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The Potential of *Salvinia molesta* as a Copper Phytoremediation Agent based on Gene Expression Analysis

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ABSTRACT

This research explores the rapid environmental impact of the batik industry, particularly concerning batik waste pollution, especially from the heavy metal copper (Cu). Untreated batik waste can have adverse effects on the environment. The study highlights the potential of the water fern plant (*Salvinia molesta*) as a heavy metal phytoremediator, specifically for Cu, by activating phytochelatin synthase. The research aims to determine the most effective waste concentration for Cu absorption by water ferns, measure the reduction in Cu levels after phytoremediation treatment, evaluate post-phytoremediation water quality, and observe PCS gene expression in the roots and leaves of water ferns. A static method is employed with variations in waste concentrations. Data analysis utilizes one-way ANOVA for Cu level reduction and changes in water quality. The results indicate that the most effective wastewater concentration for absorbing heavy metal copper (Cu) using water fern (*Salvinia molesta*) is 2%. The highest reduction in heavy metal copper (Cu) concentration after phytoremediation treatment with water fern (*Salvinia molesta*) is 41.48%. Water quality post-phytoremediation treatment using water fern (*Salvinia molesta*) at all concentrations exhibits improvement with an increase in dissolved oxygen (DO) levels. The expression of the phytochelatin synthase (PCS) gene in the leaves and roots of water fern (*Salvinia molesta*) confirms the role of the PCS gene in binding heavy metal copper (Cu) to the plant's vacuole.

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1. Introduction

Batik is one of Indonesia's cultural heritage that needs to be preserved. Conservation efforts for batik can be achieved by increasing production in national batik production centers, such as batik industry centers. According to Suhendra (2013), the demand for batik has been increasing since its recognition by UNESCO in 2009, which has boosted the reputation of batik and increased batik entrepreneurs' turnover by up to 50%. The batik industry has expanded to meet the growing market demand, not only in Yogyakarta but also in various regions across Indonesia. While this has had positive impacts

on society, it has also led to negative consequences, particularly environmental pollution due to untreated disposal of batik waste into the environment.

Pollutants in batik liquid waste come from various processes, ranging from preparation, coloring, to finishing. The coloring process contributes the highest pollutant load, depending on the type of dye used and the quantity of batik products produced (Sulaeman et al., 2001). Reactive dyes contain Cd, Cu, and Pb, while Naphthol contain Zn, and ergan soga dye contains Cr or Cu (Eskani et al., 2005). Copper (Cu), as a microelement, is essential for both terrestrial and aquatic organisms, but only in small amounts. This metal will be continuously absorbed by aquatic biota if its presence in the water is consistently available, leading to biomagnification and, ultimately, endangering human health.

This risk can be minimized if batik craftsmen are willing to treat batik waste before disposing of it into the water. However, most batik craftsmen dispose of the waste directly into drainage channels that flow into rivers. Only a few craftsmen have individual wastewater treatment plants (IPAL), which collect and treat the waste with zeolite adsorbents before disposal. According to the Local Regulation of the Special Region of Yogyakarta No. 7 of 2016, the concentration of heavy metal copper (Cu) in water should not exceed 0.5 mg/L.

The fate of heavy metals in water is influenced by processes of adsorption or desorption, depending on organic and inorganic substances (Bilinski et al., 1991). Furthermore, according to Darmono (1995), influencing inorganic factors include soil acidity, organic matter, temperature, texture, minerals, clay, and other element concentrations. pH is a crucial factor determining metal transformations. Generally, a decrease in pH increases the solubility of heavy metals, except for Mo and Se.

Plants have the natural ability to absorb metals in varying amounts because some heavy metals are essential elements for plant growth. Some plant species have hyper-tolerant properties, meaning they can accumulate metals at high concentrations in their root and shoot tissues, making them hyper-accumulators. According to Oomen et al. (2009), hyperaccumulator plants can accumulate heavy metal concentrations 10-100 times higher than non-hyperaccumulator plants without showing clear toxicity symptoms. Water fern (*Salvinia molesta*) is one plant with the potential to be a heavy metal phytoremediator in wastewater treatment. By utilizing its fast growth and the morphology of its long, finely hairy roots submerged in water, it is hoped that this plant can be used for heavy metal absorption in water. According to Widiarso (2011), the selection of *Salvinia molesta* as a phytoremediator is based on the consideration that *Salvinia molesta* can grow in water with low nutrient levels. Additionally, morphologically, *Salvinia molesta* has relatively small leaf diameters (average of 2-4 cm) but has dense and long roots. Based on this, *Salvinia molesta* is expected to actively absorb pollutants without obstructing light penetration into the water. From this, *Salvinia molesta* is suspected to be developed as an adsorbent for copper (Cu) heavy metal found in batik liquid waste.

The mechanism of plants in heavy metal detoxification generally involves extracellular and intracellular methods (MacNair, 1997). External mechanisms include chelator exudation to bind metals, exudation of substances that alter the pH of the rhizosphere, and ion exchange on cell membranes to bind metal ions. Intracellular mechanisms include changes in cell membrane or other structural proteins to reduce metal attacks, changes in sensitive enzymes to prevent metal inhibition, changes in metal ion influx/efflux to reduce metal concentrations in cells, production of substances that bind metals in cells and make them non-toxic, and metal transport to vacuoles where detoxification occurs. Cellular detoxification involves compartmentalizing subcellular metal into vacuoles by transporters that carry metals from the cytosol by ligands with high affinity (including potential ligands such as amino acids and organic acids, and two classes of peptides, phytochelatin and metallothionein), or bound to the cell wall.

Phytochelatin synthase is activated by other heavy metal ions such as Cd2+, Cu2+, Ag+, Hg2+, and Pb2+. Genes involved in phytochelatin synthesis have recently been identified in Arabidopsis. Since non-mobile metals are less toxic than free ions, the binding of metals by phytochelatin is considered part of a higher plant detoxification mechanism. After complexing, the phytochelatin-metal complex is transported to the vacuole, the final storage compartment where they are stable due to the acidic vacuole pH, preventing re-oxidation (Chandra & Shivastava, 2003). The mechanism of metal binding by the phytochelatin synthase gene to the vacuole is not yet clearly understood. If the PCS gene is overexpressed in the roots or leaves, it is suspected that PCS plays a role in binding metals to the

plant's vacuole. Based on the above problem background and field studies, this research is aims to determine the most effective waste concentration for Cu absorption by water ferns, measure the reduction in Cu levels after phytoremediation treatment, evaluate post-phytoremediation water quality, and observe PCS gene expression in the roots and leaves of water ferns.

2. Methods

2.1. Tools and Materials

The tools used in this research include plastic containers, jerrycans, microtubes, tubes, PCR tubes, blue tips, yellow tips, white tips, aluminum foil, mortar, micropipette, autoclave, microcentrifuge, water bath, analytical balance, vortex, spectrophotometer, PCR, electrophoresis equipment, UV transilluminator, thermometer, pH meter, and AAS.

The materials used for the research include leaves and roots of the *Salvinia molesta* plant, well water, batik waste, ddH2O (double-distilled water), absolute ethanol, RNA isolation materials (Total RNA Mini Kit from Geneaid®), cDNA synthesis (RevertAid RT Reverse Transcription Kit from Thermo®), PCR mix, agarose, Ethidium Bromide (EtBr), loading dye, DNA ladder (1kb), aquades, and TAE 1X (Tris-Acetate-EDTA buffer).

2.2. Procedures

2.2.1. Plant Preparation and Waste Collection

Salvinia molesta plants were collected from one of a local field in Sleman Regency and batik liquid waste was obtained from one of the batik industries in Sleman Regency. The plants were acclimatized in a large plastic container with well water medium, maintaining a pH of 6-7, for one week. Waste samples were collected using jerrycans each time preliminary and actual tests were conducted.

2.2.2. Preliminary Test

Salvinia molesta, each weighing 25 grams, was placed in a test container containing 5 liters of sample fluid. The use of 25 grams of *Salvinia molesta* per liter of sample fluid follows the standard plant size for remediating 1 liter of sample fluid, requiring 5 grams of fresh weight (Permatasari, 2010). Static phytoremediation was conducted, where the water remained stationary during the treatment. The preliminary test was conducted twice. The first preliminary test involved variations of waste concentrations at 100%, 80%, 60%, 40%, 20%, and 0% (negative control). The second preliminary test involved waste concentrations at 15%, 10%, 5%, 2.5%, 1.25%, and 0% (negative control). The preliminary test lasted for six days, and changes in morphology were observed.

2.2.3. Actual Test

Based on the morphology of the plants surviving without showing significant toxicity symptoms until the sixth day of the preliminary test, new concentrations were established for the actual test. The new concentrations for the actual test were waste variations at 2.5%, 2%, 1.7%, 1.4%, 1.25%, and 0% (negative control), positive control 2.5%, and positive control 1.25%. The negative control represented plants without waste treatment, while the positive control represented waste without plants. Positive control was intended to produce the desired effects, while the negative control was expected not to yield the desired effects. Changes in leaf color morphology were then observed for six days.

2.2.4. Analysis of Cu Levels in Water

The measurement of copper (Cu) metal levels was performed on initial batik liquid waste and the fifth treatment before and after the experiment. This was done to determine the optimum reduction in metal levels, if any, in each treatment. A 200 mL water sample was taken, and the copper content was measured using Atomic Absorption Spectrophotometry (AAS) at the Balai Laboratorium Kesehatan (BLK) Yogyakarta.

2.2.5. Total RNA Isolation

Total RNA isolation was carried out using the Total RNA Mini Kit from Geneaid. Sample materials, consisting of fresh roots and leaves without experiencing toxicity effects, were taken from each treatment, totaling 50 mg, then finely ground in 500 µl RB Buffer. The sample was then placed in a 1.5 ml tube and homogenized using a vortex. The tube was incubated for 10 minutes at 60ºC. After

incubation, the solution was transferred to a new tube equipped with a filter column. The solution was centrifuged for 1 minute at a speed of 1000 g.

After centrifugation, the filter column was discarded, and ethanol absolute was added to half the volume of the filtrate, followed by vigorous shaking. The RB column was attached to the tube, and the sample was centrifuged again for 1 minute at a speed of 16,000 g. The liquid was discarded, and the tube was reattached.

Washing was performed with 400 µl of W1 Buffer added to the middle of the RB column. The solution was centrifuged for 30 seconds at a speed of 16,000 g. The liquid was discarded, and the tube was reattached. Another wash was done with 600 µl of Wash Buffer, previously diluted with absolute ethanol (1 buffer: 4 absolute ethanol), added directly to the middle of the RB column. The solution was centrifuged for 30 seconds at a speed of 16,000 g. The liquid was discarded, and the tube was reattached. An additional 600 µl of Wash Buffer was added, and the solution was centrifuged again for 30 seconds at a speed of 16,000 g. The liquid was discarded, and the tube was centrifuged again for 1 minute at a speed of 16,000 g.

The RB column was transferred to a new 1.5 ml tube, and 50 µl of ddH20 was added to the center of the column. The tube was incubated in a water bath for 10 minutes at 70ºC. Then, centrifugation was performed for 1 minute at a speed of 16,000 g. The obtained total RNA solution was then spectrophotometrically analyzed to determine its purity and concentration.

Once total RNA was obtained from the isolation process, within less than 60 minutes, the cDNA synthesis process was immediately carried out. The synthesis was performed using the RevertAid RT Reverse Transcription Kit from Thermo. The synthesis started by mixing 1 µl total RNA with oligo (dT) primer (1 µl); ddH2O (10 µl); reaction buffer (4 µl); riboLock nuclease inhibitor (1 µl); dNTP mix (2 µl); and revertaid (1 µl) sequentially. The solution was incubated on a thermal cycler at 42ºC for 60 minutes, terminated at 70ºC for 5 minutes, and temporarily stored at 4ºC indefinitely. Then, the solution was retrieved and stored at -20ºC to halt cDNA synthesis.

2.2.6. Primer Design and PCR

Primer design for the PCS gene started by downloading DNA sequences from the available PCS gene on GenBank. The gene was copied in FASTA format and used as a reference to find conserved regions. Primers were designed for both forward (F) and reverse (R) directions using Primer 3. The primer design was made with the criteria of GC percentage for each primer between 40-60%, a maximum 3ºC difference in melting temperature between the two primers, and a length of each primer between 16-28 bases.

PCR or cDNA amplification was carried out by preparing 6.5 µl ddH2O, 2.5 µl primer F, 2.5 µl primer R, 12.5 µl master mix, and 1 µl DNA template. The solution was added to the PCR thermal cycler (Bio-Rad) with initial heating conditions at 94°C for 3 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds. These conditions were repeated for 30 cycles. After 30 cycles, a final heating was done for 5 minutes at 72°C, and the solution was temporarily stored at 4°C indefinitely.

2.2.7. Electrophoresis

Agarose solution is prepared by mixing 0.25 grams of agarose into 25 mL of 1X TAE heated in the microwave for 1 minute. After the agarose solution solidifies into a gel (± 1 hour), the comb is removed from the mold and placed in the electrophoresis chamber. 1X TAE buffer is poured into the electrophoresis tank until the gel surface is submerged. The loading mixture is prepared by mixing 5 µL of the master mix sample and 1 µL of loading dye. The DNA marker is also prepared by mixing 1 µL of DNA ladder (1 kb) with 1 µL of loading dye and 4 µL of ddH2O. The voltage and operation time used in the study are set at 100 V for 45 minutes. After completion, the machine is turned off, and the gel is removed. The gel is observed under a UV transilluminator in a darkroom. Documentation is carried out using a camera connected to a computer device. UV light illuminates the DNA bands, which can be captured by the camera and viewed on the computer monitor. The visualized gel images are stored in the computer hard disk.

2.2.8. Water Quality Measurement

The parameters utilized for water quality measurement include Dissolved Oxygen (DO), pH, and temperature. DO measurement through titration is carried out at the Yogyakarta Health Laboratory (BLK), pH is determined using universal pH meters, and temperature is measured using a thermometer. Temperature and pH measurements are conducted daily over a six-day treatment

period. Meanwhile, DO measurements are performed three times, specifically on days H0, H4, and H6 during the treatment.

2.3. Data Analysis

The results of copper (Cu) metal concentration, pH, temperature, and Dissolved Oxygen (DO) measurements were analyzed using a One-Way ANOVA with a 95%. If the obtained results are significant, further analysis will be conducted using the Duncan Multiple Range Test (DMRT) to examine the factors of each waste concentration treatment. Meanwhile, the analysis of PCS gene expression in the roots and leaves of *Salvinia molesta* plants in reducing copper (Cu) heavy metal concentrations is qualitatively described.

3. Results and Discussion

3.1. Initial Characterization of Batik Liquid Waste

Waste collection from the batik industry for initial concentration characterization consists of two samples. The first sample is the first rinse waste containing additional soda ash, while the second sample is the second rinse waste containing additional TRO or Turkish Red Oil. Both industrial waste samples use additional chemicals during the fabric rinsing process, where soda ash is added to remove remaining wax from the fabric in the first rinse, and TRO is added to enhance the color of batik fabric in the second rinse. The addition of different chemicals requires an initial measurement of copper levels in both first and second rinse waste to determine which has a higher concentration. The measurement with a higher copper concentration will be used as the test material. The results of the initial characterization of batik liquid waste are presented in Table 1.

Table 1. Initial characterization of copper (Cu) heavy metal concentrations, temperature, and ph in batik liquid waste before treatment

Parameter	Concentration of Waste 1	Concentration of Waste 2
pH	8,86	9,02
Temperature (ºC)	27,7	27,43
Cu Concentrations (mg/L)	0,49	0,39

From Table 1, it can be observed that both waste samples, whether the first or second rinse, contain the heavy metal copper (Cu). The higher copper content is found in the first rinse sample at 0.49 mg/L. The higher Cu content in the first rinse is due to the dyes used in the batik process; more dyes are washed away in the first rinse compared to the second rinse. The dye used in the batik-making process is naphtol, a synthetic dye. According to Budiyanto et al. (2017), synthetic dyes contain heavy metals such as Cd, Cr, Pb, Co, Cu, Hg, Ni, Mg, Fe, and Mn. Heavy metals are used to enhance the bonding strength between the dye and the fabric. According to Jahan & Datta (2015), Copper Sulfate (CuSO4) and Alum are intentional mordants added during the first dyeing process. Mordants are metal salts that create an affinity between the fabric and the dye. The simultaneous use of mordants and dyes indicates excellent color fastness and can enhance color brightness.

3.2. Acclimatization and Preliminary Test

Acclimatization is conducted to condition the plants to adapt to a new environment. Before acclimatization, both kiambang roots and leaves are thoroughly washed to remove any remaining paddy mud, ensuring that the kiambang is clean. This is to prevent the kiambang from carrying pollutants from its original environment. The acclimatization period is one week, during the primary growth phase, as new leaf buds start to appear on the sixth day. The emergence of buds under the leaves marks the transition to the secondary growth phase.

The expected growth phase of kiambang is during the primary growth phase, where kiambang experiences good growth in roots, stems, and leaves. It requires abundant nutrients during this growth phase. The goal is that, with the addition of waste, the metal concentration will be well absorbed by the kiambang plant. The results of acclimatization in kiambang plants include the morphological characteristics of light green leaves, indicating that they are still in the primary growth phase. The leaves are undamaged (intact), not overly large, not taking the leaves with buds, having long roots, and thick root hairs. Long roots and thick root hairs will sink deeper into the water, allowing for more

metal absorption.

Preliminary testing in this study aims to obtain an overview of the samples to be tested, namely to determine the concentration of batik liquid waste that does not cause toxicity to kiambang plants. Copper in batik liquid waste is a stressor for kiambang plants, so copper toxicity can have a negative effect on the plant. According to Rosidah et al. (2014), copper stress can disrupt essential mineral absorption and cell division, damage cell wall tissue, inhibit root and shoot growth, and polymerize lignin. Other parameters commonly used to determine plant responses to stress include root growth, localization of accumulation in the root, and leaf color. Leaf color is a parameter used to indicate the presence or absence of toxicity effects because leaf color is closely related to disturbed or undisturbed cellular activity and plant metabolism.

The preliminary test consists of six treatments, one of which is a negative control (without waste), and the other five treatments involve variations in the use of batik liquid waste concentrations. The total volume for each treatment is 5 liters (variations in batik liquid waste concentration added to water up to 5 liters) with the addition of 25 g of previously acclimatized kiambang. In the first preliminary test, the researcher tried variations of 100%, 80%, 60%, 40%, and 20% batik liquid waste concentrations from the total volume of 5 liters to determine the optimal concentration for kiambang growth. In the results of the first preliminary test, all plants in each treatment experienced a color change in the leaves on the first day. The leaves turned dark black, even changing to white, except for the control group, where the leaves remained green. This indicates that all concentration variations are still too high for kiambang plants, causing excessive absorption of waste and pronounced toxicity effects. The plants eventually died before six days, except for the control group, which survived. Seeing that the control group remained green without a change in leaf color, the second preliminary test was conducted with lower concentrations of batik liquid waste.

The batik liquid waste concentration variations in the second preliminary test are 15%, 10%, 5%, 2.5%, and 1.25%. The results of the second preliminary test show a gradual color change in the leaves on different days. On the first day after treatment, only the 15% and 10% concentrations began to show some blackening of leaves, but green leaves were still present. By the fifth day, at concentrations of 15%, 10%, and 5%, almost all leaves turned black. On the sixth day, when the preliminary test period ended, concentrations of 2.5% and 1.25% showed in this picture below.

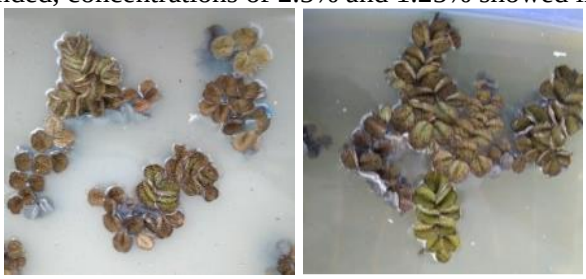

Image 1. Preliminary test photo on the sixth day with 2.5% waste concentration (left) and 1.25% (right)

The 2.5% concentration is used as the actual test threshold, and the 1.25% concentration is set as the lower threshold. The new concentrations created for the actual test are waste concentrations of 2.5%, 2%, 1.7%, 1.4%, and 1.25%. In addition to the negative control (without waste) in the actual test, two positive controls were added, namely controls with waste but without plants. Positive controls were created with waste concentrations of 2.5% and 1.25%. The results of Cu analysis with AAS in water before and after phytoremediation are presented in Table 4. From this data, it is observed that there is a difference in Cu concentration before and after treatment with water ferns (phytoremediation).

From the data above, it can be seen that only the 2.5% waste concentration did not experience a decrease during the six-day exposure period. All other concentrations experienced a decrease in Cu levels. The lack of a decrease in Cu levels at 2.5% concentration will be discussed later in the environmental parameter discussion. Water ferns (*Salvinia molesta*) at waste concentrations of 2%, 1.7%, 1.4%, and 1.25% are suspected to reduce the Cu concentration in water and accumulate it in plant organs. This is in line with the opinion of Setyaningsih (2007), stating that the absorption and accumulation of heavy metals by plants can be divided into three continuous processes: (i) metal absorption by roots, (ii) metal translocation from roots to other parts of the plant, and (iii) metal localization in specific cell parts to prevent interference with the plant's metabolism.

3.3. Reduction of Copper Heavy Metal Concentrations

The extent of the decrease in heavy metal Cu levels in water through phytoremediation using water ferns is shown in Table 2 below.

Table 2. Decrease in Cu concentrations in water during six days of phytoremediation treatment

Types of Tests	Percentage Reduction (%)
Waste 2,5%	-26,07
Waste 2%	34,39
Waste 1,7%	7,78
Waste 1,4%	10,75
Waste 1,25%	41,48

Table 2 shows that the highest percentage decrease is 41.48%, which occurs at a waste concentration of 1.25%. The magnitude of the decrease in metal levels is related to the plant's ability to absorb and utilize metals for growth. According to Mangkoediharjo & Samudro (2010), heavy metals given to plants in certain amounts can help accelerate plant growth as a positive response, but at certain levels, it can inhibit plant growth or even lead to plant death as a negative response. At a waste concentration of 2.5%, there was no decrease in metal levels, but an increase was observed with a percentage of -26.07%. The 2.5% waste concentration is suspected to be the maximum concentration that can inhibit the growth of *Salvinia molesta*. A high concentration of Cu in water can lead to toxic effects on plants, which can subsequently affect the phytoremediation rate and the quality of the resulting water. Thus, in this study, *Salvinia molesta* is presumed to absorb the heavy metal Cu the most but can still grow optimally at the maximum waste concentration of 2%.

3.4. Measurement of Water Quality Parameters

The results of copper (Cu) metal concentration, pH, temperature, and Dissolved Oxygen (DO) measurements were analyzed using the One Way ANOVA test, and significant results were obtained for Cu, pH, and DO. Subsequently, the significant results were further examined using the Duncan Multiple Range Test (DMRT). The One Way ANOVA test results indicated a significant difference at a 5% level. The outcomes of the DMRT are presented in Table 3.

Table 3. Results of further testing with DMRT on copper (Cu) metal concentration

		Subset for alpha = 0.05		
Perlakuan	N	1	2	3
Kontrol	2	.0000000		
Konsentrasi 2%	2		.0156500	
Konsentrasi 1.4%	2		.0176000	
Konsentrasi 1.25%	2		.0246500	.0246500
Konsentrasi 1.7%	2		.0259500	.0259500
Konsentrasi 2.5%	2			.0316500
Sig.		1.000	.085	.204

From Table 3, it is evident that the copper (Cu) concentration experiences the most significant reduction in the treatment with a waste concentration of 2%. The obtained value is 0.01565, which is close to the control (no Cu concentration, indicating no reduction in copper metal concentration). This suggests that the highest percentage reduction in copper metal concentration does not necessarily mean that the waste concentration used is the most effective. Other factors, such as pH and DO parameters, still play a role in reducing the copper metal concentration.

The pH values in the waste before phytoremediation treatment tend to be more basic, and only the control is neutral. This is because the waste originates from discharges containing inorganic substances such as carbonate compounds. It was explained in the initial waste characterization that soda ash is added during the rinsing process. Another name for soda ash is Sodium Carbonate (Na2CO3). However, after phytoremediation using *Salvinia molesta*, only the 2.5% concentration shows a decrease in pH, but it has the highest copper (Cu) concentration. This is in line with Darmono's opinion (1995) that an increase in pH will reduce the solubility of metals from carbonate form to hydroxide form, forming bonds with particles in the water body. The lower the pH value (more acidic), the higher the metal concentration, as lower pH values correspond to higher [H+].

Thus, the solubility of metal ions in water becomes higher.

The Dissolved Oxygen (DO) values from H0, H4, and H6 of all treatments undergo changes. Treatments using varying waste concentrations do not meet the quality standards as required. Dissolved Oxygen (DO) is an indicator of water freshness and plays a crucial role in water quality assessment. The increase in DO is caused by the increased number of living leaves, leading to a high rate of photosynthesis and the production of a significant amount of oxygen. On the other hand, plants grown in high-concentration waste often experience leaf damage, leading to a lower DO content in wastewater. Plants with damaged leaves produce less oxygen through the photosynthesis process. The oxidation and reduction processes by plants are essential in reducing pollution loads in water naturally (Salmin 2005).

3.5. Primer Design and Expression of Phytochelatin Synthase Gene

The DNA template used for reference is the PCS gene sequence from *Salvinia minima* available on NCBI, sourced from Estrella-Gomez et al. (2007). The primer design resulted in forward primer (F) and reverse primer (R) as follows:

Table 4. Results of primer design for the PCS gene of *Salvinia minima*

Primer Design Code	DNA Sequence	Product Length	GC (%)	Tm (°C)	Secondary Structure
7 F	CCTTGGAGAT GGTTTGATGA	242	45	52,2	Lemah
249 R	AGTTCCAGTT TGACCAAGTT		40	52	Lemah

From Table 4 above, it can be observed that both in silico-designed primers meet the criteria for good primers. Both the forward and reverse primers meet the established reference criteria, including primer length, %GC, Tm (melting temperature), and primer interactions (dimers). The forward and reverse primers have %GC of 45 and 40, respectively. The recommended %GC is 40-60. According to Maitriani et al. (2015), the %GC can affect the DNA strand binding. A higher %GC will result in stronger DNA strand binding because GC contains more nucleotide bonds, thus affecting the Tm value. The Tm of the designed primers is 52.2°C and 52°C. The difference in Tm values between the two primers is 0.2°C. The acceptable difference in Tm values between two primers is not more than 5°C. This ensures obtaining the appropriate and specific annealing temperature in the PCR process (Maitriani, 2015).

Secondary structures can be in the form of hairpins or dimers. Both forward and reverse primers have weak secondary structures. Dimers indicate hybridization between identical primer bases. If dimers are present in the primer, DNA polymerase can bind to identical regions and extend in both directions. This can result in a decrease in amplification efficiency, and the produced products may not be as desired (Maitriani, 2015). Hairpins are similar to dimers, but in hairpins, the ends of the primers complement each other. The formation of hairpin structures in primers should be avoided, although it is challenging to obtain primers without hairpin structures. The weak secondary structures of both primers with $\Delta G = 0.67$ kcal/mol are tolerable.

The success and purity of cDNA synthesis can be verified using PCR. If the PCS gene is overexpressed in both leaves and roots, it indicates that the PCS gene plays a role in metal binding to the plant vacuole in both roots and leaves. The results of PCS gene expression can be seen in Figure 2.

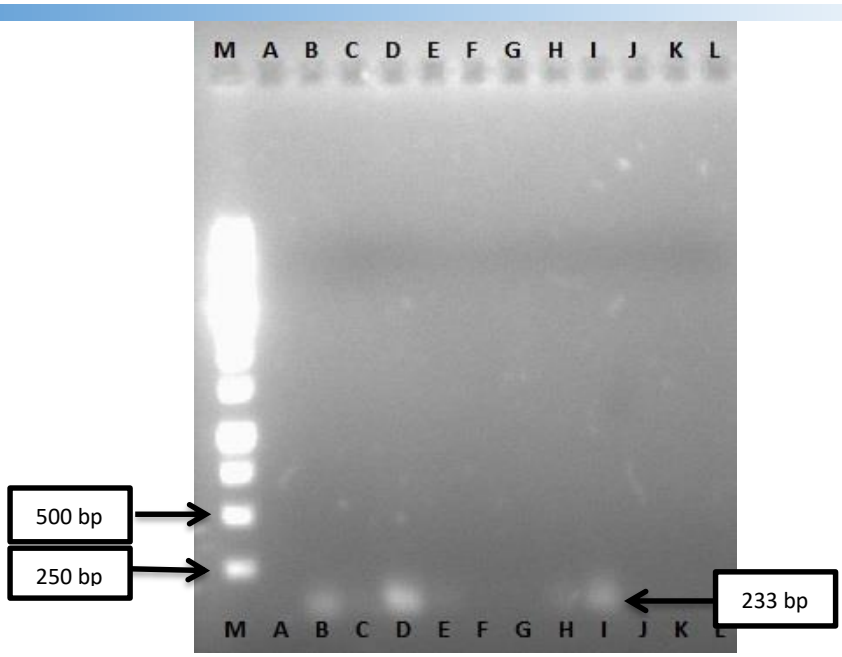

Image 2. Agarose gel electrophoresis (1%) of water fern PCR, 2.5% leaf (A), 2% leaf (B), 1.7% leaf (C), 1.4% leaf (D), 1.25% leaf (E), control leaf (F), 2.5% root (G), 2% root (H), 1.7% root (I), 1.4% root (J), 1.25% root (K), and control root (L). M is the DNA marker 1 Kb Ladder

Image 2 shows the results of agarose gel electrophoresis from the PCR. It can be observed that the amplicon is found in leaves with 2% waste concentration (B), 1.7% (C), 1.4% (D), and 1.25% (E). In roots, the amplicon is found with waste concentrations of 2% (H) and 1.7% (I). The gene is not expressed in leaves and roots with a concentration of 2.5% (A & G), in leaves and roots of the negative control (F & L), and in roots with waste concentrations of 1.4% (J) and 1.25% (K). The expression of the gene in some leaves and roots is sufficient evidence that the PCS gene does play a role in metal binding to the plant's vacuole because in control leaves and roots that were not exposed to waste, there is no mechanism in plants that binds metals to the plant's vacuole, so there is no gene expression.

On the other hand, leaves and roots with a 2.5% waste concentration show no gene expression. This has been explained earlier that the 2.5% waste concentration is the maximum concentration that actually inhibits plant growth. Plants with excessively high waste concentrations do not bind metals to the vacuole, but the toxic effects of metals inhibit growth and can even cause death in plants.

Conversely, concentrations below 2.5% all experience a decrease in metal levels, as evidenced by gene expression in all leaves with concentrations below 2.5%. Metals are suspected to have been successfully bound to the vacuole in the leaves. The lack of expression in plant roots at concentrations of 1.4% and 1.25% is suspected to be due to the very high total RNA concentration, causing the primers not to bind specifically. According to Hidayati (2005), under normal conditions, the concentration of heavy metals (such as Zn, Cd, or Ni) in roots is 10 times higher than the concentration in the shoots. However, in hyperaccumulator plants, the concentration of metals in the shoots exceeds the concentration in the roots. The shoot is the entire part of the plant above the ground surface, so it is suspected that the concentration of metals in the leaves is higher than in the roots. Therefore, DNA bands in leaves are thicker compared to roots.

Regardless of whether or not DNA bands appear and the thickness of the DNA bands, in this study, the size of the DNA bands is also obtained based on the image, all of which are below the marker. This means that the size of the obtained DNA is less than 250 bp. Based on the calculations, the size of the obtained DNA fragment is 233 bp, while the target product size from PCR is 242 bp. The difference in the size of these DNA fragments can be caused by the species used for the study being different from the species used in primer design. The species used for primer design is *Salvinia minima*. Both species are still within the same genus, so the difference in DNA size can occur due to genetic variations, especially in the phytochelatin synthase gene.

4. Conclusion

The most effective concentration of wastewater in absorbing heavy metal copper (Cu) using water fern (*Salvinia molesta*) is at a concentration of 2%, the greatest reduction in the concentration of heavy metal copper (Cu) after phytoremediation treatment using water fern (*Salvinia molesta*) is 41.48%, the water quality after phytoremediation treatment using water fern (*Salvinia molesta*) at all concentrations shows an improvement with an increase in dissolved oxygen (DO) levels, and the expression of the phytochelatin synthase (PCS) gene in the leaves and roots of water fern (*Salvinia molesta*) proves that the PCS gene indeed plays a role in binding heavy metal copper (Cu) to the plant's vacuole.

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