EFFECT OF NUTRIENT AND INOCULATION OF PB-TOLERANT BACTERIA ON REMOVAL OF PB FROM SIMULATED WASTEWATER BY *Pistiastratiotes&Eichhorniacrassipes*

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Abstract -The potential of removal capacities of selected aquatic plants P. stratiotes and E. crassipesfrom simulated wastewater were determined by using Continuous Vertical Inlet Flow (CVIF) technique. Under present investigation, both indigenous aquatic plants were used to evaluate the effect of nutrient (0, 1 and 2-fold nutrient) and inoculation of Pb – tolerant bacteria (Bacillus cereus 1-NMeHI-Cr2)on removal of Pbfrom simulated wastewater. Both species were cultivated in simulated UMS Lake water by adding 1.5 mg/L concentration of Pbat 0, 1 and 2 fold nutrient levels for 15 days experimental period. Then, the preferential nutrient level was selected to examine the effect of inoculation Bacillus cereus 1-NMeHI-Cr2 on the removal capacities of Pb. The concentration of Pb in wastewater and plants were analyzed by Inductively Coupled PlasmaAtomic Emission Spectroscopy(ICP-OES). The results showed that both indigenous aquatic plants performed extremely well in removing Pb with (99.6%) at 0-fold > (94.7%) at 1-fold and (85.4%) at 2-fold nutrient levels after 15 days experimental set up. The inoculation of Pb-tolerant B. cereus 1-NMeHI-Cr2 (T1) onto the roots of P. stratiotes and E. crassipeshad accelerate the removal capacity within 4 day period with 99.6% followed by (T2) without inoculation with (99.4%). The results also shown that both plants have a similar distribution pattern of Pb in plant tissues as roots > leaves > stalks. The physical changes and morphological symptoms of both plantswere also discussed in this paper.

Keywords: Phytoremediation, P. stratiotes, E. crassipes, Pb, Removal Capacities, Bacteria Inoculation, Plant Tissues

I. INTRODUCTION

Heavy metals are recalcitrant in the environment and are considered as one of the major environmental problem. Increasing of industrial pollutants in contrast with organic materials into water bodies can contribute to a variety of toxic effects such as muscular stiffness, loss of appetite, kidney damage, and can cause cancer in the digestive tract to human health [6]. Occurrence of toxic metals in these different components of the environment will affect the lives of local people that depend upon these water sources for their daily [16]. Phytoremediation, the use of plants to remove pollutants offers a promising technology for heavy metal removal from wastewater [12][14] and had been done to remediate soils, sludge, sediments and water contaminated with organic and inorganic contaminant. This technology offers cost effectiveness, non-intrusive and safe alternative to conventional clean-up techniques [17].

In the last few decades many scientists in different part of the world has worked out to improve the phytoremediation uptakes of heavy metals with the presence of nutrients concentration and inoculation of bacteria to roots. These addition of nutrients and interactions of microbes are implicated to play an essential role in plant metal uptake. In particular, the plant-associated beneficial microbes can enhance the efficiency of phytoremediation process directly by altering the metal accumulation in plant tissues and indirectly by promoting the shoot and root biomass production. This process is a positive synergy for both sides, once the plants supply nutrients for microorganisms which turn grow and thus enhance the capacity of degradation by plants or increasing the phytotoxicity of the contaminated area [9].

*P. stratiotes*and *E. crassipes*or commonly known as water lettuce and water hyacinth were chosen in this study as they can easily be obtained in our environment and act as biological filters by accumulating heavy metals in their tissues. This plant also has high absorption capacity of heavy-metal and nutrient qualify thus make it suitable use in wastewater treatment [7][8].The objective of the present study is to evaluate the effect of nutrient and inoculation of Pb-tolerant*B. cereus* 1-NMeHI-Cr2 bacteria on removal of Pb from simulated wastewater by *Pistiastratiotes* and *Eichhorniacrassipes*using CVIF reactor.

II. MATERIALS AND METHODS

A. *Experimental Design*

The methodology of this research was divided into two main research section, which were the effect of nutrients at 3 different concentrations level (0,1& 2-fold) and theeffect of preferential nutrient level with and without the inoculation of Pb-tolerant *B. cereus* 1-NMeHI-Cr2.

B. *Experimental set-up*

Continuous vertical-inlet flow (CVIF) reactor consisted of 4 glass tanks was set up vertically with the lowest glass tank (45 x 45 x 30 cm) containing a water pump to circulate the simulated wastewater continuously. The second (35 x 35 x 15 cm) and third (25 x 25 x 10 cm) tank was filled with *P. stratiotes*and *E. crassipes*. The fourth tank (11 x 11 x 13 cm) is the central outlet of the wastewater. The second, third and fourth tanks had protruding outlets on all sides which enable the water to circulate vertically (Figure 1).

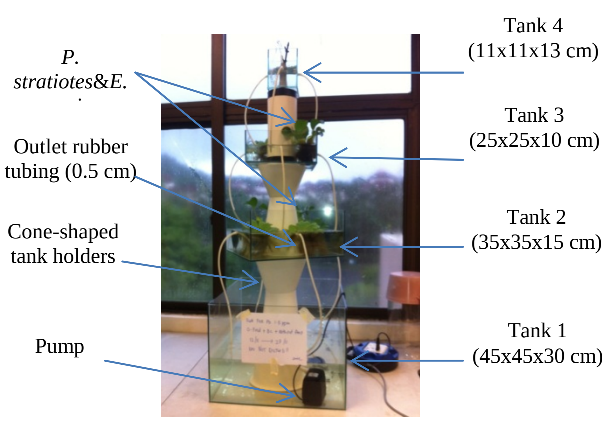


Figure 1.Experimental set-up on the effect of nutrient and inoculation on metal removal by *P. stratiotes* and *E. crassipes* using CVIF reactor.

C. *Removal Capacities of Pb by P. stratiotes and E. crassipes with the Effect of Nutrients*

P. stratiotes and *E. crassipes* were obtained from a non-contaminated site and cultivated in an aquaria filled with 40 L of water. The macrophytes were washed under running tap water to eliminate remaining debris. Heavy metal Pb was added at 1.5 mg/L with addition of nutrient solution at 0- fold, 1- fold and 2-fold. A nutrient solution containing 28 and 7.7 mg/L of total nitrogen and total phosphorus were used as a standard nutrient solution (1- fold) [11]. The following experiments (in duplicate) were performed.

Experiment I — Approximately 50g (fresh weight) of *P. stratiotes* and *E. crassipes* were cultivated in the second and third tank CVIF of 40L Lake UMS water. Heavy metal Pb was added in the concentration at 1.5 mg/L. No nutrient solution (0-fold) was added.

Experiment II — Approximately 50g (fresh weight) of *P. stratiotes* and *E. crassipes* were cultivated in the second and third tank CVIF of 40L Lake UMS water. Heavy metal Pb was added in the concentration at 1.5 mg/L. A 1-fold nutrient solution was added.

Experiment III — Approximately 50g (fresh weight) of *P. stratiotes* and *E. crassipes* were cultivated in the second and third tank CVIF of 40L Lake UMS water. Heavy metal Pb was added in the concentration at 1.5 mg/L. A 2-fold nutrient solution was added.

Experiment IV — Approximately 1.5 mg/L of Pb was added in the CVIF reactor. No plants were grown in the second and third tank of CVIF reactor (Control).

D. *Removal of Pb by P. stratiotes and E. crassipes(Effect of Inoculation Pb-Tolerant Bacteria)*

1. Microorganisms used

Pb-tolerant strain *B. cereus* 1-NMeHI-Cr2 was used in this study is known to have high removal capacity for Pb of plant based wastewater treatment systems[13]. This strain was obtained from the culture collection of the Faculty of Science & Natural Resources, Universiti Malaysia Sabah.

2. Media Preparation

Nutrient agar and broth were used for culture and maintenance of the bacteria strain used. Approximately 20 mL of the autoclaved media were poured into pre-sterilized disposable petri dishes in laminar flow. The media were left to cool and solidified at room temperature before being cultured overnight at 30^0C to ensure they are free from contamination prior analysis.

3. Inoculation of *B. cereus* onto Roots of *P. stratiotes and E. crassipes*

The roots of *P. stratiotes and E. crassipes* were inoculated with (*B. cereus 1*-NMeHI-Cr2). The netlike roots of both plants were rinsed several times with sterile distilled water.

For roots inoculation, bacterial cultures were grown for 18h and harvested at hour 8. The harvested cells were by centrifuged at 6000 rpm for 10 min at 10^0C. The bacterial cells were then washed twice with sterile distilled water and resuspended in biological saline (0.85 % KCl). The roots of the plants were inoculated by submerging in a bacterial suspension containing 10^8 cells mL^{-1} for approximately 10 min as described by [4].The following experiments (in duplicate) were performed.

Experiment T_1 Approximately 50g (fresh weight) of *P. stratiotes* and *E. crassipes*were cultivated in 40L Lake UMS water. Heavy metal Pb was added at 1.5 mg/L concentration. The roots of *P. stratiotes*&*E. crassipes* were inoculated with rhizospheric bacteria (*B. cereus* 1-NMeHI-Cr2) using CVIF reactor.

Experiment T_2 Approximately 50g (fresh weight) of *P. stratiotes* and *E. crassipes*were cultivated in 40L Lake UMS water. Heavy metal Pb was added at 1.5 mg/L concentration. Non inoculation of *B. cereus* 1-NMeHI-Cr2 were done using CVIF reactors.

Experiment T_c Approximately 1.5 mg/L of Pb was added in CVIF reactor. No plants were grown in second and third tank of CVIF reactor (Control).

Before inoculation of Pb-tolerant bacteria, the roots were submerged in ampicillin solution to kill the originally rhizospheric bacteria. Then the preparation samples 1-2 cm of inoculation roots were cut and dipped in distilled water twice. The samples were mounted on SEM specimen stubs for viewing under a Field Emission Scanning Electron Microscope. A SEM image of roots *P. stratiotes* and *E. crassipes*with and without inoculation of Pb-tolerant bacteria are shown in Figure 2 (a)-(d).

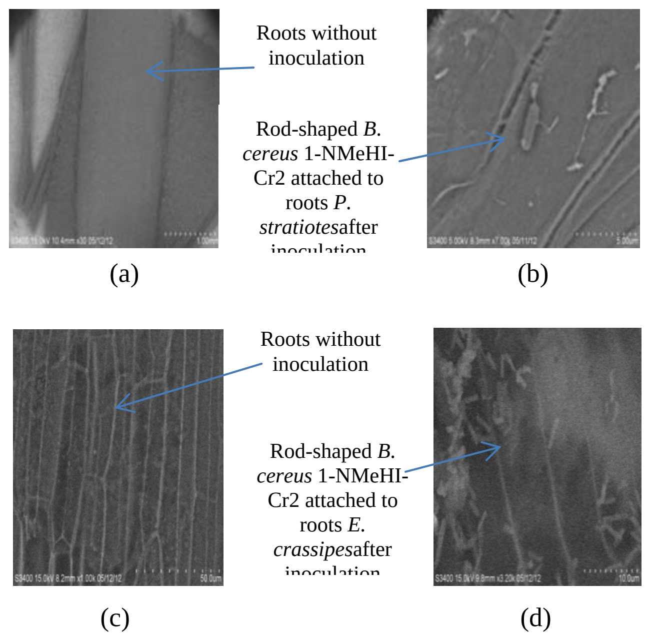


Figure 2.SEM root images of *P. stratiotes*and *E. crassipes*with various treatments: without inoculation (a) and (c), with inoculation of *B. cereus* 1-NMeHI-Cr2 (b) and (d).

E. Sampling and Preparation of Simulated Wastewater and Aquatic Plants

1. Wastewater

Initial concentration ofPb in the simulated wastewater were determined. Duplicate water samples (40 ml) each from the reactor were taken every day until 15 days of experimental sets. The samples were filtered using 0.45um membrane filter (Whatman) and preserved at 4^0C for prior analysis.

2. Aquatic Plants

The concentration of heavy metals in *P. stratiotes* and *E. crassipes* were determined before and after the heavy metal removal experiments during the 15 days period. Plants sample were collected and cleaned under the running tap water and distilled water to remove debris from the roots and leaves section. The plants were cut into different parts namely roots, leaves and stalks before being dried at 60-75^0C to a constant weight and pulverized to facilitate wet digestion.

3. Digestion

2 g of dried samples were digested using the acid digestion process with 25 ml HNO_3 in *Kjeldahl* block digester at 120^0C for 2 hours. The digested samples were filtered with 0.45um membrane filter (Whatman) and stored at 4^0C [1].

4. Determination of Heavy Metals Concentration

The concentrations of Pb in the simulated wastewater were determined before, during and after the experiments within 15 days period. The plant samples (roots, leaves and stalks) were analyzed for heavy metals on two occasions, first on initiation date of removal experiment and second after 15 days removal experiment. All heavy metals were analyzed with Inductively Coupled Plasma Spectroscopy (ICP-OES). Standard calibration curve for each metal was established to determine their concentrations.

5. Removal Capacities and Uptake of Pb by Both Aquatic Plants

The removal percentage and the uptake of Pb in both plant parts were calculated according to the formula (i) and (ii) respectively.

i) Removal percentage (%) = $\frac{T_i - T_f}{T_i} \times 100$

where:
T_i = Total metal initial (mg/L)
T_f= Total metal final (mg/L)

ii) Uptake of metal in plant parts (ug/g) = $\frac{C_p - T_V}{D_W}$

where:
C_p = Concentration in plant parts (ug/mL)
T_V = Total volume (mL)
D_W = Weight of biomass (g)

III. RESULTS AND DISCUSSION

1. Effect of Different Nutrient Levels on Removal Capacities of Pb with *P. stratiotes* and *E. crassipes*

Figure 3 show changes in metal concentration in simulated wastewater at different nutrient levels and different times upon exposure experiments. Experiments containing *P. stratiotes* and *E. crassipes* for single Pb 1.5 mg/L at 1 and 2-fold nutrient levels show gradual drop until day 15 except for 0-fold nutrient show a sudden drop until day 7 of cultivation. It is important to highlight that the final concentration of Pb exposed to 1.5 mg/L at 0-fold nutrient was below 0.0005mg/L as compared to other experiments. Thus the data suggested that, both aquatic plants exposed to 0-fold nutrients had uptake highest concentration of Pb. The removal percentage of Pb at 3 nutrient levels studied can be shown in Table 1.

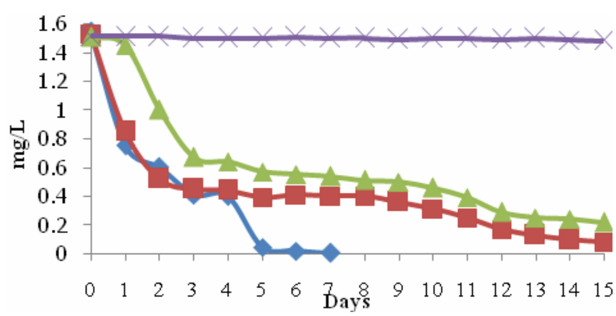


Figure 3.Pb concentration vs time at 1.5 mg/L cultivated at different nutrients levels (0–fold, 1-fold, 2-fold)

Table 1.Removal Percentage of Pb at different nutrient levels andphysical changes observed during 15 days cultivation

Pb (mg/L)	Addition nutrient	Removal Percentage (%)	Observed physical changes on plants								
			Roots				Leaves				Stalks
			Fragile		Decay		Yellowing		Wilting		Wilting
			P	*E*	*P*	*E*	*P*	*E*	*P*	*E*	*E*
1.5	0	99.6	-	-	-	-	-	-	-	-	-
	1	94.7	-	-	-	-	-	-	-	-	-
	2	85.4	√	-	√	-	√	-	√	-	-

**P = Pistiastratiotes* **E = Eichhorniacrassipe*

As clearly shown in Table 1, the removal percentage of Pb at 1.5 mg/L are in descending orders as both plants shown a great potential in removing Pb when cultivated under 0- fold (99.6%)> 1-fold (94.7%)> 2- fold (85.4%) nutrient levels. The physical changes such as roots become fragile, wilting and decaying of the leaves were observed visually in *P. stratiotes*cultivated at 2-fold nutrient levels at 1.5 mg/L (Figure 4). On contrary, *E. crassipes* was observed healthy during 15 days of cultivation periods. The increase in metal concentrations in the experiment with increasing nutrient levels up to 2-fold may be attributed to the release of this metal from dead plant tissues into the solution medium during the experiment.

(a) (b)

Figure 4. Physical changes observed in *P. stratiotes* cultivated in different nutrient levels at 1.5 mg/L (a) yellowing and wilting leaves and (b) fragile roots

2. Uptake and Distribution of Pb in Plant Parts of *P. stratiotes*and *E. crassipes* at Different Nutrient Levels

Figure 5 showed the uptake and distribution of Pb at 1.5 mg/L in plant parts of *P. stratiotes*and *E. crassipes*cultivated in 0-fold, 1-fold and 2-fold nutrient levels. The results showed that roots of *P. stratiotes* cultivated at 1.5 mg/L with 1-fold nutrient level had highest uptake biomass with 20.1 ug/g whereas the leaves cultivated at 2-fold nutrient levels showed the lowest uptake with 3.7 ug/g. The results revealed as the nutrient levels and concentrations added increased, the rate of biomass uptake by *P. stratiotes* was decreased except for roots cultivated at 0-fold nutrient. The experiment data showed that *P. stratiotes* under nutrient poor condition (0-fold) can uptake higher Pb as compared to other nutrient levels. The results were justified whereby the removal percentage was proved to be higher when this plant was cultivated in nutrient poor condition with 99.6%. The distribution pattern of Pb in *P. stratiotes* was observed higher in roots as compared to leaves by 2 times for all nutrient levels studied. The similar pattern can be observed whereby the highest uptake of Pb by *E. crassipes*can be shown in roots. Meanwhile, stalks cultivated in 2-fold nutrients showed the lowest uptake with only 6.2 ug/g. The difference distribution of Pb in *E. crassipes* was observed higher in roots followed by leaves and stalks. The results also revealed that, *E. crassipes* cultivated in poor nutrient condition had highest uptake of Pb as compared to rich nutrient levels.

Figure 5. Uptake and Distribution of Pb at 1.5 mg/L in plant parts of *P. stratiotes* and *E. crassipes* cultivated at three different nutrient levels.

3. Inoculation Effect of Pb Tolerant *B. cereus*1-NMeHI-Cr2 on the Removal of Pb from Simulated Wastewater

Figure 6 show experiment containing *P. stratiotes* and *E. crassipes* in CVIF reactor for T1 (with inoculation) and T2 (without inoculation) shows a simultaneous drop until day 5 and day 7 of exposure. It is important to note that the final sample of T1 at day 5 in CVIF reactor is lowest with 0.007 mg/L as compared to T2 (0.009 mg/L). The results revealed that the inoculation of *B. cereus*1-NMeHI-Cr2 (T1) towards both plants had great effect in reduced the Pb concentration in CVIF reactor upon the time. The removal percentages of Pb by both aquatic plants are shown in Table 2.

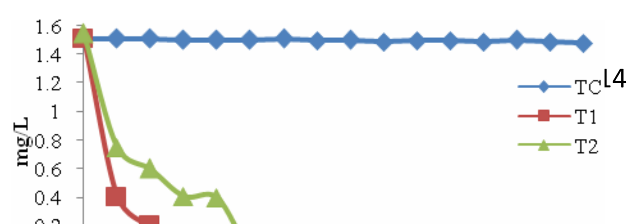


Figure 6.Pb concentration at 1.5 mg/L vs. time with *P. stratiotes* and *E. crassipes* in CVIF reactor : TC= treatment control (no plants added), T1= With inoculation of *B. cereus*1-NMeHI- Cr2, T2= without inoculation of *B. cereus*1-NMeHI-Cr2.

Table 2. Removal Percentage of Pb by *P. stratiotes* and *E. crassipes* with and without inoculation of *B. cereus*1-NMeHI-Cr2 and physical changes observed during 15 days cultivation.

No.	Experiment	Removal Percentage (%)	Observed visual physical changes on plants								
			Roots				Leaves				Stalks
			Fragile		Decay		Yellowing		Wilting		Wilting
			P	*E*	*P*	*E*	*P*	*E*	*P*	*E*	*E*
1.T1	With inoculation of *B. cereus* 1-NMeHI-Cr2	99.6	-	-	-	-	-	-	-	-	-
2.T2	Without inoculation of *B. cereus* 1-NMeHI-Cr2	99.4	-	-	-	-	-	-	-	-	-

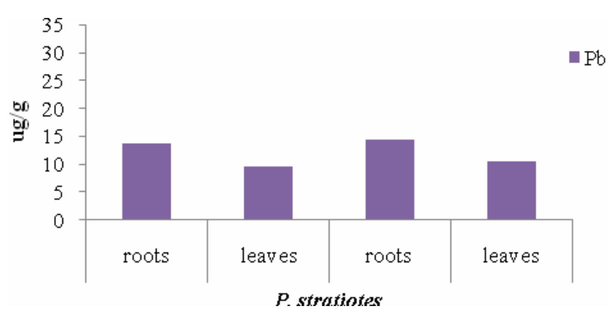
**P = Pistiastratiotes* **E = Eichhorniacrassipe*

As clearly shown in Table 2, the removal percentage of T1 (with inoculation *B. cereus*1-NMeHI-Cr2) recorded the highest percentage with 99.6% within 5 days of cultivation. This removal percentage is 0.2% highest than T2 (without inoculation of *B. cereus*1-NMeHI-Cr2). The experiment data revealed that the effect of inoculation *B. cereus* 1-NMeHI-Cr2 towards both plants roots had showed a great potential in removing Pb with only take 5 days of cultivation as compared to T2. Both plants were appeared healthy within the exposure times of experiments.

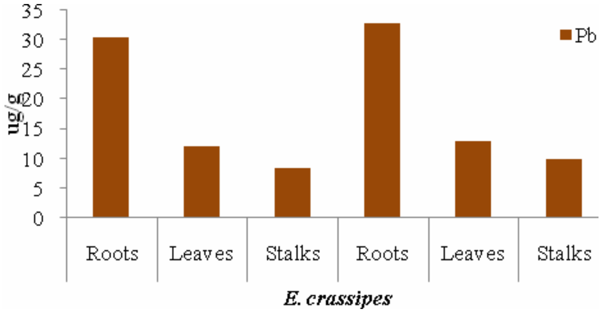
The inoculation of locally isolated Pb-tolerant *B. cereus* 1-NMeHI-Cr2 into the roots system of both plants in CVIF reactor had evidently enhanced in removal of Pb. According to [15], native rhizospheric bacteria had aid in the process of Pb removal. In addition, the characteristics of *B. cereus* component which is Gram positive bacteria cells that contains teichoic acids and acids associated to the cell wall, whose phosphate groups are the key components for the uptakes of metals. According to [3], the interaction of heavy metals and surface biological structures are inevitable. This surface accumulation occurs through chemical reaction such as complexation and ion-exchange with structural compound presents in the surface of rhizospheric microbes [2]. This finding is in line with [2], reported that plant biomass and metal acquisition by (*Trofoliumrefens*) grown in multi contaminated soil highly increased as compared to control when the plant roots were inoculated with *B. cereus*. [2]also had determined that Indole acetic acid (IAA) are production by *B. cereus* 6.2 ugmL^{-1}. This indicates that the bacterial effectiveness in reducing metals may be due to the involvement of IAA production plus the bioaccumulation ability exhibited by the bacterial cells.

4. Uptake and Distribution of Pb in *P. stratiotes* and *E. crassipes* With and Without Inoculation of *B. cereus*1-NMeHI-Cr2

Figure 7 showed the uptake of Pb in roots leaves and stalks by *P. stratiotes*and *E. crassipes*cultivated in CVIF reactor. As clearly shown in Figure 7 (I), the highest uptake of Pb can be observed in roots inoculated with *B. cereus*1-NMeHI-Cr2 with 14.35 ug/g whereas the lowest uptake of Pb can be shown in leaves P. *stratiotes*without inoculation (9.55 ug/g). The similar pattern distribution can be found in *E. crassipes* Figure 7 (II) whereby the highest uptake of Pb can be observed in roots *E. crassipes*with 32.9 ug/g with inoculation while the lowest uptake was observed in stalks without inoculation of *B. cereus*1-NMeHI-Cr2. The results also show that both plants were able to store high amount of Pb in roots as compared to leaves and stalks. The distribution of Pb in both plants shown in descending order as roots> leaves in (*P. stratiotes*) and roots> leaves> stalks in *E. crassipes* respectively.



(I)



(II)

Figure 7. Uptake and distribution of Pb at 1.5 mg/L in plant parts of *P. stratiotes* (I) and *E. crassipes* (II) with and without inoculation of *B. cereus*1-NMeHI-Cr.

In the present study, the effects of inoculation *B. cereus1*-NMeHI-Cr2 towards both plants roots are shown highly effective in uptake of Pb from simulated wastewater. Generally, plants and bacteria can form specific association in which the plant provides bacteria with a specific carbon source, thus this induces bacteria to reduce the phytotoxicity of contaminated water. In this study, *P. stratiotes* and *E. crassipes* form non specific association with *B. cereus*1-NMeHI-Cr2 that can stimulate the microbial community, which in turn the metabolic activity can degrades the Pb. These biochemical mechanisms increase the remediation activity of bacteria associated with plant roots [10]. It is reasonable to assume that inoculation of *B. cereus*1-NMeHI-Cr2 can increase plant biomass and thereby to stabilize and remediate the simulated wastewater. Previous studied by [5], reported that the inoculation of *Bacillus* eliciting plant growth promotion include auxin production, increase uptake available, bio-control abilities and induction of systematic resistance. Hence this finding suggested that the inoculation of *B. cereus*1-NMeHI-Cr2 towards both plants roots had displayed a good uptake of Pb. It is therefore concluded that both plants can be used as phytoremediation plants for removal of Pb in industrial wastewater.

IV. CONCLUSIONS

In this study, the effect of nutrient and inoculation of Pb-tolerant on metal removal of Pb from simulated wastewater had suggested that the effect of 0-fold nutrient and inoculation of *B. cereus* 1-NMeHI-Cr into the roots system in both plants had evidently enhance in removal of Pb. The results also shown that both plants have a similar distribution pattern of Pb in metal tissues as roots> leaves>stalks (*E. crassipes*) and roots> leaves (*P. stratiotes*). Studies with regard to aquatic plants combination with rhizospheric microbes to be used in engineered wetland treatment ponds should be seriously undertaken for developing more efficient and natural economic approach in removing heavy metals from contamination water. The ability of *P. stratiotes* and *E. crassipes* to remove heavy metals especially Pb had indicates their potential in treatment of metal polluted water. However, the plants must be harvested regularly to avoid the plants from wilting and releasing heavy metals back into the environment.

V. ACKNOWLEDGMENT

The study was supported by a grant (FRG0195-SG-1/2010) from the Ministry of Higher Education Malaysia.

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