Conference Paper

The Role of Heavy Metals-Resistant Bacteria *Acinetobacter* sp. in Copper Phytoremediation using *Eichhornia crasippes* [(Mart.) Solms]

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Abstract

Phytoremediation is a bioremediation process using plants and microorganisms to extract, sequester, or detoxify heavy metals. *Eichhornia crassipes* [(Mart.) Solms] is a well-known phytoremediating plant that has the ability to remove heavy metals from water by accumulating them in their tissues. *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 are copper resistant bacteria isolated from industrial waste in Rungkut, Surabaya. The aim of this research was to study the effect of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 inoculation in copper phytoremediation process using *Eichhornia crassipes*. Bacterial isolate with colony form unit of 10^8 was inoculated into the rhizosphere of *Eichhornia crassipes* in water containing 10 mL · L^{-1} and 20 mL · L^{-1} copper. Copper removal in contaminated water and copper accumulation in the plant roots was analyzed using atomic absorption spectrophotometer. The results showed that inoculation treatment enhanced the potency of the plant to reduce copper from 94 % concentration level in the medium without bacterial inoculation to 98.3 % and 97 % in medium inoculated with *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2, respectively. *Eichhornia crassipes* inoculated with *Acinetobacter* sp. IrC1 accumulated up to six fold higher copper concentrations in roots compared with un-inoculated controls. The roots of *Eichhornia crassipes* accumulated 596 mg · kg^{-1} and 391 mg · kg^{-1} in medium containing 5 mL · L^{-1} and 10 mL · L^{-1} copper without inoculation, while, the upper part of the plants accumulated up to 353 2.5 mg · kg^{-1} and 194 1.5 mg · kg^{-1} in medium inoculated with *Acinetobacter* sp. IrC1, respectively. The findings of the study indicated that *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 can improve the phytoremediation potential of *Eichhornia crassipes*.

Keywords: *Acinetobacter* sp.; copper; *Eicchornia crassipes*; phytoremediation, removal

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1. Introduction

Heavy metal contamination in water as a result of industrial activities is a serious environmental problem. Industries often discharge waste waters either directly or indirectly which is endangering the life of organism [1]. Heavy metals are considered as the most dangerous elemental pollutants because of their toxicities to human health [2]. There are two types of heavy metals is essential and non-essential. Non-essential heavy metals are toxic because it is a carcinogen that can cause cancer and other dangerous diseases especially for children. Non-essential heavy metals is also toxic if it is in high concentration. [3]. One of the most immediate concern and abundant metal is copper. Copper contamination is usually caused by a variety of sources including agriculture, electropolating, mining, leather tanning, chemical industry, and the textile industry. [4].

Various methods have been used to treat heavy metal pollution, which is chemically treatment such as precipitation, adsorption, nanofiltration, and reverse osmosis, but these methods are expensive, require high energy, and not environmental friendly because it can not remove all the heavy metals from the environment. [5]. Therefore, it should be considered a method that is eco-friendly, inexpensive, and efficient to resolve the problem of heavy metal pollution.

Phytoremediation is a waste treatment method that is eco-friendly and can solve the problem of environmental pollution because some plants have the potential to remove heavy metals from the environment [6]. Phytoremediation is the use of plants and microorganisms to reduce the concentration of heavy metals in the environment [3]. Phytoremediation is a detoxification method which manipulate the characteristics of plants to accumulate heavy metals in plant biomass. It is resulting of decreasing in heavy metals concentration in the environment [7]. The success of phytoremediation process depends on the ability of plants to grow in heavy metals contaminated area and to remediate the pollutants through several mechanisms such as accumulation the heavy metals in the plant tissues.

Eichhornia crassipes [(Mart.) Solms] frequently used in phytoremediation process because it can grow and produce high biomass in an environment that contains high concentration of heavy metals without showing many of the symptoms of toxic [8]. *Eichhornia* crassipes is a hiperacumulator plant that can accumulate heavy metal 100,000 times greater than other plants inside its tissues [9]. Therefore the use of *E. crassipes* as agents of phytoremediation can be a solution to overcome the metal pollution in the aquatic environment because the plant is not only hiperaccumulator plant but also easily available, low cost, effective, and eco-friendly methods [10]

Recently, phytoremediation associated with bacteria has appeared as a more successful approach to remove heavy metals from contaminated sites [11]. Plant growth promoting rhizobacteria is microorganisms which growing in the root zone and closely associated with roots or a diverse group of free-living bacteria which grow in the root area of plants. The bacteria can improve the growth and development of the host when the plant was grown in heavy metal contaminated sites by reducing toxic effects of heavy metals on the plants. [12, 13]. Previous study showed that inoculation of *Bacillus licheniformis* [(Weigmann 1898) Chester 1901] in the rhizosphere of *Brassica juncea* [(L.) Vassiliĭ Matveievitch Czernajew] enhanced cadmium accumulation in plant tissues [14]. The addition of *Microbacterium arabinogalactanolyticum* [(Yokota et al. 1993) Takeuchi and Hatano 1998], *Sphingomonas macrogoltabidus* (Takeuchi et al. 1993), and *Microbacterium liquefaciens* [(Collins et al. 1983) Takeuchi and Hatano 1998] significantly increased the plant uptake of nickel compared with un-inoculated controls [15].

E. crassipes is found growing in almost all the aquatic environment in Indonesia. Some bacteria can be isolated from heavy metal contaminated sites and introduced into the rhizosphere of the plant to enhace phytoremediation processes. *Acinetobacter* sp. IrC1 and *Acinetobacter*. sp. IrC2 are copper resistant bacteria isolated from industrial wastewater in Indonesia that have high potency to be accumulated [16]. The aim of this research was to study the effect of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 inoculation in increasing the efficiency of *E. crassipes* to remove and accumulate copper.

2. Materials and methods

2.1. Bacterial strains, media and growth

Acinetobacter sp. IrC1 and *Acinetobacter* sp. IrC2 are copper-resistant bacteria with the Minimum Inhibitory Concentration (MIC) of (6 to 7) mM $CuSO_4$, under the accession number of JX009133 and JX 009134, respectively. Bacterial isolate was grown in Luria Bertani (LB) agar containing the following contents (per L): tryptone: 10 g, yeast extract 5 g, NaCl 10 g, and glucose 0.1 g. Cells were incubated at 37 °C on a 200 rpm shaker [16] (1 rpm = 1/60 Hz). Growth was monitored by measuring optical density at 600 nm. Amount of 100 μLcells culture with appropiate dilution were spreaded on agar medium to determine the value of colony form unit (CFU). Bacterial inoculum with CFU of 10^8 would be ready for inoculating treatment in phytoremediation processes.

2.2. Phytoremediation experiment

Some of *Eichhornia crassipes* was collected from Kelapa-Dua Lake, Tangerang, Indonesia. Approximately (250 to 300) g of each plants were selected and put into containers containing 8 L water for acclimatization during 2 wk before being exposed to copper contaminants. Each plant was put into containers containing 10 mg · L^{-1}, 20 mg · L^{-1}, and without copper, with three replicates for each plants at each treatment, respectively. Amount of 800 mL of bacterial inoculums 10^8Colony Form Unit (CFU · mL^{-1}) was inoculated into the rhizosphere of *E. crassipes* in the container as inoculated treatment. The plants also were grown in water without copper addition as an un-inoculated control. The volume of water in each container was kept to be constant and the change in volume due to evaporation was compensated by the addition of water. Water solution was collected every two weeks during 28 d for copper analysis. Physiological appearance of leaves was also being observed including color and clorosic leaves for assesment the effect of copper toxicity toward the plant.

2.3. Copper content analysis

The root of plant was collected, washed with water, and dried in oven for 24 h at 70 °C. Amount of 0.2 g of dried root sample was grounded and suspended in 10 mL of water. The suspension was disrupted with HNO_3 at 100 °C. Copper content in dried root sample was analized using atomic absorption spectrophotometer for assesment the potency of *E. crassipes* to accumulate copper. Water sample from the container was also collected and destrupted with the same way for measuring the copper removal efficiency by *E. crassipes*. The removal efficiency was calculated as (Ci-Cf/Ci) x 100 % where C_i is the initial concentration and C_f is the remaining copper concentration in the solution [17].

3. Results and discussion

3.1. Bacterial inoculum

Acinetobacter sp. IrC1 and *Acinetobacter* sp. IrC2 are heavy metals-resistant bacteria isolated from industrial sewage in Rungkut, Surabaya, Indonesia. The bacteria have high resistance to some heavy metals such as copper, zinc, cadmium, mercury, and lead might be due to the selective pressure by pollution of industrial wastewater at Rungkut, the location from where the strain has been isolated. Chan et al. [18] reported that *Acinetobacter* can ussually isolated from polluted environment. *Acinetobacter* species are important biotechnological tools, and have been utilized extensively in the synthesis of enzymes and other life-sustaining macromolecules and for degradation of recalcitrant compounds [18]. Genus *Acinetobacter* was a member of bacterial community in rhizosphere of *E. crassipes* and in wetland water samples [19]. It was expected that inoculation of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 to

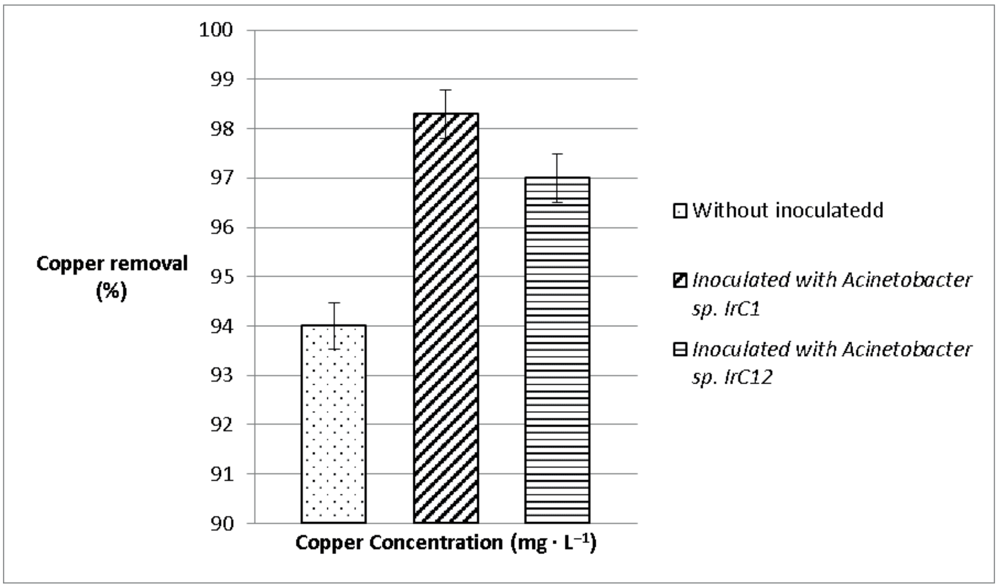

Figure 1: The effect of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 inoculation to remove copper in medium containing 10 mg · L^{-1} $CuSO_4$.

rhizosphere of *E. crassipes* improving the plant to accumulate and remove copper from solution.

3.2. Copper removal

The potency of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 to reduce copper concentration in water containing 10 mL · L^{-1} $CuSO_4$ can be seen at Figure 1. The results showed that bacterial inoculation treatment enhanced the potency of the plant to reduce copper from 94 % concentration level in the medium without bacterial inoculation to 98.3 % and 97 % in medium inoculated with *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2, respectively. Previous study showed that the ability of *E. crassipes* without bacterial inoculation to remove and accumulate cadmium, chromium, copper, lead, and nickel was 24 % to 80 % of total heavy metals [20]. Sharma et al. [21] reported that *Eichhornia crassipes* showed increased removal efficiency of heavy metals through the activity of its rhizopheric bacteria. The bacteria facilitate the uptake of essential elements.

According to Jing et al. [15], plant and bacteria can form specific associations in which the plant provides the bacteria with a specific carbon source that induces the bacteria to reduce the phytotoxicity of the contaminated sites. Rhizosphere plays a significant role in heavy metals phytoremediation in contaminated area. Microbial populations have potential to enhance phytoremediation processes. Bacteria can augment the remediation capacity of plants or reduce the phytotoxicity of the contaminated sites. Bacteria affect the mobility and availability of heavy metals in the plant through

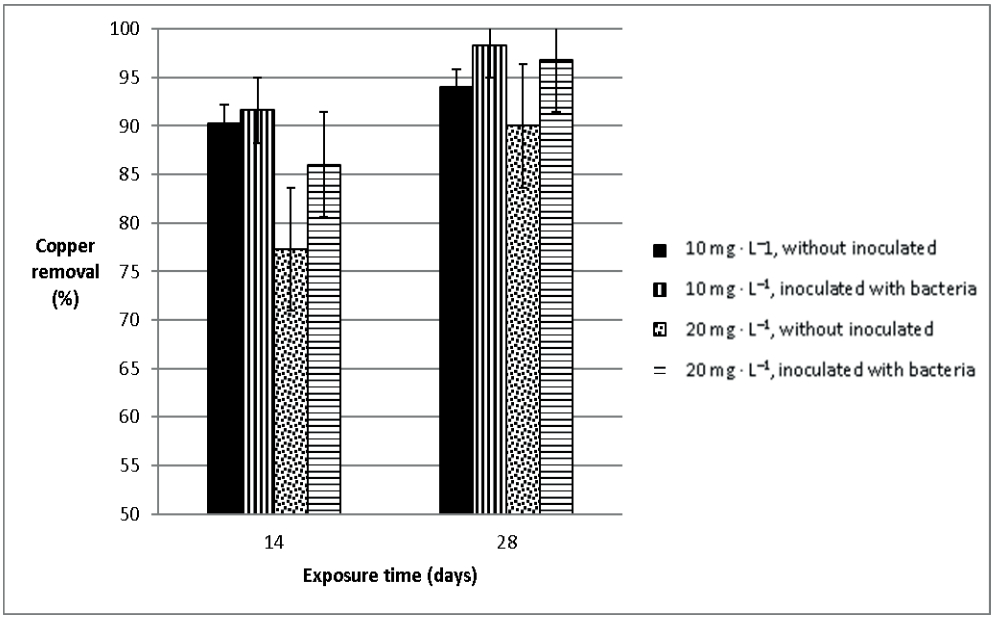

Figure 2: The potency of copper removal by *Eichhornia crassipes* in water containing 10 mL · L^{-1} and 20 mL · L^{-1} $CuSO_4$ without and with inoculation of *Acinetobacter* sp. IrC1.

release of chelating agents, acidification, phosphate solubilization and redox changes. Babalola [22] reported that plant roots provide exudate such as free amino acids, proteins, carbohydrates, alcohols, vitamins, and hormon for bacteria which are important sources for bacterial nutrient. These biochemical mechanisms increase the remediation activity of bacteria associated with plant roots [15].

According to Hussein [14] bacteria that interact specifically with plant roots in rhizosphere inducing phytoremediation either by forming symbiotic relationships with the plant or by microbial biotransformation of organic compounds, thereby indirectly facilitate their removal. It suggested that *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 induced copper phytoremediation in *E. crassipes* not only by forming symbiotic relantionship but also by bacterial bioaccumulation. *Acinetobacter* sp. IrC2 showed that multiple resistance to heavy metals and had abillity to accumulate copper, zinc, lead and cadmium [23]. Previous study showed that copper resistance mechanism of *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 were facilitated through the bioaccumulation of copper inside the cell up to 364.66 mg · L^{-1} of copper [16] It also was reported that the bacterium removed copper up to 52.98 % [24].

Copper removal by *Eichornia crassipes* inoculated with *Acinetobacter* sp. IrC1 in medium containing 10 mM and 20 mM increased during observation periode for 28 d (Figure 2).

The effect of *Acinetobacter* sp. IrC1 inoculation treatment in copper removal by *E. crassipes* can be seen at Figure 3. Copper removal of the plants with inoculation treatment was higher than without inoculation treatment. The coper removal percentage of

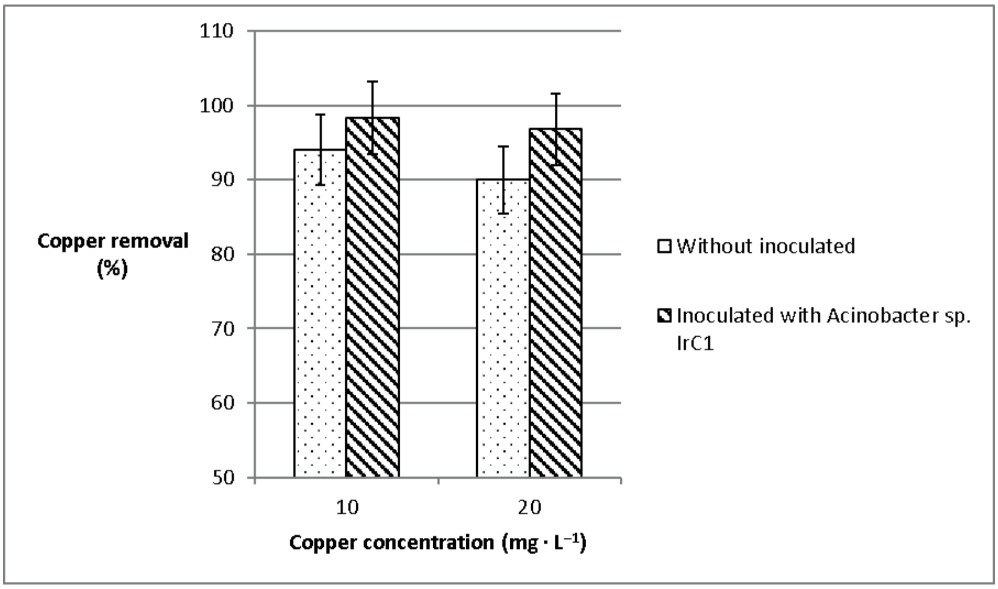

Figure 3: The effect of *Acinetobacter* sp. IrC1 inoculation treatment in copper removal by *Eichornia crassipes*.

plants growing in medium containing 10 mg · L^{-1} $CuSO_4$ were higher than that of 20 mL · L^{-1} $CuSO_4$. The copper removal percentage of *Acinetobacter* sp. IrC1 and *E. crassipes* in medium containing 20 mM decreased might be due to the saturation of the available binding sites of the bacteria and the plants to remove copper. Increasing the concentration of heavy metals lowered copper removal percentage due to its toxic effects. The metal toxic influence the blocking of functional groups, displacement and substitution of essential metal ions from biomolecular, conformational modifications, denaturation and inactivation of enzymes as well as disruption of cellular and organellar membrane integrity [25]

3.3. Copper Accumulation

The effect of *Acinetobacter* sp. IrC1 inoculation to the ability of *E. crassipes* to accumulate copper in medium containing 10 mg · L^{-1}, 20 mg · L^{-1}, and without copper can be seen at Figure 4. Inoculation with *Acinetobacter* sp. IrC1 significantly enhanced the potency of *E. crassipes* to accumulate copper. *Eichhornia crassipes* inoculated with *Acinetobacter* sp. IrC1 accumulated up to six fold higher copper concentration in roots compared with un-inoculated controls. The roots of *Eichhornia crassipes* accumulated 596 mg · kg^{-1} and 391 mg · kg^{-1} in medium containing 5 mL · L^{-1} and 10 mL · L^{-1} copper without inoculation, while, the upper part of the plants accumulated up to 3 532.5 mg · kg^{-1} and 1941.5 mg · kg^{-1} in medium inoculated with *Acinetobacter* sp. IrC1, respectively.

Previous studies showed that bacterial inoculation enhanced the ability of *E. Crassipes* to accumulate heavy metal. Application of cadmium or lead resistant microorganisms improved the ability of *Solanumnigrum* to accumulate heavy metals and increased

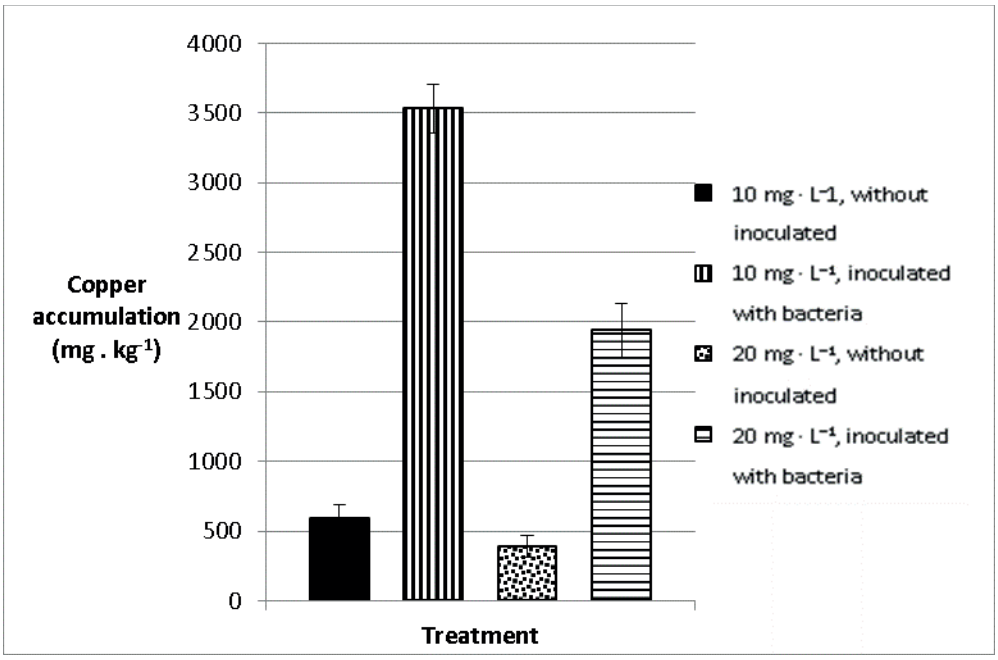

Figure 4: The effect of *Acinetobacter* sp. IrC1 inoculation to the ability of *Eichhornia crassipes* to accumulate copper in medium containing 10 mL · L^{-1}, 20 mL · L^{-1}, and without copper.

plant yield [26]. *Eichhornia crassipes* inoculated with *Ochrobacterium anthropi* and *Bacillus cereus* increased mangan accumulation in roots by 2.4 and 1.3 fold compared to un-inoculation, respectively [27]. Huang et al. [28] reported that inoculation of multiple heavy metals *Burkholderia* sp. strain LD-11 significantly enhanced copper and/or lead accumulation especially in the roots of the plants grown in the polluted sites. Inoculation with the bacteria improved germination, promoted elongation of roots, and enhanced the dry weight of the plants in the presence of copper. Plant-growth-promoting bacteria especially with the resistance to multiple heavy metals are helpful to phytoremediation processes. *Acinetobacter* sp. IrC1 is also a multiresistant bacteria so it must be very helpful for improving phytoremediation processes.

Beneficial effects of plant growth promoting bacteria in phytoremediation are to promote the plant to uptake and accumulate copper [29]. *Eichhornia crassipes* has affinity towards heavy metals because it contains high cellulose and functional groups such as amino (-NH2), carboxil (-COO-), hydroxil (-OH-), sulfahydryl (-SH) that binds heavy metals. [30]. The efficiency of phytoremediation process enhace the plant-associated beneficial microbes by alternating the metal accumulation in plant tissues and by promoting the shoot and root biomass production. [31]. *Acinetobacter* sp. IrC1 might also promote copper phytoremediation processes in *Eichhornia crassipes* by absorbing the copper inside the cells. Previous study reported that copper resistance mechanisms of *Acinetobacter* sp. IrC1 was facilitated through the bioaccumulation of copper inside the cell which was marked by the alteration in the colour of the colonies into blue in high concentration [16]. CopA gene encoding copper accumulation has been successfully amplificated in plasmid of *Acinetobacter* sp. IrC1 [32].

A group plants termed hyperaccumulators are considered to be the best candidates for taking up toxic metals, tranporting them, and accumulating them [33].The highest ability of *E. crassipes* to accumulate copper was 1941.5 mg $\cdot$ kg^{-1} in medium inoculated with *Acinetobacter* sp. IrC1 (Figure 5). Bacterial inoculation treatment enhaced the potency of plant to accumulate copper more than 5.00 mg $\cdot$ kg^{-1} dry weight in plant roots. It indicated that inoculation of *Acinetobacter* sp. IrC1 affected *E. crassipes* to become a good accumulator. Kabeer, et al. [19] reported that plants is recognized by good accumulator because its ability to take heavy metals up more than 5.000 mg $\cdot$ kg^{-1} dry weight and its ability to bioconcentrate the element in its tissues [19].

High copper concentration affected physiological appearance of *E. crassipes* (data is not shown). *Eichhornia crassipes* with bacterial inoculation grew well in medium containing 20 mg $\cdot$ L^{-1} without significant changes in physiological appearance compared to un-inoculation controls, whereas, the leaves growing in medium without bacterial inoculation was becoming clorosic. Hu et al. [34] reported that high concentration of copper caused chlorophyll degradation and induced oxidative stress in chloroplast because of inhibition of chloroplast development and chlorophyll biosynthesis in the presence of high level of copper. The result was similar with the previous study in sunflower plants. Total chlorophyll content decreased in sunflower plants under nickel and lead treatment [17].

Bioaccumulation of copper in *E. crassipes* could result in the loss of pigment concentration significantly. High concentration of copper can lead to chlorophyll degradation. Excess copper can induce oxidative stress in chloroplast which bring damage to membranes by impacting the normal physiological function of proteins, lipids and membran [34]. Accumulation of heavy metals inside the plant in high concentration may induce toxicity by modifying essential protein structure or replacing essential elements. It can be seen from chlorosic symtomp on leaves, growth impairment, browning of roots, and disruption of photosyntesis process [35]. Cellular enzymes and proteins contain several mercapto ligands that can structurally chelate metals, and thereby cause these proteins to lose their functional property. Heavy metals also generate oxidative stress that is mediated through generation of free radicals [36].

4. Conclusions

The findings of this study indicated that *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 inoculation enhanced the efficiency of phytoremediation using *E. crassipes*. Inoculation treatment enhanced the potency of the plant to remove copper and accumulate it up to six fold higher copper concentration in roots compared with un-inoculated controls.

Bacterial inoculation induced the plant to grow well without physiological changes as a result of copper toxicity. *Acinetobacter* sp. IrC1 and *Acinetobacter* sp. IrC2 are promising bacterial inoculant to improve the phytoremediation processes using *E. crassipes.*

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