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Inoculation of Soil With Cadmium-Resistant Actinomycetes Flora Reduces Cadmium Accumulation in Rice (Oryza Sativa L.)

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Abstract-The microorganism and other amendments were immobilized in pellet carrier as microbial reverse screening model and were applied to the simulated Cd contaminated soil. Microbial flora(*Streptomycete* XW8, *Actinomycetes* XW3, *Actinomycetes*XW5) reduces Cd accumulation in rice when combined with biochar, humic acid and Carbon silicon functional liquid fertilizer. Microbial flora(*Bacteria* XW6, *Actinomycetes* XW3, *Actinomycetes* XW5) has highest TF and raises the bioavailability of Cd in soil. But *Bacteria* XW6 activate Cd in soil, which is a premium candidate for application in phytoremediation Cd farmland contamination. The compatibility of microbial flora had a significant effect in Cd reduction.

Keywords: Cd farmland contamination, screen model, microbial flora, bioremediation, amendments

1. Introduction

Heavy metal contaminated farmlands remediation techniques include purification (remediation of contaminated soil by plants and microorganisms), passivation (absorption of heavy metal elements by minerals), and hazard avoidance (conversion of contaminated soil by "alien soil") , by high efficiency and low cost functional materials.

Microbial remediation of heavy metal involves bioaccumulation, biosorption, biomineralization and biotransformation. [1]. Continuous flow reactor, are feasible methods for both CRM screening and formula optimization. However, the screening use of these two methods is limited to a lot of media and poisonous substances.

In soil polluted by heavy metals, actinomycetes are the most abundant, many of which have the bioremediation of repairing heavy metals and promoting plant growth[2].

The development of treatment strategies for Cd-contaminated farmlands is urgent , because the Cd contamination has been a serious concern in recent years worldwide.

Bioremediation has attracted increasing interests for its eco-friendliness. Currently, functional screening of Cd-remediation microorganisms(CRM) is associated with low throughput, prohibitive costs and unsafe strains[3]. Studies carried out in actinomycetes strains were focused on setting the optimum conditions to remove heavy metals from the edible part of an agricultural product grown in the soil. The application of microbial flora immobilized cells to pollution area is still in its preliminary stages.

In this study, joint immobilization of microorganism-Cd-amendments in alginate beads is a screening model for stimulating plant-microorganism-Cd-soil-amendments interactions.

CRM screened by reverse model[4] from 8 kinds of heavy complex contaminated soil in Baiyin, Gansu Province, China, can tolerate 450 mg/L Cd. Pots experiments were designed to investigate the effect of immobilized gel ball made by CRM and Humic acid, biochar, attapulgite, on soil Cd availability and Cd uptake in rice.

This is the first report of the use of screen model by the immobilized gel ball embedded in complex remediation material by sodium alginate. This approach greatly speeds up the screening of CRM by scaling down the whole process of simulating ecologic state with plant, microorganism and contaminated soil together. Therefore, with this novel approach to screen , likelihood of discovering useful strains for farmland bioremediation is increased.

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Abbreviations: cadmium (Cd), Cd-remediation microorganism (CRM), Cd tolerance strains(CTS), Translocation factor (TF)

2. Materials and Methods

2.1 Material

Humic acid, biochar and attapulgite (produced in Linze, Gansu) were provided by Hebei Shijiazhuang Yubo Co., Ltd., Professor Zhou Jianbin of Nanjing Forestry University and Liu Xilin of Shanghai Lichang Environment Co., Ltd. Carbon silicon functional liquid fertilizer, presented by Sheng Jianwei of Zhongqing Co., Ltd

Samples were collected from Minqin village, Baiyin, Gansu. Potted Cinnamon soil was collected from the east bank of Ye river in Pingshan county, Hebei Province. Latitude 38°4 ' north, longitude 114 ° 9 ' east.

Nutrient Agar(NA), Nutrient Broth(NB), Gauss No1 medium, Potato Dextrose Agar (PDA), Columbia blood plate, Murashige & Skoog Medium (MS) purchased from Haibo biotechnology Co. Ltd China.

2.2 Method

Microbial screening model is consists of seed, soil, heavy metal and amendments. The matrix solution was prepared by mixing alginate and attapulgite-biochar-humic acid in media in the following way. The heavy metal contaminated soil and its leached liquid were used in the bioencapsulation process. Heavy metal contaminated soil leaching solution were made according to mass ratio of soil and water 1:1, sodium alginate, xanthan gum, heavy metal pollution of soil, attapulgite, biochar, humic acid, NB, MS and Gauss No1 medium to join in the leaching solution in proportion, mixing to join in mixture quality score is 1-2% $CaCl_2$. The matrix mixture was transferred to the conical flask with rice seed soaked in water for 12h then sterilized in 2% sodium hypochlorite solution for 15min, the conical flask were incubated at 30 °C for 48h–96 h. Cd tolerance strains(CTS) were isolated from germinated rice rhizosphere, the growing colonies were repeatedly inoculated onto a new NA, PDA, Gauss No1 medium plate containing the same and increased level of Cd concentration respectively. Finally, CTS were selected for further studies.

Bioencapsulation process . All the material used for the bioencapsulation process[5] was previously sterilised and the process was carried out under aseptic conditions. Three grams of sodium alginate were dissolved in 100 ml of media composed of MS, NB and Gauss No1 medium in 1:1:1 and stirred for 30 min to obtain a homogeneous solution (viscosity of alginate at 3% solution: 34 centipoise). The composition of alginate-attapulgite-biochar-humic acid matrix was given as follows mass ratio 1:1:1:1, 0.4 g, respectively, were added to the alginate solution. The matrices were then stirred for 30 min for homogeneity. The matrix mixture was transferred to the syringe (50 ml). The matrix solution dropped into sterile calcium chloride (0.1 moL). The collected beads were placed on a filter paper in a Petri dish. Before application, Microorganism in calcium alginate gel beads were cultured for 24h, 150rpm/min at 28°C.

Morphological characteristics of actinomycetes. Inoculate actinomycetes along the line between the cover slide and the culture medium, Remove cover slides at 2d、5d、7d、10d、15d regularly. The morphology of basal mycelia, aerial mycelia, spore filaments, spore emitting mode and color morphological characteristics were observed under the microscope. Soluble pigment in different media were checked.

Hemolytic Activity Test. Transparent ring around the lawn is hemolytic positive in Columbia blood plate.

Determination of cadmium tolerance by disk diffusion method. Minimal inhibitory concentration (MIC) of CRM were assessed by the Kirby Bauer disk diffusion method on NA, Gauss No1 medium respectively.

Pot experiment. Potted soil samples were collected from the surface layer (0–20 cm in depth) of a cinnamon soil rice field on the east bank of Ye river in Pingshan county, Hebei Province, China. The pH of the soil is 6.8 and the chemical properties are as follows: organic matter (OM)18g/kg, cation exchange capacity (CEC) 1.52 g/kg, total N 2.71 g/kg, available N 130.74mg/kg, available P 296.46mg/kg, available K 95.37 mg/kg.

The content of heavy metal Cd added into the soil for this test was 0.5mg/kg. Super hybrid rice seed Y liangyou 900(T900) were used in the present study. The seeds of the rice were surface-sterilized in 2% sodium hypochlorite for 5 min and rinsed several times with sterile distilled water. The seeds were then soaked for 24 h at room temperature, germinated for 2 days, and sown in normal, Cd-free soil. After 30 days, seedlings with similar appearance and biomass were carefully transplanted into each pot (fifteen plants/pot).

A completely randomized design was used to determine the inoculation of CTS on the growth and Cd uptake of rice plants grown in the Cd-contaminated soil. Triplicate pots were used for each treatment, with each pot (36 cm in diameter and 30 cm in height) containing 20.0 kg of soil. The strains immobilized in glue beads were cultivated in the sterile medium for 24 h then immobilized glue beads was adequately mixed with the soil in each pot. Soil of No2 No3 No4 were inoculated

with living immobilized glue beads follow as Tab1, CK(No1) was inoculated with same amount of non-strains immobilized glue beads, Before sowing, All treatments were applied with 630kg/hectare humic acid, 4800kg/hectare biochar, 75L/hectare Carbon silicon functional liquid fertilizer.

Soils in the pots are left to stabilize for 2–3 weeks to achieve stabilization.

Table 1: The treatment of pot experiment

Trial No	1	2	3	4
Actinomycetes XW3	-	-	+	+
*Actinomycetes*XW5	-	-	+	+
Bacterium XW6	-	+	-	+
Streptomycete XW8	-	+	+	-

Determination of Cd, Translocation factor (TF)

The extraction method of soil active Cd follow Hani[6]. Soil available Cd is extracted by 1mol ammonium acetate from soil with a solid-liquid ratio of 1:5, 23 °C, 200r/min, 1.5h, centrifuged at 5000r/min for 15min, then filtered and supernatant was taken.

According to GB 5009.15-2014 [7], the pre-treatment samples of plants were processed by microwave digestion to extract the full Cd of plants.

The calculation formulas of the TF of the treated rice are as follows [8]:

TF= Cd content in the aboveground part of the plant/Cd content in the underground part of the plant

The performance of graphite furnace atomic absorption spectrometer (PE AA800) was adjusted to the best state: wavelength 283.3nm, 228.8nm slit 0.5nm, lamp current 5mA, drying temperature 120 °C, 20s. Cd standard liquid was absorbed 10.0μg/L, 20.0μg/L, 40.0μg /L, 60.0μg /L, 80.0μg /L, 10μL each, and injected into the graphite furnace. The absorption value was measured and the linear regression equation was calculated.

SPSS 22 statistical analysis software single factor ANOVA was used to analyze the correlation among data.

3. Result

3.1 Heavy metal pollution index of soil in Baiyin, Gansu province, China

Eight heavy metals data in soil of village Minqin in Baiyin, Gansu are shown in table 2, The two sampling points are 50 meters apart from each other, sample collecting zone is 60 meters away from and parallel to East groove. the distance between these two samples is close, but the data difference is large, especially copper, chromium. The heterogeneity of farmland soil pollution [9] also make it tricky to remediate. Content of Cd and As in Baiyin is above risk intervention value in 6.5<pH<=7.5 of national standard , so the farmland in this area will be forbidden to tillage[10]. Other soils were selected for pot experiment in this study.

Table 2: Heavy metal index of soil of Baiyin, Gansu

	Cd	Pb	Ni	Hg	As	Cr	Cu	Zn
site1（mg/kg）	7	254	31.4	0.400	180	56	331	495
Site2（mg/kg）	6.4	303	45.9	0.588	138	102	172	434
Degree of difference (%)	8.6	16.3	31.7	32.0	23.3	45.1	48.0	12.3
risk screen value [10]（mg/kg）	0.3	120	100	2.4	30	200	100	250
risk intervention value[10]（mg/kg）	3.0	700	no	4.0	120	1000	no	no
Pollution severity	**	*	No	No	**	No	*	*

3.2 Resistance to Cd

The diameter of bacteriostatic ring which has inhibit effect is between 11-16mm . Resistance to Cd of the *Streptomycete* XW8, *Actinomycetes* XW3, *Actinomycetes*XW5 is 450mg/kg and *Bacterium* XW6 is 350mg/kg.

While *Streptomycete* XW8, *Actinomycetes* XW3, *Actinomycetes* XW5 of Cd and *Bacterium*XW6 produced no hemolysis on whole blood agar plates. The hemolysis experiment is simple and feasible[11], and it can screen out the unsafe strains at the beginning to avoid long ineffective experiment.

3.3 Immobilized Glue Beads

At 4% sodium alginate, 0.4% attapulgite and 0.4% biochar, the pressure resistance of the glue **bead** was 33%, round and black, as shown in Fig 1 on the left.

Rice sprouted on the screening model with Baiyin heavy metals soil, as shown in Fig 1 on the right.

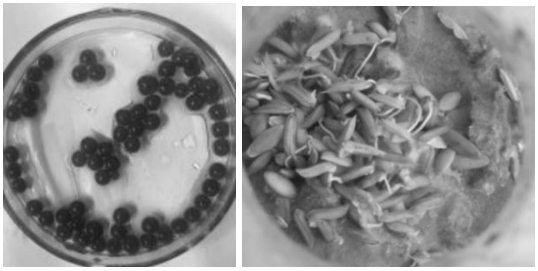

Fig.1 Immobilized glue beads left）, Microbial screening model (right)

3.4 Identification and Characterization Of CRM

The *Streptomycete* XW8 spore filaments are straight, soft, pink, oval, irregularly round (FIG. 2).The air hyphae did not form the transverse septum at the initial stage, but the transverse septum and fracture appeared only when the long spore filament was mature.Aerial hyphae are mainly gray, gray and purple. Gause No1 agar produces melanin on the back of media. According to Bergey's manual[12], XW8 was identified as Streptomyces preliminarily (Fig. 2).

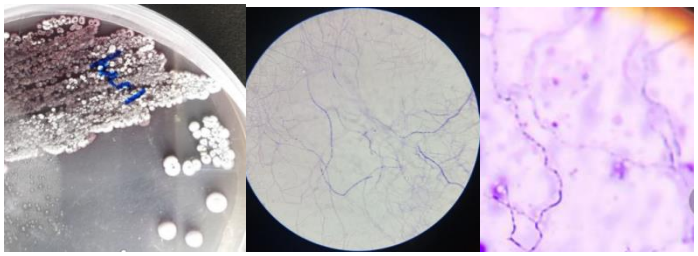

Fig2. *Streptomyces* XW8 (left colon, middle 7d gram stain 400x , right 14d crystal violet stain 1600x)

The standard $CdNO_3$ was added to potted soil, mostly in an exchange state, which was easy to be extracted by ammonium acetate, and the addition of high-concentration ammonium acetate effectively prevented the re-adsorption of Cd [13].Therefore, the active soil Cd was extracted with ammonium acetate in this cinnamon soil.

Table 3: Cd content and TF of rice under Cd stress (calculated by dry weight)

	belowground part	overground part	Soil active Cd	TF
1	1.18±0.1a	0.05±0.003a	0.084±0.006a	0.042±0.003a
2	1.8±0.2a	0.041±0.003ab	0.054±0.004ab	0.0228±0.001ac
3	0.89±0.1ab	0.033±0.002ac	0.043±0.003ac	0.037±0.002ab
4	0.035±0.002ab	0.58±0.04a	0.040±0.003ac	16.57±1.01a

For each column, values not marked with the same letter in superscript are significantly different at $p<0.05$ (Duncan's).

All the indexes of No3(*Streptomycete* XW8, *Actinomycetes* XW3, *Actinomycetes*XW5) were significantly different from the control($P<0.05$), there were significant differences in the content of soil available Cd and Cd in the overground part between No3 with other groups. The TF of the No3 group was much higher than that of the other groups. Compared with the No2(*Bacterium* XW 6, *Actinomycetes* XW3, *Actinomycetes*XW5), only one strain of No3 was different, indicating that *Bacterium* XW6 activate Cd, Soil available Cd content are almost the same in No2 and No3., so soil active Cd is not the determinant of crop cadmium safety.

4.Discussion

A single component cannot has all the mechanism, microbial flora complement each other and improve their resistance to pollutant. Bioremediation effect of No3(*Streptomycete* XW8, *Actinomycetes* XW3, *Actinomycetes*XW5) is better than that of the No2(*Streptomycete* XW8, *Bacterium* XW 6), but No3(*Streptomycete* XW8, *Actinomycetes* XW3, ActinomycetesXW5) and No2(*Streptomycete* XW8, *Bacterium* XW6) is superior to No4(*Bacterium* XW6, *Actinomycetes* XW3, *Actinomycetes*XW5), *Streptomycete* XW8 is supposedly the best one, particularly when applied in combination with *Actinomycetes* XW3, *Actinomycetes* XW5.

Actinobacteria would be excellent candidates for these microbial flora because of their proven versatility and abundance in the environment, microbial flora are robust, stable, and with synergistic activity to remove Cd and complex pollution[14].

For simulating complex interactions among plants and microorganisms and soil entrapped in a gel matrix, the immobilization reverse screening model is high-efficiency and low-cost to get CRM[15].

Microbial flora (*Bacterium* XW6, *Actinomycetes* XW3, *Actinomycetes* XW5) raises the bioavailability of Cd in rice soil. Clay mineral types, microorganisms, humus, pH, heavy metal properties, soil enzymes and human activities all affect the bioavailability of heavy metals in soil. Bioremediation of heavy metals seems to be more efficacious when microbial flora are compatible.

- Silicon can form a hard silicified cell on the surface of stems and leaves, reducing heavy metal pollution. Phytase in Carbon silicon functional liquid fertilizer can increase the activity of soil phosphatase and increase the content of phosphorus[16], which passivate Cd to phosphate by biomineralization. Biochar and humic acid [17-18] increased soil organic matter and nutrient as well as the survival of the place, to ensure the distribution of microbial colonization in time [19]. The application of multiple amendments is the reason for the success in this study.
- Quantification index is Single set of goals of soil and agriculture product,Unquantifiable index will be changed with space-time such as long-acting, ecological safe and biodiversity. Control to quantification index is not guaranteed for control of unquantifiable index. How to integrate the goals at different levels of complex giant system, will be challange for us.

5. Conclusion

1.*Bacterium* XW6 is a premium candidate for application in phytoremediation field trials.

2.Simple experimental model is useful for simulating complex interactions among plants and bacteria and soil entrapped in a gel matrix.

3.Microbial flora(*Streptomycete* XW8, *Actinomycetes* XW3, *Actinomycetes* XW5) reduces Cd accumulation in rice when used together with biochar, humic acid and Carbon silicon functional liquid fertilizer.

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