Isolation and Screening of Rhizobacteria from *Scirpus Grossus* Plant after Lead (Pb) Exposure

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Abstract: Phytoremediation is one of method which can be applied to remediate the contaminated environment. In most cases, microorganisms bacteria and fungi, living in the rhizosphere closely associated with plants, may contribute to mobilize metal ions by increasing the bioavailable fraction. Some studies have evidenced that heavy metal-resistant bacteria can enhance metal uptake by hyperaccumulator plants. Lead-resistant bacteria which could help to increase the lead uptake by *Scirpus grossus* was isolated and screened. The samples were taken from plant roots after being exposed in a range finding test by spiking analytical grade of $Pb(NO_3)_2$ solution in variation of Pb concentrations. The results of rhizobacteria isolation showed that there were several colonies having resistance to grow and survive in contaminated environment even the host plant had withered. Only a few of rhizobacteria colonies were affected by high concentrations of lead exposure during screening test. The screening test was conducted by growing the isolated colonies on plates containing tryptic soy agar (TSA) medium containing of 200, 400 and 600 mg/L Pb solution including the plate with only TSA media without any lead exposure acting as a control medium, and incubating them at 30°C for 72 hours. Isolation of bacteria from rhizosphere had found 47 colonies including several colonies from the withered plants. These all 47 colonies then become 28 after characterization by using color and colony morphology, followed by Gram stain, catalase, oxidase and motility test. The screening test of lead resistant bacteria colonies resulted 3 groups which is scored high, medium and low. The screened colonies will then be used for further study.

Key words: Bacteria screening, rhizobacteria isolation, phytoremediation.

1. Introduction

Several methods have already been used to clean up the environment from these kinds of contaminant, but most of them are costly and difficult to get optimum result. Currently, phytoremediation is an effective and affordable technological solution used to extract or remove inactive metals and metal pollutants from contaminated soil. Phytoremediation is the use of plants to clean up a contamination from soils, sediments and water. This technology is

environmental friendly and potentially cost-effective. Plants with exceptional metal-accumulating capacity are known as hyperaccumulator plants [1]. Phytoremediation takes the advantage of the unique and selective uptake capabilities of plant root systems, together with the translocation, bioaccumulation, and contaminant storage/degradation abilities of the entire plant body [2].

Several species of plants have the ability to uptake contaminant from their growing environment such as *Scirpus grossus*. Many species of plants have been successful in absorbing contaminants. The one of phytoremediation category, phytoextraction can be used to remove heavy metals from soil using its ability

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to uptake metals which are essential for plant growth (Fe, Mn, Zn, Cu, Mg, Mo and Ni). Some metals with unknown biological function (Cd, Cr, Pb, Co, Ag, Se, Hg) can be accumulated [1]. The uptake process mechanisms might include phytoextraction, phytostabilization, rhizofiltration and phytovolatilization [3-6] and these processes influenced by factors such as the plant species, root zone and properties of the medium [7-10]. In most cases, microorganisms such as bacteria and fungi within the rhizosphere which are closely associated with plants, might be contributing to mobilizing metal ions by increasing its bioavailable fraction. According to Ref. [4], their role in eliminating organic contaminants is more significant compare to inorganic compounds. Studies have also evidenced that heavy metal-resistant bacteria can enhance metal uptake by hyperaccumulator plants. Sheng, et. al. [11] reported that total Pb uptake by rape plants was significantly enhanced by heavy metal-resistant endophytic bacteria. The application of the heavy metal-resistant endophytic bacterial strains has effectively increased the bioavailability of Pb in the rhizosphere soils and promoted the growth of plants. Consequently, it increased the total Pb uptake of plants even under nonsterile conditions.

Procedures of bacteria isolation and screening include isolating and growing the bacteria colonies on special growth agar (Tryptic Soy Agar) media, colony characterization, Gram stain and through chemical/biochemical tests such as catalase, oxidase and motility. The biochemical test consists of gram stain, catalase test, oxidase test and motility test. Gram stain is used to classify bacteria on the basis of their forms, sizes, cellular morphologies, and gram reactions. And the later test was screening the lead resistant bacteria colonies by growing each on tryptic soy agar (TSA) medium in the presence of variation concentration of lead (Pb).

The aim of this study is to isolate and determine the lead resistant bacteria from rhizosphere *Scirpus*

grossus plant after exposure on a range finding test. Range finding test is the first step of phytotoxicity test and was conducted to establish and determine the concentration for phytotoxicity test [12]. The resulting concentration was used as a baseline study in conducting the next step [13]. The screening and isolation of lead resistant bacteria was performed to obtain species having high resistance against lead containing environment and therefore might have the ability to enhance lead bioremediation process.

2. Materials and Methods

2.1 Bacteria Isolation

The range finding test was conducted on a single exposure system by using several concentration of lead as the contaminant. The variation of lead concentration are 0 mg/L (control), 50 mg/L, 100 mg/L, 200 mg/L, 350 mg/L, 500 mg/L, 650 mg/L and 800 mg/L, by diluting analytical grade of $Pb(NO_3)_2$ salt (R & M, UK) in deionized water. For treating the simulated wastewater, the plants of *Scirpus grossus* species were grown in a bed of inert granular substrate (sand or pea gravel) which were sieved (0.5 cm) to remove coarse fragments and obtain similar size [2, 14]. Three plants were planted in each concentration of lead. The observation about the number of plant withered was conducted within 7 weeks and by the end of the observation, the roots of *Scirpus grossus* were taken to isolate rhizosphere bacteria. About 10-15 gram of roots from each lead concentration were separated and added with 100 mL of sterilized distilled water. The mixture of roots and water was agitated for 1 hour before 0.1 mL of the solution being taken and plated on Trypticase Soy Agar (TSA) (BD, USA) for 24 hour. TSA was chosen because there was no yeast, peptone, and amino acids in media which can bind large amounts of divalent Hg, Pb, Ag, and Cu ions [15]. Several colonies were observed growing. The colonies were isolated and grown in separate Tryptic Soy Agar for more than 24 hour.

2.2 Bacteria Characterization

The characterization of rhizobacteria colonies based on the shape and colors of the colonies were examined under a microscope (Leica-DM 1000) [15, 16]. Chemical tests of Gram stain, catalase, oxidase and motility were also performed.

Bacteria are classified according to Gram staining reaction, which permits the separation of all bacteria into two large groups, those which retain the primary dye (gram-positive) and those that take the color of the counterstain (gram-negative). Gram positive bacteria have thicker and denser peptidoglycan layer in their cell walls. Iodin penetrates the cell wall of these bacteria and alters the blue dye to inhibit its diffusion through the cell wall during the colorization. The Gram negative bacteria must have an intact cell wall to produce a positive reaction. Gram negative cells which do not retain the methyl/crystal violet stained by a counterstain. Neutral red or safranin may be used as the counterstain [17]. The catalase test is to detect the catalase enzyme in the most cytochrome-containing aerobic and facultatively anaerobic bacteria, except *Streptococcus* and *Enterococcus* species. It detects the presence of catalase enzymes by the decomposition of hydrogen peroxide to release oxygen and water which is formed by some bacteria as an oxidative end product of the aerobic breakdown of sugars. If it is allowed to accumulate, it is highly toxic in bacteria and can result in the cell death. Catalase either decomposes hydrogen peroxide and oxidizes secondary substrates, but it has no effect on other peroxides [18]. The oxidase test is used to determine if an organism possesses the cytochrome oxidase enzymes. The test is used as an aid for the differentiation of *Neisseria*, *Moraxella*, *Campylobacter*, and *Pasteurella* species [19]. Motility test is conducted by using hanging drop method, a small drop of liquid bacterial culture is placed in the centre of coverslips. A slide with a central depression is inverted over the coverslip that will stick to the slide and when the slide is inverted the drop of

bacterial culture will be suspended in the well. The motile bacteria is examined microscopically. Positive result includes darting, zig zag, tumbling or other organized movement, whereas negative result if there is no movement of Brownian motion [20].

2.3 Bacteria Screening

Later the screening test for lead resistant bacteria colonies was conducted by growing each on tryptic soy agar (TSA) medium in the presence of concentration variation of lead (Pb) (0 mg/L as control, 200 mg/L, 400 mg/L and 600 mg/L). Plates were incubated at 30°C for 72 hours [21]. The observation results on the growth then were scored and grouped as high, medium and low score with respective scores of 3.50 to 5.00, 1.50 to 3.49 and 0.00 to 1.49 when compared with the control medium. High score means that the bacteria growth is almost similar to control medium, whereas low score means the bacteria growth is very slow.

3. Results and Discussion

3.1 Bacteria Isolation and Screening

There were 47 colonies isolated from *Scirpus grossus* roots which were planted in all the variation of lead concentration (0 mg/L (control), 200 mg/L, 500 mg/L, 650 mg/L and 800 mg/L). Eight colonies from rhizosphere of *Scirpus grossus* which were isolated from control crate (0 mg/L), 9 colonies from 200 mg/L, 9 colonies from 500 mg/L, 11 colonies from 650 mg/L and 10 colonies from 800 mg/L. Even in the concentration of 650 mg/L and 800 mg/L in which the plants had withered, bacteria colonies were still detected from the root isolation. The isolation results of rhizosphere bacteria colonies are summarized in Table 1.

The first step was to group the isolated bacteria colonies based on the morphology characteristic. The bacterial colony formation was described by their form, elevation, margin shape, appearance, texture and pigmentation. These characteristics were observed by using a light microscope at 4x magnification. The nature of the colony elevation is apparent when

variation of lead concentration. The plates were incubated at 30°C and observed for 72 hours. All these 28 groups then were classified on 3 group of high score, medium score and low score of visual bacteria growth, where as the high score is 3.50 to 5.00, medium score is 1.50 to 3.49 and low score is 0.00 to 1.49, as shown in Table 5. Most of the bacteria colonies were still able to grow in Pb contaminated media even the growth was decreased compared to uncontaminated media (control media). According to some studies about bacterial heavy metal resistance, it can be possibly due to the

Concentration in range finding test.

Variation concentration of Pb (mg/L)		
0	650	800

No.	Rhizosphere bacteria from variation concentration of Pb (mg/L)				
	0 (control)	200	500	650	800
1	Sand 10-1(1)2 white	C200 10-1(3)1 white	C500 10-1(1)1 white	C650 10-1(2)1 white (rhizote)	C800 10-1(1)1 pink
2	Sand 10-1(1)2 pink	C200 10-2(2)1 blue	C500 10-2(2)1 white (rhizot)	C650 10-1(2)1 yellow	C800 10-1(3)1 white
3	Sand 10-1(2)2 white	C200 10-2(2)1 white (rhizot)	C500 10-4(1)1 yellow	C650 10-1(2)2 white	C800 10-1(3)2 yellow
4	Sand 10-1(2)2 blue	C200 10-2(2)1 yellow	C500 10-4(1)2 white	C650 10-3(1)1 light	C800 10-3(1)1 white
5	Sand 10-1(2)3 white	C200 10-2(2)2 light white	C500 10-4(1)3 white	C650 10-3(3)1 blue	C800 10-3(2)1 yellow
6	Sand 10-1(3)1 pink	C200 10-4(2)1 white	C500 10-4(1)3 blue	C650 10-3(3)2 white	C800 10-3(2)2 blue
7	Sand 10-3(3) yellow	C200 10-4(2)2 yellow	C500 10-4(2)1 white	C650 10-3(2)1 pink	C800 10-3(3)1 light yellow
8	Sand 10-3(3)1 pink	C200 10-4(3)1 yellow	C500 10-4(3)1 white	C650 10-3(1)1 yellow	C800 10-5(3)1 white (rhizot)
9		C200 10-4(3)2 white	C500 10-4(3)1 yellow	C650 10-5(3)3 white	C800 10-5(3)1 light yellow
10				C650 10-5(3)1 yellow	C800 10-1(1)2 white
11				C650 10-5(3)4 white	

[illegible]

Table 3 Result of gram stain, catalase, oxidase, motility test.

No.	Group	Gram Stain	Catalase Test	Oxidase Test	Motility Test	Code
A = 4						
1	Sand 10-1(1)2 white	(+)	(+)	(-)	(+)	A1
2	Sand 10-1(1)2 pink	(+)	(+)	(-)	(+)	A1
3	Sand 10-1(3)1 pink	(+)	(+)	(-)	(+)	A1
4	C800 10-5(3)1 light yellow	(-)	(+)	(-)	(+)	A2
B = 7						
5	Sand 10-1(2)2 white	(+)	(+)	(-)	(+)	B1
6	C200 10-4(3)2 white	(-)	(+)	(-)	(+)	B2
7	C500 10-1(1)1 white	(+)	(+)	(-)	(-)	B3
8	C500 10-4(1)2 white	(+)	(+)	(+)	(+)	B4
9	C650 10-1(2)2 white	(+)	(+)	(-)	(-)	B3
10	C800 10-1(3)1 white	(-)	(+)	(-)	(+)	B2
11	C800 10-3(1)1 white	(+)	(+)	(-)	(-)	B3
C = 5						
12	Sand 10-1(2)2 blue	(-)	(+)	(+)	(+)	C1
13	C200 10-2(2)1 blue	(-)	(+)	(+)	(+)	C1
14	C500 10-4(1)3 blue	(-)	(+)	(+)	(+)	C1
15	C650 10-3(3)1 blue	(-)	(+)	(+)	(+)	C1
16	C800 10-3(2)2 blue	(-)	(+)	(+)	(+)	C1
D = 2						
17	Sand 10-1(2)3 white	(+)	(+)	(+)	(-)	D1
18	C500 10-4(3)1 white	(+)	(+)	(+)	(-)	D1
E = 4						
19	Sand 10-3(3) yellow	(-)	(+)	(-)	(-)	E1
20	C200 10-4(3)1 yellow	(-)	(+)	(-)	(-)	E1
21	C500 10-4(1)1 yellow	(-)	(+)	(-)	(-)	E1
22	C650 10-5(3)1 yellow	(-)	(+)	(-)	(+)	E2
F = 3						
23	Sand 10-3(3)1 pink	(+)	(+)	(-)	(-)	F1
24	C650 10-3(2)1 pink	(-)	(-)	(-)	(+)	F2
25	C800 10-1(1)1 pink	(-)	(+)	(-)	(+)	F3
G = 5						
26	C200 10-1(3)1 white	(-)	(+)	(-)	(+)	G1
27	C200 10-4(2)1 white	(-)	(+)	(-)	(+)	G1
28	C500 10-4(2)1 white	(-)	(+)	(-)	(+)	G1
29	C650 10-5(3)4 white	(+)	(+)	(-)	(-)	G2
30	C800 10-1(1)2 white	(-)	(-)	(-)	(+)	G3
H = 4						
31	C200 10-2(2)1 white (rhizot)	(+)	(+)	(-)	(-)	H1
32	C500 10-2(2)1 white (rhizot)	(+)	(+)	(-)	(-)	H1
33	C650 10-1(2)1 white (rhizot)	(+)	(+)	(-)	(-)	H1
34	C800 10-5(3)1 white (rhizot)	(+)	(+)	(-)	(-)	H1
I = 6						
35	C200 10-2(2)1 yellow	(-)	(+)	(-)	(-)	I1.1
36	C200 10-2(2)2 light white	(-)	(-)	(+)	(-)	I2
37	C200 10-4(2)2 yellow	(-)	(+)	(-)	(-)	I1.3
38	C500 10-4(1)3 white	(+)	(+)	(+)	(+)	I3
39	C650 10-3(1)1 light	(-)	(+)	(+)	(+)	I4
40	C650 10-3(3)2 white	(-)	(+)	(-)	(-)	I1.2
J = 2						
41	C500 10-4(3)1 yellow	(-)	(-)	(-)	(-)	J1
42	C650 10-3(1)1 yellow	(-)	(-)	(-)	(-)	J1
K = 4						
43	C650 10-1(3)1 yellow	(-)	(+)	(-)	(+)	K1
44	C800 10-1(3)2 yellow	(-)	(-)	(-)	(+)	K2
45	C800 10-3(2)1 yellow	(-)	(+)	(-)	(+)	K1
46	C800 10-3(3)1 light yellow	(-)	(-)	(-)	(-)	K3
L = 1						
47	C650 10-5(3)3 white	(-)	(-)	(+)	(-)	L1

Table 4 Bacteria colony from the rhizosphere of *Scirpus grossus* which were planted in all lead concentrations.

No.	Code	Pure culture picture	Colony picture (x40)	Gram stain picture (x1000)
1	A1
2	A2
3	B1
4	B2
5	B3
6	B4
7	C1
8	D1
9	E1
10	E2

11	F1
12	F2
13	F3
14	G1
15	G2
16	G3
17	H1
18	II.1
19	II.2
20	II.3

21	I2
22	I3
23	I4
24	J1
25	K1
26	K2
27	K3
28	L1

mechanisms of tolerance and detoxification and also may be caused of chelating agent production that bound the metals and reduce their phytotoxicity [22]. Besides, some heavy metals have important roles in

biochemical reaction and have important portions to the cell growth even in limited biological function [22].

Several colonies from each group of the screening test result will be picked randomly for further study.

Table 5 Classification of bacteria group based on the score of bacteria growth on screening test.

No.	Score of bacteria growth observation		
	High score (3.50-5.00)	Medium score (1.50-3.49)	Low score (0.00-1.49)
1	A1=4.17	E1=3.17	A2=1.33
2	B1=4.83	E2=2.67	F3=0.83
3	B2=5.00	F2=1.83	G1=1.17
4	B3=5.00	G2=2.00	I2=0.17
5	B4=5.00	G3=2.17	J1=0.0
6	C1=5.00	I1.1=1.67	K3=1.17
7	D1=5.00	I1.2=2.17	
8	F1=4.33	I1.3=2.17	
9	H1=5.00	I3=3.17	
10	I4=3.50	K1=1.50	
11		K2=3.17	
12		L1=3.17	

These classified bacterial groups will later be tested for their maximum inhibition concentration (MIC) and continued with biosorption test to further screen the lead resistant bacteria before being identified.

4. Conclusions

Range finding test on *Scirpus grossus* has shown that lead (Pb) concentration of 650 and 800 mg/L had withered the plant. Isolation of bacteria from rhizosphere had found 47 colonies including several colonies from the withered plants. These 47 colonies were further reduced to 28 after characterization through color and colony morphology, and followed by gram stain, catalase, oxidase and motility test. The screening tests of lead resistant bacteria colonies resulted in 3 groups which were scored high, medium and low. Several colonies from each group of this screening test will be picked randomly to be applied on future tests in determining the inhibition concentration (MIC) and biosorption capacity in order to further screen for the lead resistant bacteria.

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