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# Potential of a combination of *Heliconia psittacorum* and its associated bacteria for phytoremediation

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**Abstract.** Remediation has recently received a lot of attention to combat or deal with contaminated soil, water, or air. There are a few types of remediation technologies, such as microbiological remediation and phytoremediation, in which microbial remediation uses microorganisms as the agent, while phytoremediation uses flowers as the agent. *H.psittacorum* is a flower with phytoremediation and ecologically favorable potential. The study is aimed at learning more about the microorganisms that could help improve soil and water quality. Microbiological remediation uses microorganisms as the agent, while phytoremediation uses flowers as the agent to overcome contamination problems. *H.psittacorum* was found to be able to remove copper, zinc, and phosphorus from soil and groundwater. As a result, *H.psittacorum's* phytoremediation capacity was measured in soil and root samples. Microorganism awareness within the root pattern increased to  $4.4 \times 10^9$  cfu/ml, whereas the soil pattern increased to  $1.5 \times 10^9$  cm/ml. The concentration and identity of bacteria in roots and soil revealed information about these bacteria's ability to remove pollutants, while the water quality assessment demonstrated *H.psittacorum's* potential. These seven bacteria have been identified as having a historical mechanism that has proven to be effective in removing, transferring, and stabilizing pollutants. In addition, a water quality assessment demonstrated *H.psittacorum's* phytoremediation potential reduced copper, zinc, and phosphate concentrations in the water from 0.64 to 0.068 mg/l, and 0.513mg/l to 0. The concentration and identity of bacteria in roots and soil revealed information about these bacteria's ability to remove pollutants.

## 1. Introduction

Water pollution caused by these development sectors, such as the agricultural and industrial sectors, has become a serious concern worldwide, particularly in the aquaculture business. The basic biogeochemical cycles rely heavily on soils and sediments. However, the land may eventually lose its ability to recover from such disturbances, resulting in long-term changes with often unintended consequences. Traditional waste-contamination technologies are limited because they can be expensive in terms of both capital and operating costs, and they may also contribute to the creation of secondary waste, which poses treatment challenges.

As a result, phytoremediation has been advocated as a viable generation method because of its low cost and environmental benefits [1]. Phytoremediation is derived from the Greek terms phytion (connected to plants) and remedy (cleansing) [2]. There are several types of remediation techniques, including microbial remediation and phytoremediation. Microbiological remediation employs bacteria as the agent, whereas phytoremediation employs plants [3]. In this study, *H. psittacorum* was used as a



variety of phytoremediation plants. The genus of these plants evolved into Heliconia, a member of the *Heliconiaceae* family, and developed in the neotropical regions of South America [4]. There are around 182 Heliconia species on the planet, with 37 of them growing naturally in Brazil. *H. psittacorum* is also known as "*sepit nuri udang*" in Malay and "*false bird of paradise*" in English. The plant's inflorescences have a variety of colours that range from yellow to orange, crimson, and purple, and frequently contrast with the leaves. The leaves, on the other hand, are wide and broad, with a variety of green colours that characterise them as garden tropical plants [5]. *Heliconia sp.* is a flowering plant that is widely grown as an attractive plant. The rhizomes of this plant can also be utilised to propagate it. Because of its rhizome qualities, this plant will have every other possibility as a phytoremediation plant in a built-in marsh.

Plant soil contains a variety of microorganisms, including bacteria, fungus, actinomycetes, protozoa, and algae. Microorganisms (bacteria) are the most studied and plentiful in soil, as soil serves as a useful host for microorganisms. The genetic diversity of the microbial population has a major impact on plant growth [6]. The study of bacteria concentration and identification results in an indirect promotion of phytoremediation in which microorganisms enhance the biomass production of *H. psittacorum* to restrict pollutants and identify the bacteria using biochemical assays and a commercially available API Kit.

## 2. Methodology

### 2.1. Raw material

Plant samples of *H. psittacorum* and water samples from man-made shrimp aquaculture ponds were collected from Tronoh and the nearby area of Universiti Teknologi PETRONAS (UTP), Perak (Figure 1). In this experiment, a healthy *H. psittacorum* with similar characteristics (like the same plant size and number of leaves) was selected. The plant chosen for this experiment must have a stem that is 30-50 cm long, roots that are up to 10 cm long and have root hair, a sufficient anatomical state with straight growth, appropriate stem thickness and bright green in colour, with no necrosis. The plants were introduced into the system (each bay had individual plants, to have a significant plant root effect on the wastewater treatment). During transit, all samples, including soil attached to the root, were wrapped in aluminium material to prevent sunlight penetration and photodegradation of the chemicals.

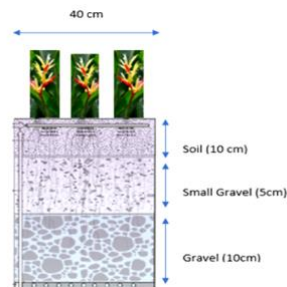


**Figure 1.** The *H. psittacorum* is typical of the Amazon, has flowers that are transformed leaves and reach a height of up to four feet.

### 2.2. Experimental Design

The study was carried out within an existing experiment plant (As Built Constructed Wetlands – CW) created at Universiti Teknologi PETRONAS (UTP). The entire experiment was carried out in two rectangular basin baffled tanks, with one of the baffles serving as a control with no *H. psittacorum*. The rectangular basin tanks were made of concrete and measured 90cm x 40cm x 25cm (Length x Width x Height). Detention times were varied every 5 days by splitting the tank into four baffled compartments and installing sample sites on the tank's border for each compartment. Each baffle has

three layers, as shown in Figure 2. The first layer is 10 cm of gravel (10–20 mm), the middle layer is 5 cm of gravel (1–5 mm), and the top layer is 10 cm of nutrient-free soil. Healthy *H. psittacorum* plants with similar criteria (such as plant size and leaf number) were then planted inside each reactor.



**Figure 2.** Experimental set up of CW reactor

The shrimp pond wastewater was supplied into the beaker tanks using plastic tubing and peristaltic pumps (Master Flexx). To avoid algae growth in the channeling tubes, each 6 m long plastic tube was painted black.

### 2.3. Chemicals

Sodium sulfate, nutrient agar, nutrient broth, 3% hydrogen peroxide, urea broth, Methyl red voges proskauer (MR-VP) broth, oxidase reagent, sodium sulfate agar, Mannitol salt agar, Simmons citrate agar, SIM medium, triple sugar agar, methyl red indicator, Barrit reagent A, Barrit reagent B, nitrogen reagent A, nitrogen reagent B, API kit strips (API 20 NE, API staph), bichinchoninate copper (Cu) powder, ZincoVer5 reagent powder, PhosVer 3 phosphate powder.

## 3. Media Preparation

### 3.1. Preparation of Agar Medium

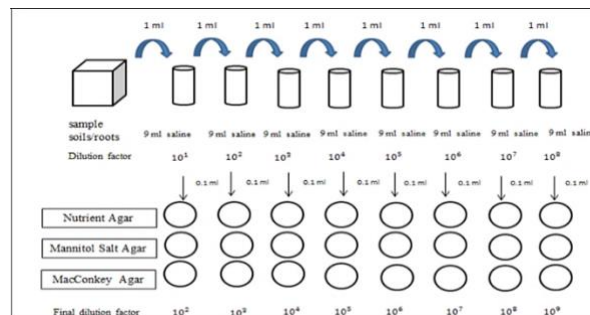
14 grams of nutrient agar (Oxoid) powder was suspended in 500 ml of distilled water. The agar was autoclaved at 121°C for 15 minutes and poured 10ml of melted agar into every petri dish. A quality control process of 18 to 24 hours was done to detect any contamination. These methods were repeated to make a differential agar to separate the bacteria into gram-negative and gram-positive bacteria. In the case of gram-negative bacteria, 25 grammes of MacConkey agar (Oxoid) powder was suspended in 500 ml of distilled water, while in the case of gram-positive bacteria, 55.5 grammes of mannitol salt agar (Oxoid) powder was suspended in 500 ml of distilled water. 16.25 grammes of triple sugar agar (Oxoid), 6.07 grammes of Simmons citrate agar (HiMedia), and 9 grammes of sim medium (HiMedia) were suspended in 250 ml of distilled water for biochemical tests. These methods were repeated to make a differential agar to separate the bacteria into gram-negative and gram-positive bacteria.

### 3.2. Plant Soils and Roots Sampling and Processing

Plant, root, and soil samples were collected from *H. psittacorum*. Each sample had three repetitions (triplicates) of plants 1, 2, and 3, resulting in six soil and root samples. The roots should then be sliced into 10-cm pieces and dried on a paper towel. Roots and adhering soil were both weighed into 5 gram samples. Both samples were then well mixed in 45 mL of 0.85 percent saline buffer solution for one to two minutes [7]. After blending, the suspensions should be allowed to rest for a few minutes to allow bacteria to move from plant tissue into the solution to homogenise it [8].

### 3.3 Performing Serial Dilution

10-fold serial dilutions were prepared for the suspension of roots and soil samples with slightly modified methods suggested by Baldani et al., (2014) [9]. After the bacterial suspension was homogenized, 1 ml of the bacterial suspension was transferred into a test tube consisting of 9 ml of saline water. The process was repeated for another 7 test tubes in serial dilution. 0.1 ml of the diluted sample was spread onto the surface of prepared nutrient agar and differential agar of mannitol salt agar and MacConkey agar respectively, as shown in Figure 3.



**Figure 3.** Serial dilution in nutrient agar, mannitol salt sugar & MacConkey agar.

### 3.4. Bacteria Colonies

After 18-24 hours of incubation at 37°C, plates of nutrient agar were observed to obtain the overall concentration of bacteria. Meanwhile, plates of MacConkey agar and mannitol salt agar were observed to differentiate between gram-negative and gram-positive bacteria. The colony unit should be from a range of 30 to 300 colonies and the concentration of the bacterial culture was determined by using eq. 1 as follow [10]:

$$\text{cfu/ml} = (\text{no. of colonies} \times \text{dilution factor}) / \text{volume of culture plate} \quad (1)$$

### 3.5. Bacterial Identification Using an API Identification Kit (Biomeriux, France)

Two different API kits were used. API 20 NE is used to identify gram-negative bacteria that are not fastidious or enteric. API Staph identifies Staphylococci, whereas API Strep identifies *Streptococci* and *Enterococci*. The mixture was then vigorously agitated to disseminate the inoculum until it was homogeneous. The bottom perforations of the strips were then filled with 5 ml of saline to prevent them from drying out during incubation. To prevent the formation of an air bubble, the strips must be slanted. Apply mineral oil to the indicated ADH, LDC, ODC S, and URE tests after filling the strips. The strip was labelled with a name and incubated for 18-24 hours at 37°C. Following the development of the test, identification was obtained using the numerical profile based on the chart provided. The data was then analysed on the website <http://biomeriux.com> by manually entering a 7-digit numerical profile.

### 3.6 Water Quality Testing

The Hach Test was used to examine three parameters: copper, zinc, and phosphate. Bicinchoninate copper (Cu) powder pillow reagent was used for copper. The ZincoVer5 reagent powder pillow was also used for zinc analysis, whilst the PhosVer 3 phosphate powder pillow was added for phosphorus analysis.

## 4. Result and Discussion

### 4.1. Bacteriological Analysis on Differential Agar

4.1.1. *Concentration of Bacteria in H. psittacorum.* Two samples of soil and roots of triplicate *H. psittacorum* plant from a shrimp aquaculture pond were prepared and serially diluted. The bacteria culture was grown on nutrient agar media to identify the bacteria concentration. A total concentration of bacteria from the original culture was observed and recorded as shown below.

**Table 1.** Bacteria concentration of Soils and Roots in *H. psittacorum*.

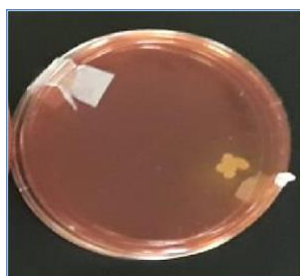
Sample	Bacteria concentration (cfu/ml)			Total Bacteria Concentration (cfu/ml)
	Plant 1	Plant 2	Plant 3	Average
Soil	$1.4 \times 10^4$	$1.1 \times 10^4$	$2.0 \times 10^4$	$1.5 \times 10^4$
Root	$3.9 \times 10^5$	$1.3 \times 10^{10}$	$2.3 \times 10^8$	$4.4 \times 10^9$

Bacterial concentrations in the soil of plants 1, 2, and 3 did not differ significantly (Table 1). Because of the short interval between sampling times for all of the plants, the concentrations of bacteria in the soil of plants 1, 2, and 3 are practically comparable at  $1.4 \times 10^4$  cfu/ml,  $1.1 \times 10^4$  cfu/ml, and  $2.0 \times 10^4$  cfu/ml, respectively. Bacterial accumulation and absorption are larger than in soil, with  $1.5 \times 10^4$  cfu/ml in the root tuber, demonstrating root tuber bacterium-legume symbiosis significant for phytoremediation [11]. Table 2 shows the concentrations of gram-negative and gram-positive bacteria in the medium.

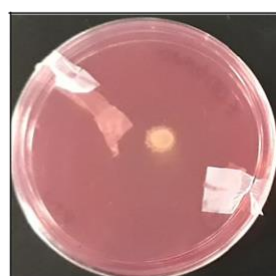
**Table 2.** Dilution Plate of MacConkey agar and Mannitol Salt Agar

Media	Dilution Plate (no of colony growth)							
	$10^1$	$10^2$	$10^3$	$10^4$	$10^5$	$10^6$	$10^7$	$10^8$
MacConkey agar	0	0	0	0	0	0	0	1
Mannitol salt agar	0	0	0	0	0	0	1	0

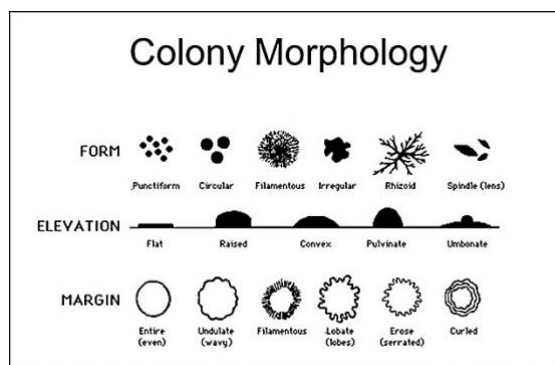
Based on Figure 4, a dilution plate of  $10^8$  MacConkey agar showed the formation of a single clumping colony. While, based on Figure 5, a single large colony was observed in the dilution plate of  $10^7$  mannitol salt agar. The colonies were isolated for further identification using biochemical tests. [12]. Figure 6 adopted from colony morphology descriptions (Simpson 2017) [13] [14].



**Figure 4.** Colony growth on MacConkey Agar



**Figure 5.** Colony growth on Mannitol Salt Agar



**Figure 6.** Colony Morphology Descriptions

**Table 3.** Colony Morphology of Bacteria Isolated from *H. psittacorum*

Media	Bacteria	Shape	Margin	Elevation	Colour
Nutrient agar	A	Circular	Entire	Flat	Colourless
	B	Circular	Entire	Flat	Yellow
	C	Circular	Entire	Flat	Orange
	D	Filamentous	Undulate	Raised	Colourless
MacConkey agar	E	Punctiform	Eros	Dome Shaped	Colourless
	F	Filamentous	Undulate	Raised	Colourless
Mannitol salt agar	G	Rhizoid	Filamentous	Umbonate	Colourless

**Source.** Colony Morphology Descriptions (2017) [17].

#### 4.2. Identification of Bacteria using Biochemical Tests

The biochemical tests that were performed on isolates were the triple sugar iron test, citrate test, urease test, motility test, MR-VP test, oxidase test and catalase test. After 18 hours of incubation at 37°C, all the results of biochemical tests were observed and recorded as in Table 4.

**Table 4.** Analysis of Biochemical Tests from Seven Bacterial Isolates

Identified Bacteria	Biochemical Test								Gram bacteria (Unknown/+ve/-ve)
	Triple Sugar ion/Gas/S	Citrate	Urease	Motility	MR	VPOxidase	Catalase		
A	K/K/-/-	+	-	-	-	-	+	+	Unknown
B	A/A/-/-	-	-	-	-	-	-	+	Unknown
C	K/K/-/-	+	-	+	-	-	-	+	Unknown
D	K/K/-/-	+	-	+	-	-	+	+	Unknown
E	A/A/-/-	-	-	+	+	+	+	-	-ve
F	A/A/-/-	-	-	+	+	+	+	-	-ve
G	K/A/-/-	-	-	-	-	-	-	-	+ve

**Notes.** (+) sign indicate positive results, (-) sign indicates negative result. For TSI, (A/A) indicate acidic fermentation, (K/K) for alkaline or no fermentation.

According to the TSI test results in Table 4, all seven species of bacteria had a negative form of gas and *S. Pseudomonas* are the organisms that are expected to pass the TSI test (K/K) [15]. *Shigella* and *Serratia* are organisms that pass the TSI test (K/A). As shown in Table 4, a gram-negative bacillus can use citrate as its only carbon source. *Klebsiella pneumoniae*, *Enterobacter species*, and *Serratia marcescens* were identified as potential organisms with citrate positivity. The bacteria produce carbon dioxide, which then combines with the medium to form sodium carbonate, an alkaline chemical that

elevates pH and causes the colour to change from green to blue [16]. *Escherichia coli* and *Shigella spp.* were two organisms that tested negative for citrate.

A positive urease test would convert the faint orange tint to pink within 8-24 hours of incubation. As indicated in Table 4. *Flagella*, which are normally associated with gram-negative bacteria, are found in the majority of motile bacteria [17]. The *Vibrio species* (gram-negative motile curved rod) and the *Aeromonas species* are both likely positive motile organisms, but they are not motile. The MR test proved positive for microscopic organisms E and F, as stated in Table 4 [18]. *Enterobacter aerogenes* was found as a negative MR test organism, while *Escherichia coli* was recognised as a probable positive MR test organism.

#### 4.3 Identification of Bacteria using API Kit.

4.3.1. *API 20 NE*. Bacteria A through D grew on nutritional agar with unknown Gram bacteria, according to the biochemical tests in Table 4. Bacteria E and F thrived on MacConkey Agar with gram-negative bacteria. The bacteria E was identified as a result of *Pseudomonas luteola*. Although its natural habitat is unclear [19].

4.3.2. *API Staph*. API Staph is a standardised system for identifying *Staphylococcus*, *Micrococcus*, and *Kocuria bacteria*. Bacteria A–D from nutrient agar containing unknown Gram-positive bacteria and bacteria G containing Gram-positive bacteria were tested using this API Staph kit, since *Staphylococcus* was a Gram-positive bacterium. Additionally, bacteria B and C were believed to be *Staphylococcus* according to their catalase positivity and oxidase negativity. Using the API Staph test kit, bacteria B was identified as *Staphylococcus xylosus*. Meanwhile, *Staphylococcus saprophyticus* was discovered as bacteria C [20].

#### 4.4. Water Quality Monitoring (Hach Method)

4.4.1 *Zinc*. According to the data in Table 5, an average zinc before treatment was 0.173 mg/l. After treatment, the averages in both samples 1 and 2 were reduced to 0.068 mg/l. While the average sample 3 was reduced to 0.042 mg/l. The amount of zinc reduction in samples 1 and 2 was higher compared to the sample of water in plant 3. Both samples 1 and 2 might accumulate an almost similar amount of zinc before treatment. The amount of zinc in sample water reduced after treatment implied that *H. psittacorum* has heavy metal translocation potential [20], and besides, zinc is one of the essential micronutrients for the plant.

**Table 5.** The Detection Value of Zinc in Water Sample

	Before treatment	After treatment		
	Raw samples (mg/l)	Sample 3(mg/l)	Sample 2(mg/l)	Sample 1(mg/l)
1	0.22	0.04	0.08	0.1
2	0.21	0.05	0.12	0.08
3	0.11	0.05	0.08	0.07
4	0.17	0.05	0.08	0.06
5	0.16	0.02	0.04	0.04
6	0.17	0.04	0.04	0.06
Average(mg/l)	0.173	0.042	0.068	0.068

4.4.2 *Phosphate*. Phosphate is one of the macronutrients in plants and is sometimes associated with phosphate-solubilizing bacteria to increase plant growth, as shown in Table 6. Phosphate levels in samples 1, 2, and 3 were lowered to 0.478 mg/l, 0.068 mg/l, and 0.293 mg/l, respectively, after treatment. According to Brix et al., (2012) [21], wetlands planted with *Heliconia* have a stronger



ability to collect nitrogen (N) and phosphorus (P). According to Oliver et al., (2016) [17], the type of microbial population that was most frequently detected in water was faecal indicator organisms (FIOs) such as *E. coli*. During this study, no strain of *Escherichia coli* was isolated, but the majority of the bacteria isolated, including *Pseudomonas luteola*, *Serratia liquefaciens*, *Serratia marcescens*, *Enterobacter cloacae*, *Staphylococcus xylosus*, *Staphylococcus saprophyticus*, *Enterococcus faecium*, were pathogenic and could cause disease. Although these bacteria may aid in the absorption of contaminants in water, the water is not safe to consume because it could be harmful to one's health.

**Table 6.** The detection value of phosphate in water sample.

	Before treatment	After treatment		
	Raw sample (mg/L)	Sample 3 (mg/L)	Sample 2 (mg/L)	Sample 1 (mg/L)
1	0.52	0.64	0.42	0.19
2	0.44	0.4	0.61	0.68
3	0.55	0.63	0.89	0.44
4	0.56	0.42	0.15	0.1
5	0.50	0.33	0.67	0.22
6	0.51	0.45	0.1	0.13
Average (mg/l)	0.513	0.478	0.068	0.293

## 5. Conclusion

Taking everything into consideration, the grouping of microorganisms invading roots and soil was determined by sequential weakening. The concentration of microscopic organisms was found to be highest in attachments as compared to the soil. According to the results, the total concentration of microorganisms in the roots is  $4.4 \times 10^9$  cfu/ml, which is higher than the soil's  $1.5 \times 10^4$  cfu/ml. Microbes in roots and soil have a peaceful relationship with soil microscopic organisms.

This collaboration would benefit both plants and microbes, increasing the viability of phytoremediation. The isolates of *Pseudomonas luteola*, *Serratia liquefaciens*, *Serratia marcescens*, *Enterobacter cloacae*, *Staphylococcus xylosus*, *Staphylococcus saprophyticus*, and *Enterococcus faecium* were isolated from the two soil and root tests based on recognisable proof of microbes from biochemical testing and API pack confirmation. These microorganisms have a relationship with plants, either by assisting in plant development or by being ready to transport contaminants from root to shoot.

Furthermore, physicochemical water quality effects revealed *H. psittacorum* was ready to reduce the amount of copper, zinc, and phosphate in a crude example of shrimp water. The viability of the phytoremediation pattern is dependent on the cooperation of water, organisms, and plants.

Phytoremediation techniques are less suitable for large-scale implementation because hyperaccumulators take longer to mature and produce less harvestable biomass. As a result, hereditary design can be brought closer to creating transgenic plants with high biomass creation qualities, greater metal gathering, and adaptability to a variety of climatic circumstances.

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