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The study of bacteria populations in phytoremediation of cadmium using *Eichhornia crassipes*

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Abstract. The method of phytoremediation using *Eichhornia crassipes* plants was able to overcome the problem of cadmium (Cd) heavy metal pollution. This study aims to analyze the population of bacteria in Cd phytoremediation in groundwater using *E. crassipes.* The treatment in the study included T1 of 3 mg/L, T2 of 7 mg/L and T3 of 9 mg/L. The parameters observed were Cd concentration, a total of bacteria numbers, change in pH and total suspended solids (TSS). Based on the results of the study, the T1 treatment was the best absorbs of Cd in groundwater when using *E. crassipes* was 82%. It was the highest compared to other treatments which presented T2 of 65% and T3 of 73%. The highest bacteria population in T1 treatment. For pH and TSS, all treatments on the 12th day relatively of similar. In addition, this study also produced five types of isolates based on the results of characterization, including M1, M2, M3, M4, and M5. This shows that the *E. crassipes* will be inhibited from absorbing Cd at high concentrations, also affecting bacteria growth.

1. Introduction

Rapid industrial development causes side effects in the form of environmental problems. The reason is that the waste thrown out contains harmful heavy metal contamination [1]. One of the many heavy metals produced from industry is cadmium (Cd). In addition, Cd can naturally be sourced from burning household waste and burning fossil fuels, as well as the use of artificial phosphate fertilizers [2].

Cd is a toxic heavy metal not only able to pollute the environment, but also human health because this element is at high risk for blood vessels and long-term can accumulate in the body, especially in the kidney and liver. According to the WHO, the human body is only capable to tolerating the Cd content of 400-500 µg per person per week [3].

Cd disposed of the environment will undergo biotransformation and bioaccumulation processes when entering living organisms such as plants, animals, and humans [4]. This metal enters the body through contaminated food. In the body of aquatic organisms, the amount of metal will continue to increase due to the bio-magnification process [5, 6].

Physical and chemical countermeasures are two choices for overcoming the adverse effects of Cd heavy metal pollution, but these two methods are ineffective and expensive [7]. Therefore, the biological method known as phytoremediation is considered more effective and environmentally friendly because it uses plants to absorb heavy metals [8]. One plant often used in the phytoremediation process to deal with heavy metal pollution is *Eichhornia crassipes*. This plant has the properties of hyper-accumulator in absorbing metals and fast to grow in polluted water [9, 10]. *E. crassipes* can grow in industrial wastewater containing high nitrogen and phosphorus element. Moreover, this plant is adept to improve the quality of waste, because of its ability to absorb heavy metals through roots or the entire surface of plant parts [10, 11].

In the phytoremediation mechanism of heavy metals in water, bacteria interact with plants or bacteria metabolism products associated indirectly with plants through water intermediaries. But, it can also be the other way around, plants metabolism products contact with bacteria [2].

Bacteria have a mechanism that causes changes in the mobility of metal elements; it makes easier to absorb by plants [12, 13]. The type of bacteria that can change metal ions are *Escherichia coli*, *Pseudomonas putida*, *Thiobacillus ferrooxidans*, *Shewanella alga*, and *Acinobacter sp*. The group of sulfate-reducing bacteria is *Desulfovibrio* and *Desulfotomaculum* [14, 15].

The microbiology process will dissolve or precipitate heavy metals so that will increase. Furthermore, it will be used by plants in the phytoremediation process of heavy metal waste that occurs in water. Therefore this research is a microbiological study on phytoremediation of heavy metals using *E. crassipes*.

2. Materials and methods

2.1. Sampling and acclimatization of E. crassipes

The *E. crassipes* were taken from the lake near the campus of Hasanuddin University, Makassar, and then conducted acclimatization process for 7 days in the acclimatization pond. The groundwater used for phytoremediation was obtained from Experimental Farm, Faculty of Agriculture, Hasanuddin University.

2.2. Treatments and experiments of phytoremediation

This study used a static phytoremediation method, which means that the treatment used silent or non-flowing water and the *E. crassipes* with relatively the same size. There were three types of treatment, namely T1 3 mg/L, T2 7mg /L and T3 9 mg/L. Each treatment was duplo. Groundwater samples were collected every 3 days until the 12th day to analyze the concentration of Cd, total microbial numbers, total suspended solids (TSS) and pH value.

2.3. Cadmium analysis

The measurement of cadmium heavy metal concentration in water samples used for phytoremediation treatment on Instrument of atomic absorption spectrophotometer was operated with flame C_2H_2 in water. Before further analysis, the first step is to calibrate the instrument. Output pressure of acetylene was operated on 80-100 kPa, the pressure of air was operated on 300-600 kPa. The velocity of air to flame was operated at 50 L h^{-1} for Cd. Burner type used is burner with wide 100 mm and high in 6 mm. 100 mL standard solution is made as a basis for determining cadmium concentration in water samples. Sample solution is poured in flask and then the absorbance was measured in wave lengths of 228.8 nm for Cd, with the principle that the amount of light absorbed is directly proportional to the level of the substance.

2.4. Enumeration of total bacteria

Plate count is a method for enumeration the total number of microbes. Phytoremediation treatment used groundwater samples through were serially diluted to10^{-1} to 10^{-4} with a physiological solution of NaCl 0.9%, and then inoculated in a petri dish containing the medium nutrient agar. The samples were incubated at room temperature for 48 hours at 37 ^{o}C. The growing colonies were counted in number and determined the number of colonies based on macroscopic and microscopic morphological characteristics. Futhermore, conducted a characterization with several biochemical and physiological tests.

2.5. Measurement of total suspended solids (TSS)
Measurement of total suspended solids was carried out using the gravimetric method. Water samples were filtered with filter papers that were previously weighed. The residue was dried at 105 ºC until it reached a constant weight. The increase in mass on filter paper represents the total level of suspended solids. The estimated value of the total suspended solids was determined by the following formula:

$$Total\ suspended\ solids\ (TSS) = \frac{a-b}{c}\ x\ 10^6$$

where, a = weigh of disk + solid (g)
b = weight of empty disk (g)
c = volume of sample used (ml)

2.6. pH measurement
The determination of pH utilized a pH meter, which previously calibrated using pH 4 buffer and pH 7 with a stabilization time of 15 minutes. The electrode was rinsed with distilled water, dried and dipped in a solution of the treated water sample. Then, pH value was measured.

3. Results and discussions
3.1. Groundwater characterization
Characterization of groundwater sample is carried out to know the initial conditions for treatment of phytoremediation experiments, both physical and chemical characteristics; shown in Table 1. The results of the characterization applied to determine the physical factors and nutrient content of groundwater in supporting the occurrence of phytoremediation process; *E. crassipes* requires certain macronutrients in its growth such as nitrogen, phosphorus, potassium and other element [16]. In addition, also analyzed Cd content in groundwater and *E. crassipes*. The result shows there was no Cd in both groundwater and *E. crassipes*.

Table 1. Groundwater physical - chemical characteristics.

Parameter	Value
pH	6.8
Electrical conductivity	0.113 μS/cm
Total suspended solid (TSS)	6 mg/L
Turbidity	0.92 FTU
Total organic carbon (TOC)	1.74%
Phosphorus	1.87 %
Nitrogen	0.93%
Phosphate	0.12%
Potassium	0.08 %

3.2. Analysis of cadmium absorption
Observation of Cd in phytoremediation treatment showed that at the treatment T1 on the 3rd to 6th day, the absorption of Cd was still low. Absorption increased on 9th day to reach 2.4 mg/L. On 12th day, the amount of Cd absorption had begun to decline and reached 2.48 mg/L. The T2 treatment was the same in treatment T1, which the absorption of Cd occurred gradually starting from 3rd to 12th day with a total of 4.6 mg/L. The T3 treatment indicated the same results in all treatments. On 3th day, absorption remained low and increased on 6th day. The decrease in absorption happened again on the 9th to 12th day with the absorption of Cd of 6.6 mg/L (Figure 1).

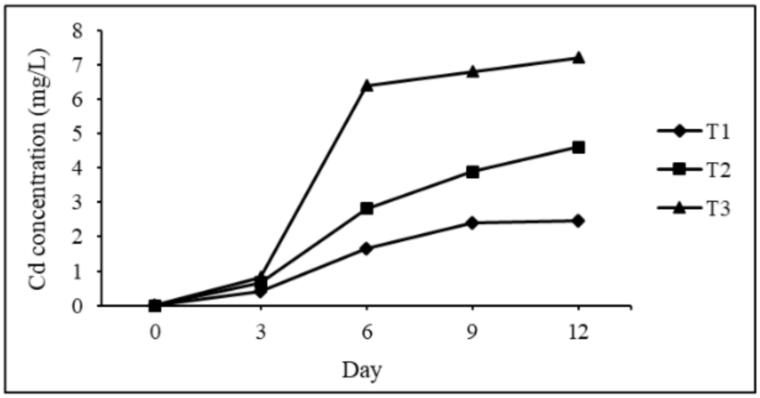

Figure 1. Absorption of Cd in groundwater phytoremediation of the treatment with Cd concentration each was T1 of 3 mg/L, T2 of 7 mg/L and T3 of 9 mg/L.

This implies initially *E. crassipes* still needed to adapt to the environment that had Cd, then on 9^{th} day, this plant had already adapted to its environment so that the absorption of Cd began to increase [16]. *E. crassipes* began to experience tissue physiology disorders due to heavy metal contamination and the influence of environmental factors such as the availability of nutrients to change water pH and the presence of microorganisms. It affected and inhibited the metal absorption process [17]. High Cd absorption existed in T2 treatment. The high content of heavy metals would be toxic to plants, which affected the absorption of substances contaminated with heavy metals. There were many factors affecting the ability of plants to absorb metals in large quantities and in a fast time. In general, the amount of heavy metal absorbed by plants was proportional to the concentration of heavy metals in the waste [7].

3.3. *Bacteria populations and number of isolate types*

In T1 treatment, the total number of microbes decreased on $3r^{d}$ day, then increased until the 12^{th} day, which reached 7.0 x 10^4. For the T2 treatment, the total number of microbes increased dramatically from 4.9 x10^4 to 2.3 x 10^4 on 12^{th} day. The treatment of T3 produced a total number of microbes, which continued to decline until the 12^{th} day (Figure 2).

The results of these observations revealed high concentrations of T3 inhibited bacteria growth, heavy metals are toxic to bacteria so it threatens the organism [13]. It was relevant to T1 treatment that relatively had low concentration; the number of bacteria increased to the 9^{th} day. The high concentration of Cd can be toxic to bacteria. The toxicity arise due to the inhibition of the mechanism of enzymes in the cytoplasm by metal ions. On the other hand, in certain concentrations, microbes are able to adapt to contaminated environments by carrying out a reduction mechanism of Cd. Changes in metal element mobility by microbes from inorganic metals include redox changes in inorganic metals and conversions in the type of metals from inorganic to organic. Specifically, this modification is the process of methylation and de-methylation [4, 18].

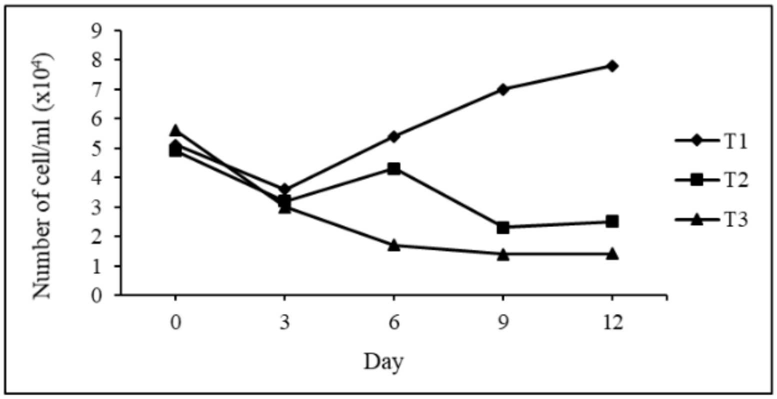

Figure 2. Number of cells in the groundwater phytoremediation of the treatment with Cd concentration each was T1 of 3 mg/L, T2 of 7 mg/L and T3 of 9 mg/L.

After the incubation process for 48 hours, five types of microbial isolate colonies were identified. Based on the macroscopic-morphological characteristics of the color and structure of colony growth were whitish-yellow, yellow, whitish-green, brown and yellowish-brown. The complete characteristics of the five isolates including biochemistry are listed in Table 2.

Table 2. The macroscopic and microscopic morphology of colony isolate.

Types of mikrobia isolate	Characteristics of Colony				Microscopic	
	Colour	Elevation	Morphology	Shape	Gram staining	Shape
M1	Cream	Umbonate	Filamentous	Irregular-shaped	+	B
M2	Yellow	Raised	Filamentous	Irregular-shaped	+	B
M3	Green	Convex	Undulate	Circle	-	R
N4	Brown	Flat	Undulate	Irregular-shaped	-	R
M5	Russet	Flat	Filamentous	Irregular-shaped	+	B

Observing the morphology of the microbial growth colonies is one of the initial techniques for identifying microbes. In terms of form, there were undulating and filamentous. In elevations, it found umbonate, raised, convex and flat. The shape consisted of irregularities and circles. Microscopic observation of morphology showed the presence of gram positive and negative, as well as forms such as sphere-shaped and rod-shaped. Hence, this study found five types of isolates, namely M1, M2, M3, M4, and M5.

To improve the validity of morphological observation results, conducted a biochemical test, which included fermentation tests using several sugar varnishes as subtracts and several other biochemical tests like catalase, citrate, nitrate, and sulfide are listed in Table 3. Biochemical tests are considered as one way of identifying bacteria based on metabolic ability in the substrate [18].

Table 3. The biochemical characteristics of the isolates.

Isolate		M1	M2	M3	M4	M5
Catalase		−	+	−	+	−
Citrate (utilization)		+	+	+	−	+
Urease (utilization)		−	+	+	−	−
Nitrate reduction)		+	+	+	+	+
H_2S formation		−	+	−	−	−
Motility		−	−	−	+	+
Indole		−	+	−	+	+
Fermentation	Glucose	−	+	+	+	-
	Lactose	+	-	+	+	+
	Fructose	−	+	+	+	+
	Sucrose	+	−	+	+	+

3.4. Changes of pH

The T1 treatment on 3rd to 12th day resulted in a decrease in pH up to 6.5. Decreasing pH also ensued in T2 treatment on 3rd day with a value of 6.5. The pH value of 6.5 for T3 treatment was relatively stable until the 12th day (Figure 3).

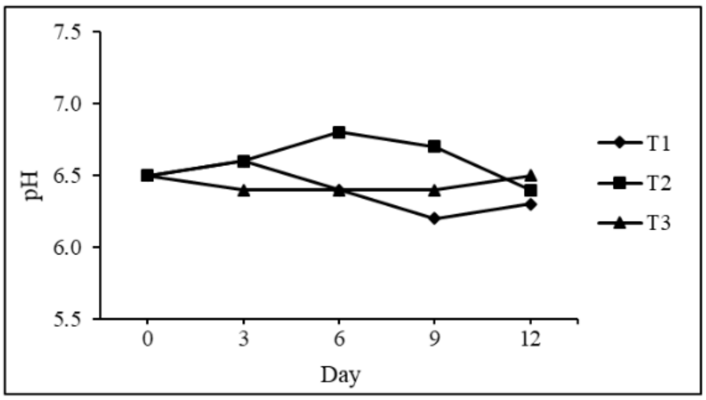

Figure 3. Changes of pH in the groundwater phytoremediation of the treatment with Cd concentration each was T1 of 3 mg/L, T2 of 7 mg/L and T3 of 9 mg/L.

Changes in pH happened during the phytoremediation process of groundwater because *E. crassipes* emitted exudates or secondary products in the form of organic acids, which affected of pH. The tendency of pH changes was likely to be produced by the alkalinity content of organic matter produced by *E. crassipes* [17, 19, 20]. Increase in pH in treatment was feasible to be influenced by the existence of biological processes in the form of interactions between plants and their environment. In addition, the concentration of CO_2 from microbial activity in water also had a great chance to increase pH because aquatic plants took CO_2 from the water during photosynthesis [16]. This shows that the presence of *E. ecrassips* that grows easily and freely in the water environment can be useful as a cleaner for heavy metal pollution.

3.5. Total suspended solids (TSS)

The TSS observations on the treatment showed that T1 had increased from the 3rd day to the 12th day with a total of 24.4 mg/L, the same as the T2 treatment. For T3 treatment there was also a rising even though the number was relatively small (Figure 4).

The remnants of the *E. crassipes* will affect TSS in water, as well as the exudates released by the roots. It is also going to dissolve in water due to microbes do the process of decomposition, produce metabolic products and it certainly influences the pH of groundwater [16, 21].

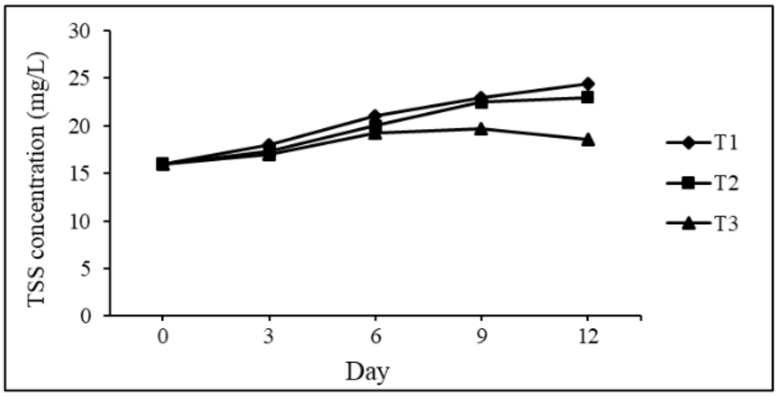

Figure 4. Total Suspended Solids in the groundwater phytoremediation of the treatment with Cd concentration each was T1 of 3 mg/L, T2 of 7 mg/L and T3 of 9 mg/L.

Overall, the concentration of Cd in the phytoremediation treatment alters the bacteria population because relates with the adjustment in pH and TSS arising from plant products in the form of organic matter. The presence of aquatic plants may transform the physical-chemical properties of water bodies [22]. *E. crassipes* which grow in polluted water, improve the physical-chemical properties of water, such as lowering pH, temperature, salinity, and turbidity [17]. *E. crassipes* absorbing Cd at certain concentrations potentially produce exudates both in roots and in other plant tissues. Exudates are useful as nutrients for microorganisms [9]. This shows that the presence of E. crassipes that grows easily and freely in the water environment can be useful as a cleaner for heavy metal pollution.

4. Conclusion

The T1 treatment proved the best Cd absorption results in groundwater using *E. crassipes* reached 82% and was the highest compared to T2 treatment that was only 65% and T3 treatment with a percentage of 73%. The T1 treatment also produced the highest bacteria population included T1 of 7.0 x 10^4, T2 was 2.3 x 10^4 and T3 was 1.4 x 10^4. Observation of pH and total suspended solids (TSS) values showed relatively the same results on 12th day of the groundwater phytoremediation treatment.

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