized plastic container and shipped to the lab in Tsinghua
103 University, Beijing by train within 3 days. When it arrived at lab, it was kept in a 4°C fridge until
104 strain isolation.

105 The soil used for *Houttuynia cordata* culture was collected from a rice cultivation field in Anhui
106 province. It was air-dried for 14 days and then shipped to the lab in Tsinghua University, Beijing by
107 train.

108 2.2 Methods

109 2.2.1 Characterization of the physicochemical and nutritional properties of the soil

110 Approximately 1 kg of the above mentioned soil was firstly screened to remove large impurities,
111 such as plants debris, gravels and plastics. It was then air-dry in the lab until no further weight loss.
112 Then, the sample was sent to the Institute of Plant Nutrition and Resources, Beijing Academy of
113 Agriculture and Forestry Sciences for characterizing the physicochemical and nutritional properties.
114 The organic matter was detected by potassium dichromate volumetric method. The total nitrogen was
115 detected by Kjeldahl method using a Kjeldahl tester. The nitrate nitrogen and ammonium nitrogen

116 were detected by a continuous flow analyzer. Mercury was detected by atomic fluorescence method.
117 Heavy metals content such as lead, cadmium and chromium were detected by atomic absorption or
118 ICP-MS.

119

120 2.2.2 Cd adsorption by living *S. marcescens* HB-4

121 *S. marcescens* HB-4 was cultivated using LB medium at 30°C, 170 rpm for 48 hours. Then the
122 fermentation broth was centrifuged at 10,000g, 4 °C for 6 minutes. The solid residue was washed by
123 deionized water and centrifuged at the same condition [Figueira et al, 2005]. The process was
124 repeated for 3 times. Then, 100 mg biomass pellet (wet weight) was added into 100 mL synthetic Cd
125 solutions containing 0.1, 0.5, 1.0 or 5.0 mg/L Cd. The adsorption was carried out at 30°C, 170 rpm.
126 The residue Cd concentration in the solution was measured by ICP-MS as described below. The
127 experiments were carried in triplicates.

128 2.2.3 Cd adsorption by deactivated *S. marcescens* HB-4 cells

129 Naturally dead *S. marcescens* HB-4 cells were obtained by adding the above mentioned 300 mg
130 biomass pellet into 100 ml deionized water leaving at room temperature for 30 days. Autoclaved *S.*
131 *marcescens* HB-4 cells were obtained by autoclaving the above mentioned 300 mg biomass pellet
132 with 100 mL deionized water. Ultrasonicated cells was obtained by ultrasonating the above
133 mentioned 300 mg biomass pellet with 30 mL deionized water for 40 minutes (power 300 W,
134 ultrasonic 2 s, intermittent 2 s). All dead/breaking cells solutions were centrifuged at 10,000g and
135 4°C for 6 minutes. The solid residue of these dead/breaking cells were added to 100 mL 100 mg/L of
136 Cd solution. Fresh *S. marcescens* HB4 biomass pellet was used as control. The adsorption process
137 was carried out at 30°C and 170 rpm. The residue Cd concentration in the solution was measured by

138 ICP-MS method as described below. The experiments were carried in triplicates.

139 Freeze dried *S. marcescens* HB-4 cells were obtained by freeze the above-mentioned biomass
140 pellet in a -20°C freezer for 2 hours and then transferred to a -80°C freezer for overnight. Then the
141 sample was dried in a vacuum freeze dryer for 24 hours. 250 mg of the freeze-dried cells were added
142 into 100 ml Cd solution with a concentration of 20, 50, 100, 150 and 200 mg/L of Cd. The residue
143 Cd concentration in the solution was measured by ICP-MS method as described below. The
144 experiments were carried in triplicates.

145 2.2.4 EDTA elution experiments

146 30 mL *S. marcescens* HB-4 cell solution after the adsorption experiment was centrifuged at
147 10,000 g 4°C for 10 minutes. The solid residue was washed by deionized water and centrifuged at
148 the same condition for 3 times. Then the solid residue was eluted by 10 mM EDTA solution five
149 times. Each elution time was 25 min.

150 2.2.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) experiments

151 50 mg (dry weight) the solid residue of *S. marcescens* HB-4 cell before and after the adsorption
152 experiment was collected by centrifugation at 10,000 g, 4°C for 10 minutes, gathering 8 mL
153 deionized water, ultrasonic disruption (power 300 W, ultrasonic 2 s, intermittent 2 s) for 30 minutes.
154 The sample was mixed with protein loading ($\times 1$) buffer at a ratio of 1:1, and boiled for 10 min at
155 100°C in a water bath to fully denature the protein. 20 μL of each sample was added to the prepared
156 gel, and the gel electrophoresis was performed at 10 V/cm [Sarraf et al, 2015].

157 2.2.6 Cd removal using *S. marcescens* HB-4 combined with *Houttuynia cordata*

158 The soil obtained from Anhui province was used for the pot experiments. Each pot contained 1
159 kg soil (containing approximately 12% moisture), with the additional of 5, 50 or 100 mg/kg of Cd,

160 respectively, and then fully mixed. The pot experiments were left at room temperature for 7 days to
161 settle down. Next, 200 mL, OD₆₀₀=7.5 fresh *S. marcescens* HB-4 cells was added. After 7 days,
162 *Houttuynia cordata* plants with 1–2 small leaves were implanted into pots. When the *Houttuynia*
163 *cordata* plant grew to 4-6 leaves, which took around 1.5 months, the plant was taken out, washed,
164 separated to aboveground part and underground part, dried in a 70°C oven until constant weight. The
165 dried plant samples were milled and the Cd concentrations were determined by Inductively Coupled
166 Plasma Mass Spectrometry (ICP-MS).

167

168 2.3 Analysis

169 2.3.1 The calculation of removal ratio and adsorption quantity

170 The percentage of Cd removal ratio (*RE*) by the biomass was calculated using equation (1):

$$RE = \frac{(C_0 - C_R)}{C_0} \times 100\% \quad (1)$$

171 where C_0 and C_R are initial and final concentration of Cd in the supernatant, respectively.

172 The adsorption quantity (*q*) were calculated using the following equation:

$$q = \frac{(C_0 - C_R) \times V}{m} \quad (2)$$

173 where C_0 and C_R are the same as in equation(1), m is the dry cell weight of HB-4 and V is
174 volume of heavy metal aqueous solution.

175 2.3.2 Scanning Electron Microscope (SEM)

176 The morphologies of *S. marcescens* HB-4 cell before and after adsorption of Cd were observed
177 by using SEM [Gharieb et al, 1995]. The model of the SEM was FEIQuanta 200 (FEI, US). 20 ml *S.*
178 *marcescens* HB-4 cell solution before and after the adsorption of Cd was centrifuged at 10,000 g 4°C

179 for 10 minutes. The solid residue was washed by deionized water and centrifuged at the same
180 condition for 3 times. Then, cells were fixed using 2.5% glutaraldehyde fixation solution, followed
181 by washing with 0.1 mol/L PBS, and dehydrating using a gradient ethanol solution, lyophilizing in
182 a vacuum dryer and gold plating.

183

184 2.3.3 Transmission Electron Microscopy (TEM) and Energy Dispersive Spectroscopy (EDS) analysis

185 The *S. marcescens* HB-4 cell before and after adsorption of Cd was also observed by using TEM
186 [Estrada et al, 1985]. The model of the TEM was H-7650 (HITACHI, Japan). 25 mL *S. marcescens*
187 HB-4 cell solution before and after the adsorption of Cd was centrifuged at 10,000 g, 4°C for 10
188 minutes. The solid residue was washed by deionized water and centrifuged at the same condition for
189 3 times. Then, cells were fixed overnight using 2.5% glutaraldehyde in pH 7.0 phosphate buffer at
190 4°C, 1% osmic acid fixation solution at 4°C for 1–2 h, followed by washing with 0.1 mol/L PBS for
191 3 times, dehydrating using a gradient ethanol solution (30%, 50%, 70%, 80%, 90%, and 100%) for
192 15 minutes each time and finally three times change of absolute ethanol. After dehydration, the
193 samples were embedded using acetone and embedding solution (1:1), solidified, ultrathin
194 sectioned and examined. Elemental analysis during TEM was simultaneously carried out by using
195 X-ray energy spectrometer. Energy Dispersive Spectroscopy [Sullivan et al, 1994] analysis was
196 performed simultaneously.

197

198 2.3.4 ICP-MS

199 The quadrupole inductively coupled plasma mass spectrometer used in this work was SOE-11
200 (Xseries2, Thermo Fisher, Germany). It was combined with a high-efficiency sample introduction

desolvating system equipped with a quartz cyclonic spray chamber. The instrument quality range: 2~255amu. Detector resolution: <1amu. Sensitivity: common elements >5Mcps/ppm. Abundance sensitivity: generally <10⁻⁶. Detection limit: the general element is lower than the ppt level. Oxide / double charge ratio: generally <3%. The argon gas utilised was of spectral purity (99.9999%). The standard solution was produced by the China National Iron and Steel Materials Testing Center Iron and Steel Research Institute, medium 10% nitric acid. The internal standard was applied to the analysis process and determined by the internal standard calibration quantitative analysis method in the ICP-MS analysis method. After the instrument is stabilized, the standard solution, the blank solution and the sample solution are sequentially determined in order.

2.4 Statistical analysis

The data were evaluated by ANOVA.

3 • Results and Discussion

3.1 Nutritional status and physicochemical properties of the tested soil

According to national standard (Soil environmental quality risk control standard for soil contamination of agricultural land, GB 15618-2018), the risk screening values and the risk intervention value of soil cadmium contamination in agricultural land were 0.6 and 3.0 mg/kg, respectively. The total cadmium content in soil from Chenzhou, Hunan Province, was 76.3 mg/kg (supporting info), which was 25.4 times higher than the risk intervention value. Lead and Chromium contents were between the risk screening value and the risk control value. But the soil obtained from Xuancheng was safe, pollution-free and suitable for plant growth. Physicochemical and nutritional properties of the soils used in this study, as shown in supporting info.

223

224 3.2 *S. marcescens* HB-4 adsorption ability at low Cd concentration

225 Fresh *S. marcescens* HB-4 cell culture was first used in Cd removal in a solution initially
226 containing 0.1 to 5.0 mg/L of Cd. As shown in Figure 1a, the residue Cd contents after two hours
227 treatment were all lower than 0.08 mg/L. The Cd limit in wastewater defined by “Integrated
228 Wastewater Discharge Standard” National Standard of China GB8978-1996 is 0.1 mg/L, while that
229 in irrigation water is 0.01 mg/L. The results suggested that when the initial Cd concentration was no
230 higher than 5.0 mg/L, the treated wastewater met the national wastewater discharge standard.
231 Furthermore, if the initial wastewater contained less than 0.1 mg/L Cd, the treated wastewater
232 (containing 0.010 ± 0.001 mg/L Cd) could be directly used as irrigation water. Actually, if the
233 wastewater contained higher than 0.1 mg/L and less than 5.0 mg/L Cd, under the same conditions,
234 two round of treatments by *S. marcescens* HB-4 adsorption would bring the Cd content down to
235 lower than 0.01 mg/L, which could be used directly for irrigation.

236 In order to facilitate practical applications, various deactivated *S. marcescens* HB-4 cells were
237 tested in Cd removal. As shown in Figure 1b, the naturally dead cells exhibited a high Cd removal
238 yield of $86.1 \pm 0.68\%$, while the live cells removed $97.12 \pm 0.02\%$ Cd. Higher than other strains
239 reported so far [Chao et al, 2016; Meseguer et al, 2016]. The Cd removal yield dropped significantly
240 to lower than 50% when the cells were autoclaved or ultrasonicated, which could attribute to
241 destroying cell structure.

242 Figure 1c shows Cd removal yield and Cd adsorption quantity against initial Cd concentration
243 by freeze-dried *S. marcescens* HB-4 powder. The yield was maintained at 84.01–91.08% at a wide
244 range of initial Cd concentrations from 20 to 250 mg/L. In comparison to Cd removal yield using

245 fresh cells, using freeze-dried *S. marcescens* HB-4 powder had a slightly lower yield. However,
 246 freeze-dried cell powder is much easy in transportation and storage and therefore easy in practical
 247 applications, Hajahmadi et al., 2015 also reported a case study of using pre-treated dried
 248 microorganism for heavy metal removal [Hajahmadi et al., 2015]. Decrease of heavy metal
 249 adsorption capability was expected as a consequence of unavoidable damage of cell structure during
 250 the drying process. However, improvement of Cd removal yield in comparison of live cells by
 251 *Drechslera hawaiiensis* was also reported [El-gendy et al., 2017].

Fig.1 Removal of cadmium by *S. marcescens* HB-4 under different conditions. (a) Low initial cadmium concentration;(b) Under different treatments;(c) Freeze-dried *S. marcescens* HB-4 cells at different initial Cd concentrations.

3.3 The mechanism of Cd removal by *S. marcescens* HB-4

The cell morphological changes of *S. marcescens* HB-4 before and after Cd adsorption were examined using SEM, as shown in Figure 2. After Cd²⁺ adsorption, the surface of the cells showed different extent of dents, wrinkles or elongation, which was caused by the toxic effect of heavy metal cadmium. In addition, a large amount of white flocculation appeared on the surface of the cells, and the cells aggregated together. This may due to the secretion of extracellular polysaccharides [Xu et al.,2017; Kranthi et al.,2018; Li et al.,2016] induced by cadmium. The results indicated that cadmium exists on the surface of *S. marcescens* HB-4 in the form of precipitates.

Fig.2 SEM images of *S. marcescens* HB-4. (a) before the treatment of 300 mg/L of Cd; and (b) after the treatment of 300 mg/L of Cd.

Figure 3(a-c) shows the TEM and EDS images of *S. marcescens* HB-4 before and after Cd adsorption. The cells were full before adsorption, but a large area of cavities appeared inside the cells

270 after adsorption. Heavy metals were mostly present in the periplasmic layer in the form of
271 aggregation (red circle in Figure 3b), and a few were concentrated in the interior of the cells (blue
272 circle in Figure 3c) and on the cell surface (arrow in Figure 3b), similar to the results reported by Naz
273 et al., [Naz et al.,2005]. In order to verify whether the black aggregates were Cd containing
274 substances, energy dispersive spectroscopy (EDS) analysis of the region was carried out. Figure 3a
275 illustrates that there was no absorption peak of Cd in the EDS spectrum, suggesting *S. marcescens*
276 HB-4 did not contain Cd before adsorption. Figure 3b and 3c illustrates the black aggregates in the
277 periplasmic layer, intracellular and cell surface contains high concentration of Cd and most of the
278 cadmium presents in the periplasm (Table 2).

Fig.3 TEM images (up) and EDS analysis (down) of *S. marcescens* HB-4. (a) Before treatment with 300 mg/L of Cd; (b) After treatment with 300 mg/L of Cd (Cd in periplasm); and (c) After treatment of 300 mg/L of Cd (Cd in cell); (d) After EDTA treatment (Cd in periplasm); (e) After EDTA treatment (Cd in cell).

Table 2 Elemental analysis of *S. marcescens* HB-4 by EDS

		C	Cl	Cu	Cd	Sum (%)	C	Cl	Cu	Cd	Sum (%)
		before and after Cd treatment					after EDTA elution				
Before	Weight/%	74.65	2.24	23.11	0.00	100					
Adsorption	Atom/%	93.57	0.95	5.48	0.00						
In periplasm	Weight/%	50.46	3.35	32.83	13.36	100	74.12	0.73	25.15	0.00	100
	Atom/%	85.20	1.91	10.41	2.41		93.68	0.31	6.01	0.00	
In Cell	Weight/%	62.36	1.62	33.30	2.71	100	61.07	1.84	33.44	3.65	100
	Atom/%	89.73	0.79	9.06	0.42		89.28	0.91	9.24	0.57	

282 Figure 3(d and e) shows Cd adsorbed *S. marcescens* HB-4 cells after EDTA elution. After
283 EDTA elution, the cell structure was intact and no sign of damage. In addition, the outer capsule of
284 the cell still existed. There was no Cd element in the EDS elemental analysis of the cells in Figure 3d,
285 confirming that Cd in the periplasmic layer was removed by EDTA. In comparison, Cd was still
286 present in the cell in Figure 3e, indicating that the EDTA elution cannot remove the heavy metal
287 particle inside the cell (Table 2). The results verified that the distribution of Cd²⁺ in intracellular,
288 periplasm and extracellular. The surface (periplasm and extracellular) adsorbed Cd was fully washed
289 out by EDTA, while intracellular Cd particles cannot be washed out by EDTA.

290

291 3.4 Protein differential analysis related to *S. marcescens* HB-4 adsorption of Cd

292 SDS-PAGE was used for the qualitative assessment of protein expression changes related to Cd
293 adsorption by *S. marcescens* HB-4. Figure 4 shows that total proteins content of *S. marcescens* HB-4
294 after Cd adsorption was negatively regulated (band (2)) in comparison to that of fresh *S. marcescens*
295 HB-4 cells (band (3)). We further cut band (2) and band (3) off for mass spectrometry (Q Exactive)
296 analysis. Here we used NCBI gene bank as a reference [<http://ieg.ou.edu/entrance.html>
297 [GeoChip5.0_180K Summary](#)], we found some proteins of HB-4 cell were depressed to express while
298 some of the proteins were motivated in the presence of Cd. The major protein differences between
299 Cd-treated and CD-untreated HB-4 are listed in the supporting information.

Fig.4 SDS-PAGE of total proteins obtained from *S. marcescens* HB-4. (1) Marker; (2) Treated by 300 mg/L of Cd; and (3) Control (No Cd).

It was shown that the expression of some DNA polymerases was significantly depressed, indicating that the presence of Cd was harmful to the HB-4 cell. However, some proteins are motivated to help HB-4 cell survive in the presence of harmful Cd, such as oxidoreductases, transporters (MFS transporter, Thiamine ABC transporter substrate-binding protein, Magnesium transporter, etc), enzymes related to energy supply (ABC transporter ATPase, Formate dehydrogenase, Phosphoesterase, etc) and membrane proteins (Outer membrane protein II). Therefore, it can be concluded that heavy metal binding protein (especially Fe binding protein), amino acid and histidine periplasmic binding proteins and protocatechuic acid are responsible for Cd removal. Other heavy metal cadmium transporters have been reported. [Uraguchi et al,2014; Wang et al,2011].

3.5 Soil remediation test of *S. marcescens* HB-4 when synergized with *Houttuynia cordata*

In order to investigate the impact of *S. marcescens* HB-4 on Cd removal by the plant, a pot experiment was carried out using a Cd-enrichment plant *Houttuynia cordata*. Each experimental

condition contained 3 replicates, 4-6 plants per pot. After cultivation period, the cultivated *Houttuynia cordata* was taken out, washed and then separated into the aboveground part and the underground part for digestion, as shown by the dotted yellow line in Figure 5. Figure 5 shows that the Cd content in the underground part was higher than that in the aboveground part. After inoculation with *S. marcescens* HB-4, the Cd contents in the aboveground part and the underground part of *Houttuynia cordata* were 23.86-34.48% and 21.37-59.13% higher than those in the control group, respectively. The Cd accumulation of the whole *Houttuynia cordata* was 28.06-44.27% higher than the control. When the Cd in the soil was 50 mg/kg, the Cd contents in the aboveground part and the underground part of *Houttuynia cordata* were 95 mg/kg and 130.5 mg/kg in the control group, respectively. The enrichment effect of *Houttuynia cordata* was better than the seven super-enriched plants listed by Kayser et al., [Kayser et al.,2000]. Microbial enrichment of heavy metals in *Houttuynia cordata* has been extensively studied. Chen et al [Chen et al.,2009], reported that rhizosphere microorganism promoted the accumulation of cadmium in *Houttuynia cordata*. *Bacillus subtilis* wb600 can facilitated Pb absorption by both root and shoot of *Houttuynia cordata* [Liu et al.,2018]. Wu et al treated sewage and heavy metal by combined nitric acid bacteria and nitrite bacteria with *Houttuynia cordata* [Wu et al, 2017]. Root microorganisms synergized with *Houttuynia cordata* showed a better adsorption of Cadmium that reported by Hou et al., 2010 [Hou et al.,2010]. The mutual interactions between rhizobia and AMF was reported to improve phytoremediation efficiency of PAHs by *S. cannabina* [Ren et al,2017], which was agreed with several investigations on phytoremediation of heavy metal contaminated soil [Karimi et al,2018; Chen et al,2013; Xu et al,2019]. Other than heavy metal pollution control, phytoremediation strategy has been successfully demonstrated for treatment of petroleum polluted soil [Gałazka et al,2015;

334 [Xun et al,2015](#)].

335 Microbial synergy with phytoremediation attracts increasing recognition as a novel method for soil
336 remediation. Further field test would be essential to obtain reliable data before large scale application
337 of the technology on the field. Bacterial control could be a challenge. The ethical and environmental
338 impact of applying a live microorganism in agriculture field should be assessed as well.

339

Fig.5 The effect of *S. marcescens* HB-4 on uptake of Cd by *Houttuynia cordata* at different initial Cd concentrations in soil

340 In summary, Figure 6 illustrated the Cd adsorption routes by *Houttuynia cordata* with the
341 synergy effect of *S. marcescens* HB-4. Cd was adsorbed by *S. marcescens* HB-4 via the synergy of
342 extracellular adsorption, periplasm accumulation and intracellular absorption. We hypothesize that
343 there might be two major pathways involved in mediating Cd transport from roots to shoots in
344 presence of *S. marcescens* HB-4. One is the direct uptake of Cd by *Houttuynia cordata*. The other
345 one is *S. marcescens* HB-4 mediated uptake of Cd. Cd can be absorbed or taken up by HB-4 by ways

346 of extracellular adsorption, periplasm accumulation and intracellular absorption. *S. marcescens* HB-4
 347 is a symbiotic bacteria to *Houttuynia cordata* and can live at the node of root and the xylem of shoot
 348 [Lu et al., 2014 ; Li et al.,2016 and 2017]. The uptake of Cd by HB-4 can translocated into
 349 *Houttuynia cordata* due to the enhanced metal transporters and related energy supply system of *S.*
 350 *marcescens* HB-4 in the stimulus of Cd.

351
 352 **Fig.6** The possible mechanism of *S. marcescens* HB-4 enhanced Cd uptake by *Houttuynia cordata*.
 353

354 4、 Conclusion

355 This study showed both fresh and freeze-dried *S. marcescens* HB-4 had excellent Cd removal
 356 capacity, which had laid the foundation for future filed applications. When the wastewater contained
 357 low Cd concentration (≤ 5 mg/L), treatment of the wastewater with *S. marcescens* HB-4 for 2 hours
 358 met the wastewater discharge standard (< 0.1 mg/L). When the initial Cd concentration was less than
 359 0.1 mg/L, the treated wastewater could be directly used as irrigation water. It was confirmed that
 360 HB-4 had good practical application potential and no external pollution. The adsorption mechanism
 361 of *S. marcescens* HB-4 on Cd was a combination of extracellular adsorption, periplasmic layer

362 accumulation and intracellular uptake. Cd was mostly present in the periplasmic layer in aggregate
363 form, with few presented in the interior of cells and attached the cell surface. The accumulated Cd in
364 the periplasm of *S. marcescens* HB-4 can be eluted by EDTA. The combination of *S. marcescens*
365 HB-4 and *Houttuynia cordata* to remedy Cd-contaminated soil showed higher Cd removal than that
366 of *Houttuynia cordata* alone. The Cd accumulation of whole *Houttuynia cordata* plant increased by
367 44.27% compared with control. This study showed that presence of *S. marcescens* HB-4 promoted
368 the absorption of Cd by plants and enhanced the ability of phytoremediation, indicating that the
369 synergistic remediation of heavy metal pollution by microorganisms and plants could be a promising
370 approach.

371

372

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