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MICROBIAL ASSISTED PHYTOREMEDIATION OF PALM OIL MILL FINAL DISCHARGE (POMFD) WASTEWATER

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ABSTRACT

This study assesses microbial assisted phytoremediation of palm oil mill final discharge (POMFD) wastewater using three local macrophyte species: Leersia oryzoides, Pistia stratiotes *and* Ludwigia peploides*. It was found respectively that* BOD_5*, COD,* NH_3*-N removal efficiencies of 84.7%, 22.3%, and 73.5% were achieved for* P. stratiotes; *88.1%, 18% and 69.2% for* L. peploides; *and 86.1%, 11.7% and 69.3% for* L. oryzoides*. The level of C, H and N in the tissue were influenced by macrophyte species and organs (*$p < 0.05$*). The bioconcentration factors (BCF) of various metals such as Mg, Ca, K, Na, Fe and Zn of the three macrophytes were* 10^{-1} *to* 10^{0} *with Fe being highly accumulated in roots of all the macrophytes (BCF=*10^{2}*). The translocation factors (TF) of most metals from root to shoot tissues were in a range of* 10^{-3} *to* 10^{0}*. In comparison with other metals, K was capable to be efficiently translocated from root to shoots in all the macrophyte species (TF=*10^{0}*). In this study,* Bacillus megaterium, Pseudomonas *spp. and* Bacillus cereus *that are usually involved in denitrification were identified in* P. stratiotes, L. pepoides *and* L. oryzoides *roots respectively. This confirms the macrophyte-microorganism interaction in remediation of POMFD wastewater.*

Keywords: phytoremediation, plant-microbe interactions, bioconcentration factor, translocation factor, POMFD wastewater.

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INTRODUCTION

In Malaysia, the palm oil industry contributes to an annual production of more than 13 million tonnes of crude palm oil, derived from plantation areas that cover 11% of the country's land. In spite of the growing productivity, the industry generates a massive amount of palm oil mill effluent (POME) which was estimated to be nearly three times that of the crude palm oil quantity (Wu *et al*., 2009). POME can cause land and water pollution if discharged untreated due to the presence of high organic content (Nwoko, 2010). The impact of POME on the environment has been of government and public concern hence, a regulation in relation to POME discharges into watercourses and lands was enacted in 1977 (Environmental Quality Act, 1974). According to this regulation, palm oil mills are required to treat their POME to reduce biochemical oxygen demand (BOD) level to less than 100 mg litre^{-1} before being discharged. In order to comply with the standard limits imposed by the government, most of the palm oil mills have incorporated a wastewater treatment system for POME remediation. An open pond system that integrates biological aerobic and anaerobic degradation has

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been widely used for POME treatment following the system's low incurring of capital, operation and maintenance costs. Nevertheless, the treated POME, known as palm oil mill final discharge (POMFD) wastewater, still shows a substantial concentration of BOD and chemical oxygen demand (COD) even after biological treatment (Mohammad *et al*., 2012). Therefore, it is imperative to treat POMFD to ensure that its discharge does not pollute the receiving watercourses.

Phytoremediation is a cost-effective technological approach based on the ability of a plant to accumulate, translocate, remove or sequester hazardous contaminants from the environment. The method has gained increasing interest for wastewater treatment. Recent studies showed that various contaminants from wastewater (*e.g*. lemon industry wastewater, metal industry effluent, dairy effluent, olive mill wastewater) were successfully treated using phytoremediation (Navarro *et al*., 2012; Curia *et al*., 2011; Tripathi and Upadhyay, 2003; Bodini *et al*., 2011). However, very limited information on phytoremediation of POMFD wastewater has been reported.

In natural environment, macrophytes play vital roles in sustaining the ecosystems. In addition, several studies have demonstrated the great ability of macrophytes to remove pollutants from various types of wastewater. *Leersia oryzoides* (L.) Sw. had been studied to remediate permethrin (Moore *et al*., 2009). *Pistia stratiotes* L. was known for its ability to improve water quality by removing nitrogen and phosphorus from eutrophic stormwaters (Lu *et al*., 2010). *Ludwigia peploides* (Kunth) P.H. Raven possesses several characteristics that make it suitable for phytoremediation, such as rapid growth, high nitrogen accumulation and ability to allocate most of its biomass and nitrogen into upper ground tissues (Hussner, 2010).

The microorganisms associated with plants and their interactions were believed to play an important role in the physiology and health of the plant. Numerous kinds of microorganisms particularly bacteria such as *Bacillus* sp. and *Pseudomonas* sp. that colonise rhizosphere can assist their host plants in removing pollutants from contaminated environment (Ajithkumar *et al*., 2001; Osem *et al*., 2007; Oliveira *et al*., 2015). The root-associated bacteria show appropriate capability to degrade, metabolise, assimilate, transform and detoxify the contaminants from the rhizosphere and/or endosphere (Oliveira *et al*., 2015).

This article discusses phytoremediation of POMFD using three local macrophyte species: *L. oryzoides, L. peploides* and *P. stratoites*. The characteristics of nutrient uptake during the phytoremediation were ascertained by measuring the percentage of C, H and N elements in the shoot, stem and root organs of the macrophyte species. The bioconcentration factor (BCF) and translocation factor (TF) values of metals for each studied organ were also determined to understand the capability of the macrophytes to accumulate and transport the elements. A molecular identification of the microbial population at the rhizosphere was also performed to obtain further understanding of the microorganisms' role particularly in the phytoremediation of POMFD wastewater.

MATERIALS AND METHODS

Macrophytes and Culture Conditions

Three selected local macrophytes species – *L. oryzoides, P. stratiotes* and *L. peploides* – were obtained from an abandoned tin mine lake at Kalumpang, Hulu Selangor, Selangor, Malaysia. The selected macrophytes species were among the abundant species found in the tin mine lake. The macrophytes were identified according to USDA plant database (http://plants.usda.gov/) and International Plant Name Index (http://www.ipni.org/). New seedlings or growing rosettes with almost similar initial weight (30 g-50 g) were selected and cleaned with distilled water to remove debris. The selected seedlings were cultured in dechlorinated tap water under shaded area with temperature, natural lighting and relative humidity ranging from 28.0°C-32.6°C, 665-1889 lux and 62.4%-83.1%, respectively for about four weeks for an acclimatisation process. The macrophytes were considered acclimatised when new adventitious shoots of rosette were produced. Healthy acclimatised macrophytes with no sign of necrosis and nutrient deficiency symptoms were used in the following study.

POMFD Wastewater

The POMFD wastewater used in this study was obtained from a final distribution pond at a treatment plant in Kuala Lumpur Kepong Berhad Palm Oill Mill, which is situated in Tanjong Malim, Perak, Malaysia. The characteristics of POMFD wastewater are summarised in *Table 1*.

Experimental Set Up

The experiment was carried out with four replicates for each *L. oryzoides, P. stratiotes* and *L. peploides* species according to a randomised complete block design. Cuboid plastic containers with dimensions 33 cm × 20 cm × 10 cm and a capacity of 6.6 litres were filled with 6.0 litres of POMFD wastewater and were added with acclimatised macrophytes that cover about 50% of the surface area. The containers added with POMFD wastewater but without macrophytes served as control (Khellaf

TABLE 1. CHARACTERISTICS OF POMFD WASTEWATER USED IN THIS STUDY

Parameter*	Range
BOD_5	504-519
COD	1 022-1 285
NH_3-N	35.2-46.9
TSS	660-750
pH	7.79-8.2
Mg	219.3-221.7
Ca	11.20-11.22
Na	5.68-5.73
K	172.55-207.8
Fe	4.68-5.32
Zn	2.35-3.00

Note: * Unit in mg l^{-1} except for pH.
POMFD - palm oil mill final discharge.
COD – chemical oxygen demand.

and Zerdaoui, 2009). The experiment was conducted under the above mentioned culture conditions for 14 days. Wastewater sampling for chemical analyses was conducted daily while plant samples were taken before and after the phytoremediation treatment for biomass (wet weight), BCF and TF determination.

Physico-chemical Analyses

Light intensity and relative humidity were measured using light meter (HD400 Extech Instruments, USA) and hygro-thermometer (Easy View20 Extech Instruments, USA), respectively. BOD_5, COD, NH_3-N, and pH were determined according to the standard method (APHA, 2005). Shoot, stem and root samples of all macrophytes were dried and digested with 2 M hydrochloric acid (HCl) and were filtered using a 0.45 μm cellulose acetate membrane filter prior to elemental analysis. The percentages of C, H and N elements were analysed using a CHNS-Op elemental analyser (FlashEA 1112 model, USA). The K, Na, Mg and Ca, Fe, Zn elements were measured using an atomic absorption spectroscopy (AAS) of AAnalyst 400 model (USA).

Bioconcentration (BCF) and Translocation Factor (TF)

In this study, the BCF and TF values were calculated as in Equations (1) and (2) (Das *et al*., 2014; Soda *et al*., 2012; Khellaf and Zerdaoui, 2009).

$$BCF = C_p/C_w \qquad \text{[Equation (1)]}$$

where C_p is metal concentration in plant tissue at harvested (mg kg^{-1}-dry) and C_w is initial metal concentration in POMFD wastewater (mg $litre^{-1}$). Larger BCF value (kg $litre^{-1}$) indicates better phytoaccumulation capability.

$$TF = C_s/C_r \qquad \text{[Equation (2)]}$$

where C_s is metal concentration in shoots tissue (mg kg^{-1}-dry) and C_r is metal concentration in roots tissue (mg kg^{-1}-dry). Larger TF value indicates better translocation capability.

Bacterial Enumeration and Isolation

The enumeration of bacteria in rhizosphere was performed according to the heterotrophic plate count (HPC) technique (APHA, 2005). Root samples were taken before and the end of phytoremediation process (Day 14). About 1 g of roots was placed in a 100 ml Erlenmeyer flask containing 30 ml of sterile saline. After vigorous shaking at 30°C for 30 min, the solution was serially diluted and plated on a solid nutrient agar (Difco) and was incubated at 25 ± 2°C for 72 hr. Each colony from the HPC that showed a different morphology was further inoculated to obtain pure isolates. The morphology of the bacteria was examined with a Hitachi UHR FESEM field emission scanning electron microscope (Hitachi, Japan).

Nucleic Acid Extraction and PCR Amplification

Pure isolates were subjected to DNA extraction using Ultraclean™ soil DNA kit (MO Bio Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer's instructions. Partial 16S rRNA fragments of the entire bacterial community were amplified by PCR from the extracted genomic DNA using the general bacteria primers, 27F (AGA GTT TGA TCC TGG CTC AG) and 1492R (ACG GTT ACC TTG TTA CGA CTT) (Yusof *et al*., 2013). The PCR programme consisted of an initial 4 min denaturation step at 95°C followed by 35 cycles of repeated denaturation at 95°C for 45 s, annealing at 56°C for 45 s, and an extension at 70°C for 1 min followed by a final extension at 72°C for 3 min. The samples were amplified using a PCR Thermal Cycler Dice (Takara, Japan).

PCR Purification and Sequencing

PCR purification was performed using Agencourt Ampure Xp, Beckman Coulter Genomics. An amount of 40 μl of the PCR product was added with agencourt onto SPRI Plate 96R for 10 min. The supernatant was then washed with 70% absolute ethanol before being dried at room temperature. The purified PCR product was viewed by electrophoresis. Samples were then sent to First BASE Laboratories Sdn Bhd (Selangor, Malaysia) for DNA sequencing. Primer 750R for soil and

environmental sample was used in sequencing. The sequences were compared to the NCBI (National Centre for Biotechnology Information) Genbank using the BLAST search tool which is available at: http://www.ncbi.nlm.nih.gov/ and Ribosomal Database Project II (RDP) through the website (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) to identify the nearest bacterial species relatives of the partially sequenced 16S rRNA genes.

Statistical Analysis

Analysis of variance (one-way ANOVA) was performed using SPSS 17.0 software to determine the significant difference in the phytoremediation performance between the macrophyte species. In this analysis, removal efficiency was the dependent variable and macrophyte species was the independent variable. A probability of 0.05 or lower was considered to be significant. A post-hoc test was also conducted using LSD (least significant difference) to identify the significant differences among the different macrophyte species in terms of pollutant removal efficiency. Two-way ANOVA was performed to compare the percentage of C, H and N in different macrophyte organs for the three macrophyte species. The independent variables were macrophyte organs of three levels (shoots, stems and roots) and macrophyte species of three levels (*L. oryzoides, L. peploides* and *P. stratiotes*). The dependent variable was the percentage of the elements. The interaction effect between the macrophyte organs and the macrophyte species on percentage of the elements can be determined with this analysis. There were three hypotheses to be proven, two from the main effects (macrophytes' organs and species) and one from the interaction effect (organs*species). An *F* value close to 1.0 indicates the lowest effect. A *p* value equal to or less than 0.05 shows a significant difference between the percentage of the elements in the macrophyte organs and in different macrophyte species. The value also implies that a significant interaction exists between both independent variables.

RESULTS AND DISCUSSION

Phytoremediation Performance

In this study, a phytoremediation of POMFD wastewater was investigated using three local macrophytes species: *L. oryzoides, P. stratiotes* and *L. peploides*. These macrophytes were chosen due to their: (1) abundance in waterlogged areas in Malaysia, (2) availability in large quantity and (3) tolerance to POMFD wastewater as observed during the preliminary study. *Figure 1* shows the phytoremediation performance of POMFD by three macrophytes - *L. oryzoides, P. stratiotes* and *L. peploides*-after 14 days of treatment. In this study, the macrophyte species used were able to remove the biodegradable organic compounds in the POMFD (*Figure 1a*). The BOD_5 removal efficiency for *L. oryzoides, L. peploides* and *P. stratiotes* were respectively 86.1%, 88.1% and 84.7% with respective mean values of 75.3 mg $litre^{-1}$, 64.7 mg $litre^{-1}$ and 72.7 mg $litre^{-1}$. However, the post-hoc result shows that there was no significant difference between macrophyte species in the BOD_5 removal (p> 0.05). Greenway (2007) reported that BOD was principally removed in a phytoremediation process by the microbiological activity associated with macrophytes. This activity provides surfaces for biofilm growth in a wetland system. It was also found that the COD removal efficiency was lower than BOD_5 (*Figure 1b*) and that there was no significant difference in COD removal between the macrophyte species (p> 0.05). The COD removal efficiency achieved by *L. oryzoides, L. peploides* and *P. stratiotes* were 11.7%, 18% and 22.3% respectively. It was believed that the recalcitrant compounds such as humic acids in POMFD contributed to the low removal efficiency of COD (Norulaini, 2001). A study by Tang *et al.* (2009) also confirmed that macrophytes do not significantly contribute to the removal of COD from wastewater in a constructed wetland without aeration and the use polyhedron hollow polypropylene balls (PBHB). The PBHB was added to the system as biofilm carrier to enhance microbial activity and subsequently pollutant removal.

The changes in NH_3-N concentration throughout the phytoremediation study are shown in *Figure 1c*. All macrophytes were able to remove NH_3-N from the POMFD wastewater with the efficiency achieved by *L. oryzoides, L. peploides* and *P. stratiotes* being 69.3%, 69.2% and 73.5%, respectively. While there was no significant difference in NH_3-N removal between macrophyte species (p> 0.05), *P. stratiotes* appeared as the most efficient macrophytes in NH_3-N removal. The NH_3-N was removed by direct uptake of macrophytes or through microbial activity in nitrification and denitrification processes (Siracusa and La Rosa, 2006). In this study, nitrification might have occurred in the aerobic micro-environments surrounding the rhizospere of the macrophytes. This is due to the diffusion of oxygen from the macrophytes roots that create oxidised areas around the rhizosphere. An oxidised area is suitable for a nitrification process in which ammonium is converted to nitrate and then taken up directly by roots (Greenway, 2007). The pH level increases gradually during the phytoremediation of POMFD for all the macrophyte species (*Figure 1d*) and there was no significant difference in pH between the macrophytes (p> 0.05). Degradation of

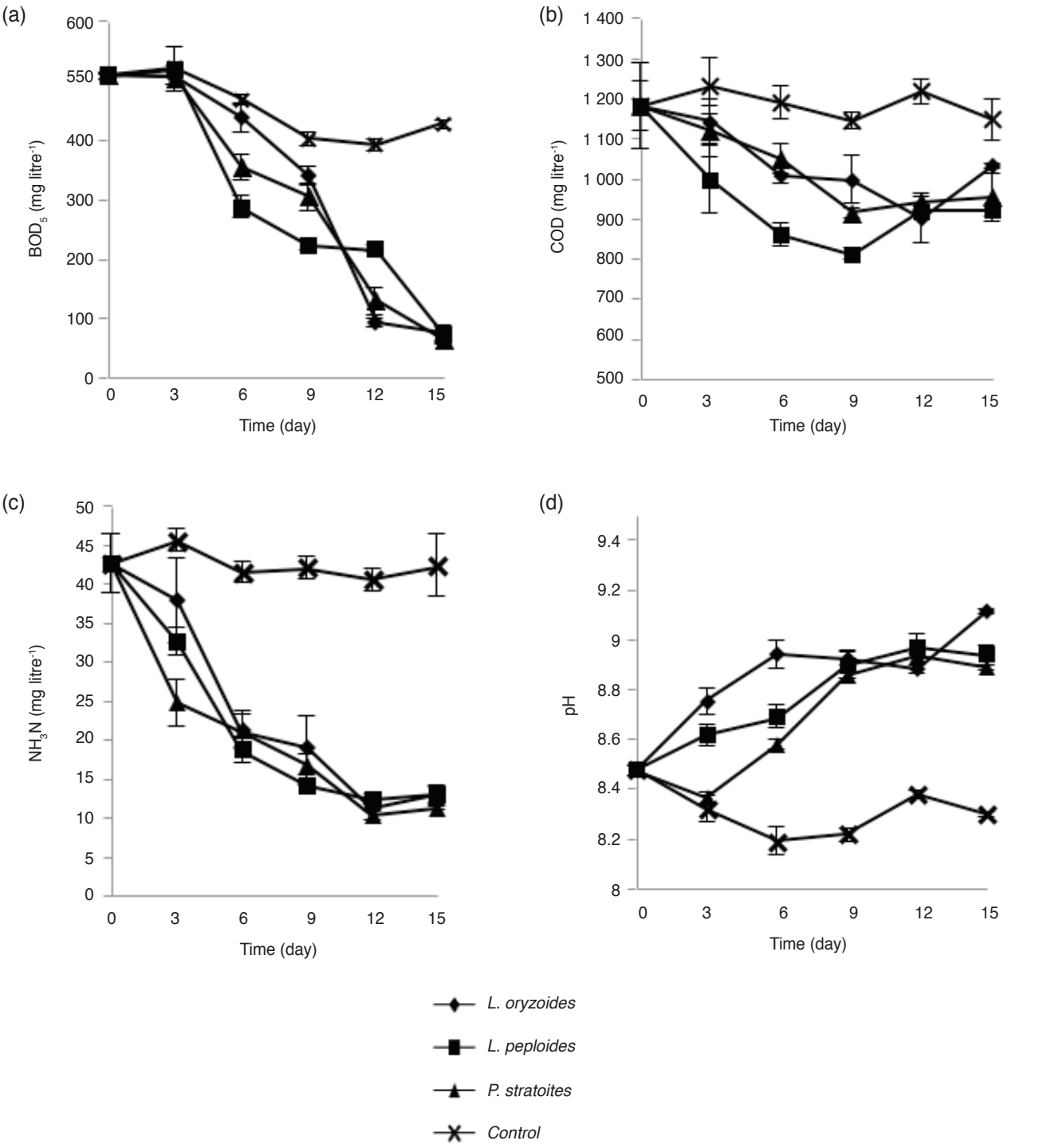

Figure 1. Changes in (a) BOD_5, (b) chemical oxygen demand (COD), (c) NH_3-N and (d) pH during phytoremediation of palm oil mill final discharge (POMFD) wastewater, N=12, p<0.05.

organic compounds by microorganism will release carbon dioxide which dissolves in water as carbonic acid (H_2CO_3). Increase in pH might be due to the loss of H^+ ions from cation exchange reaction occurs when the H^+ ions of an organic acid are exchanged with other cations in rhizosphere such as Ca^{2+}, Fe^{2+}, K^+, Mg^{2+} and Mn^{2+} (Cronk and Fennessy, 2001). Furthermore, the loss of CO_2 from water columns might also as a result of macrophytes' uptake for a photosynthesis process that could increase the pH level of POMFD wastewater. The increase of pH could also stabilise the alkalinity required in the system.

Figure 2 shows macrophytes biomass (wet weight) before (Day 0) and after (Day 14) phytoremediation of POMFD wastewater. Increase of plant biomass was observed in *L. oryzoides* after 14 days of treatment. However, a decrease of

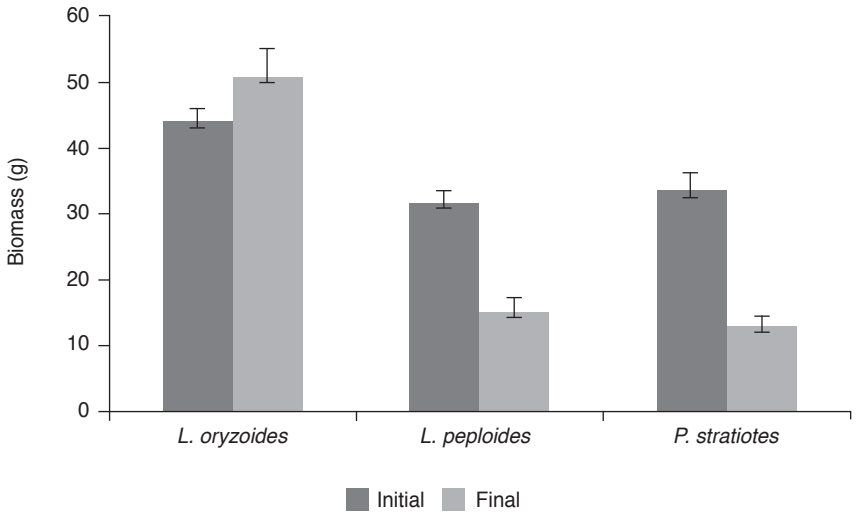

Figure 2. Macrophytes biomass before and after phytoremediation of palm oil mill final disharge (POMFD) wastewater.

macrophytes biomass occurred in *L. peploides* and *P. stratiotes* that might be due to their maximum uptake limit of nutrients. Essential elements can be toxic to plant when they cannot tolerate high amount of contaminants (Hodson and Bryant, 2012).

Carbon, Hydrogen and Nitrogen in Macrophytes Organs

Table 2 depicts the percentages of C, H and N in the shoot, stem and root organs of the macrophytes before and after the phytoremediation study, and the table also summarises the two-way ANOVA results for the percentage difference of C, H and N. The percentages of C, H and N were influenced by the macrophyte species (F = 99.23, p< 0.05; F = 37.09, p< 0.05; F = 154.09, p< 0.05) and macrophytes' organs (F = 118.78, p< 0.05; F = 102.81, p< 0.05; F = 41.08, p< 0.05). There were also interaction effects between the percentages of C, H and N and macrophyte species and the same with macrophyte organs (p< 0.05). The influence of macropyte species on the percentages of C, H, and N depended on the type of organs. In general, the percentages of C were high in all the organs of macrophytes studied were in the range of 24.8% to 42.1%. This might be due to the ample amount of C in plants in the form of carbohydrate, lipid, protein and nucleic acid molecules (Berg, 2008). There were subtle reductions in the percentages of C and H in all macrophytes after the phytoremediation. The reduction of carbon percentages might be due to the wilting and subsequent decaying process of the macrophytes. The reduction of C and H might also due to the metabolic potential of the root-associated microorganisms to utilise organic compound at the rhizosphere (Juwarkar, 2012). However, the percentage of N had increased in all macrophyte shoots with the highest being in *L. oryzoides* (3.0%). For all macrophytes, the percentages of N in shoots were higher than those in other organs (stem and roots), and this is in accordance to Greenway's (2007) finding on *Phragmites australis's* treating municipal wastewater. Translocation of assimilated ammonium from roots to shoots and stems which are then transported to shoots was believed to have resulted in high level of N in shoots.

Bioconcentration and Translocation of Metals in Macrophytes

Table 3 shows BCF and TF values of metals after the phytoremediation study.The BCF values of all metals were in the range of 10^{-1}-10^{2} of order for all studied macrophyte species. Except for Fe and Zn, BCF values of most metals (Mg, Ca, K, Na) were in the range of 10^{-1}-10^{1} for all macrophyte species. Although the accumulation of each metal was different, it was found that all studied macrophytes were capable to accumulate these metals. The BCF values for Fe and Zn were found higher in macrophyte roots than in stem and shoots for all macrophytes species. This indicates that Fe and Zn were concentrated in macrophyte roots better than in stem and shoots. It was also observed that *L. oryzoides* accumulates Fe in roots higher than *L. peploides* and *P. stratiotes*. The finding is in accordance with previous report by Soda *et al*. (2012) who claimed that Fe was retained in emergent macrophyte roots and only a small amount was transported to shoots. In *L. oryzoides, L. peploides* and *P. stratiotes*, Zn was highly accumulated in roots with BCF values of 1.2×10^{1}, 1.4×10^{1}, and 1.8×10^{1} respectively. It is likely that all macrophytes have great potential to accumulate Zn thus they may be applied to remove this metal from wastewater.

TABLE 2. CARBON (C), HYDROGEN (H) AND NITROGEN (N) PERCENTAGES IN MACROPHYTE ORGANS BEFORE AND AFTER PHYTOREMEDIATION STUDY AND TWO-WAY ANOVA RESULTS

Elements	Species	Organ	Initial	Final	Two way ANOVA					
					Species		Organ		Species*Organ	
			(%)	(%)	*F*	*p*	*F*	*p*	*F*	*p*
Carbon (C)	*L. oryzoides*	Shoot	39.90	39.99						
		Stem	37.69	36.83						
		Root	38.63	31.61						
	L. peploides	Shoot	39.11	42.06	99.23	0.000	232.21	0.000	118.78	0.000
		Stem	36.77	38.70						
		Root	40.34	32.31						
	P. stratoites	Shoot	39.60	31.53						
		Root	30.80	24.76						
Hydrogen (H)	*L. oryzoides*	Shoot	5.95	5.44						
		Stem	3.04	4.98						
		Root	5.44	4.35						
	L. peploides	Shoot	5.27	5.15	37.09	0.000	262.85	0.000	102.81	0.000
		Stem	5.03	4.89						
		Root	5.04	4.3						
	P. stratoites	Shoot	5.31	4.52						
		Root	4.49	3.34						
Nitrogen (N)	*L. oryzoides*	Shoot	0.77	3.02						
		Stem	bdl	2.57						
		Root	0.26	1.44						
	L. peploides	Shoot	0.90	2.09	154.09	0.000	24.66	0.000	41.08	0.000
		Stem	1.74	0.34						
		Root	1.41	0.77						
	P. stratoites	Shoot	1.61	1.84						
		Root	1.48	2.03						

Note: ANOVA – analysis of variance.

TABLE 3. BCF AND TF VALUES METALS FOR DIFFERENT MACROPHYTE SPECIES AND ORGANS

Metal	*L. oryzoides*				*L. peploides*				*P. stratoites*		
	BCF			TF	BCF			TF	BCF		TF
	Shoot	Stem	Root		Shoot	Stem	Root		Shoot	Root	
Mg	1.2×10^{0}	7.0×10^{-1}	1.9×10^{0}	4.0×10^{-1}	3.4×10^{0}	1.4×10^{0}	5.0×10^{0}	5.9×10^{-1}	4.4×10^{0}	7.7×10^{-1}	6.0×10^{-1}
Ca	9.2×10^{-1}	1.0×10^{1}	1.5×10^{-1}	5.3×10^{-1}	1.8×10^{-1}	4.3×10^{-1}	1.6×10^{-1}	9.6×10^{-1}	5.9×10^{-1}	5.9×10^{-1}	1.0×10^{-1}
K	2.6×10^{-1}	5.1×10^{-1}	6.9×10^{-1}	1.7×10^{-1}	5.7×10^{-1}	7.4×10^{-1}	2.2×10^{-1}	1.9×10^{-1}	7.6×10^{-1}	7.5×10^{-1}	1.2×10^{-1}
Na	1.8×10^{-1}	1.2×10^{-1}	8.0×10^{-1}	9.0×10^{-1}	3.4×10^{-1}	9.5×10^{-1}	1.2×10^{1}	2.6×10^{-1}	6.4×10^{-1}	1.3×10^{1}	6.0×10^{-1}
Fe	4.9×10^{-1}	9.3×10^{-1}	2.2×10^{2}	8.0×10^{-3}	3.6×10^{-1}	4.1×10^{-1}	1.3×10^{2}	3.0×10^{-2}	2.4×10^{-1}	1.0×10^{2}	3.0×10^{-1}
Zn	4.2×10^{-1}	8.5×10^{-1}	1.2×10^{1}	2.1×10^{-1}	2.5×10^{-1}	3.2×10^{-1}	1.4×10^{1}	2.6×10^{-1}	7.9×10^{-1}	1.8×10^{1}	1.5×10^{-1}

Note: BCF - bioconcentration factors.
TF – translocation factors.

Generally, the TF values of metals were in a range of 10^{-3}-10^{0} of order. Among other metals, K shows the highest TF value for *L. peploides, L. oryzoides* and *P. stratiotes* with respective values of 1.9, 1.7 and 1.2. This result indicates that K was translocated efficiently from root to shoots of *L.oryzoides* and *P. stratiotes*. In this study, K was highly transported to shoots where it might have taken part in many essential processes including enzyme activation, protein synthesis, photosynthesis, phloem transport, osmoregulation, stomatal regulation and tropism (Cochrane and Cochrane, 2009). A high TF value also suggested that a harvesting process can be carried out for only above-ground tissues that require less labour (Soda *et al*., 2012). The Fe shows the lowest TF values among the studied metals in

an order of 10^{-2}-10^{-3}, implying that this element was highly retained in the macrophytes' roots. This is particularly true as the BCF values for Fe in roots are greater than those in stems and shoots in all macrophyte species, which showed accumulation of the metal in below-ground tissue. It is believed that with an oxidised environment in rhizosphere, Fe could form oxyhydroxides in the roots and precipitate with other metals. As the precipitate coats the roots, translocation of metals to stem and shoots will be avoided (Soda *et al*., 2012). Zou *et al*. (2012) suggested that plants with high BCF but low TF values are suitable for remediating heavy metals through a phytostabilisation process. It was found that *L. oryzoides* accumulates Na greater in shoots and stems than other metals. It was reported that *L. oryzoides* can tolerate salt water more than other freshwater macrophytes (Flynn *et al*., 1995). The Fe was accumulated high in the roots tissue of all the macrophytes and in the shoots of *L. peploides*. The BCF values of metals were in the order of Fe>Zn>Na>Mg>Ca>K for both roots in *L. peploides* and *P. stratiotes*. Accumulation of K was found low in most of the organs except for *P. stratiotes* shoots. The K and Ca were greatly translocated from root to shoot of all the macrophytes while Fe and Zn were translocated the least. The TF values for metals were in the order of K>Na>Ca>Mg>Zn>Fe, K>Ca>Mg>Na>Zn>Fe and K>Ca>Na>Mg>Zn>Fe for *L. oryzoides, L. peploides* and *P. stratiotes*, respectively.

Identification of Bacteria Isolated from Macrophytes Root

The morphology of the bacteria colonising the roots of the macrophytes after phytoremediation shows domination of straight, rod-shaped (*Bacilli*) bacterial cells with the size of 2.33 μm × 0.7 μm (*Figure 1*). This is in accordance with the findings of Ajithkumar *et al*. (2001) and Osem *et al*. (2007), who reported that *Bacillus* sp. are involved in the phytoremediation of domestic wastewater with large bacteria population colonising the macrophyte roots. *Table 4* illustrates the number of heterotrophic bacteria in the roots of the macrophytes before and after phytoremediation. The initial bacterial number in macrophytes root was in a range of 5.71 × 10^3 – 5.71 × 10^4 CFU ml^{-1} before, and increased to 1.5 × 10^4 – 1.2 × 10^5 CFU ml^{-1} after treatment period (Day 14). In contrast, the number of heterotrophic bacteria was reduced in POMFD wastewater after the treatment, 1.16 × 10^4 CFU ml^{-1} to 4.5 × 10^2, 4.4 × 10^2 and 2.3 × 10^2 CFU ml^{-1} for *L. oryzoides, L. peploides* and *P. stratiotes*, respectively. The increasing number of bacteria in all macrophyte roots than in POMFD wastewater indicates the favourable condition of microbial growth at rhizosphere. This result supports the findings of Greenway (2007), who found that macrophyte roots play an important role as substrate for the colonisation and growth of microorganisms and are responsible for various pollutants degradation. Macrophyte roots also release oxygen and create oxidised conditions at rhizosphere, and such states stimulate the aerobic decomposition of organic matter and the growth of nitrifying bacteria (Vacca *et al*., 2005).

Results from BLAST and RDPII (*Table 5*) show that bacteria isolated from *P. stratiotes* roots were closely related to *Bacillus megaterium* (accession number: HE981752.1) and *Brevundimonas olei* (accession number: AB680017.1). The presence of *Bacillus* species in the macrophytes rhizosphere was in accordance with the bacteria morphology observed with FESEM (*Figure 1*). The bacteria isolated from *L. peploides* roots were closely related to *Pseudomonas resinovorans* (accession number: EU931560.1) and *Pseudomonas mendocina* (accession number: FJ544319.1). *Pseudomonas* species are important denitrifiers in aquatic and soil environments. Meanwhile, *Pseudomonas mendocina* was reported to have reduced nitrate by up to 96.46% (Srinandan *et al*., 2011). The bacteria isolated from *L. oryzoides* roots were closely related to *Acidovorax* sp. (accession number: AB547156.1) and *Bacillus cereus* (accession number: JX077093.1). Previous studies have shown that *Bacillus*, *Brevundimonas*, *Pseudomonas* and *Acidovorax* were denitrifiers involved in the denitrification process (Nalcaci *et al*., 2011). A denitrification process might have occurred due to the consumption of carbon from the biodegradable organic matter of the POMFD wastewater and macrophytes biomass decomposition. After the phytoremediation treatment, the immense removal of BOD_5 and NH_3-N (*Figure 1*) indicates that the denitrifiers identified in this phytoremediation might have used the biodegradable organic matter as energy source in the denitrification.

CONCLUSION

This study reveals the potential of *P. stratiotes*, *L. oryzoides* and *L. peploides* and root-associated bacterial community in remediating POMFD wastewater. The macrophyte coupled with the root-associated bacteria, play an important role in the degradation of organic compounds and NH_3-N in POMFD wastewater. The macrophytes were efficiently accumulating and translocating metal elements from POMFD wastewater to roots and from roots to shoots particularly for Fe and K, respectively.

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TABLE 4. HETEROTROPHIC BACTERIAL POPULATION ISOLATED FROM MACROPHYTE ROOTS AND PALM OIL MILL FINAL DISCHARGE (POMFD) WASTEWATER BEFORE AND AFTER THE PHYTOREMEDIATION STUDY

	Before phytoremediation		After phytoremediation	
Sample	Number of bacteria (CFU ml^{-1})	Range	Number of bacteria (CFU ml^{-1})	Range
L. oryzoides	$5.7 \times 10^4 \pm 2.4 \times 10^4$	3.0×10^4 - 7.3×10^4	$1.2 \times 10^5 \pm 2.4 \times 10^3$	1.1×10^5 - 1.2×10^5
L. peploides	$1.2 \times 10^4 \pm 3.5 \times 10^3$	8.6×10^3 - 1.5×10^4	$2.6 \times 10^4 \pm 7.1 \times 10^3$	2.0×10^4 - 3.3×10^4
P. stratoites	$5.7 \times 10^3 \pm 2.5 \times 10^3$	1.3×10^4 - 1.9×10^4	$1.5 \times 10^4 \pm 1.2 \times 10^4$	4.7×10^3 - 2.9×10^4
POMFD wastewater	$1.6 \times 10^4 \pm 4.2 \times 10^3$	1.3×10^4 - 1.9×10^4	a $4.5 \times 10^2 \pm 6.4 \times 10^2$	0.0 - 9.0×10^2
			b $4.4 \times 10^2 \pm 2.0 \times 10^2$	2.1×10^2 - 5.6×10^2
			c $2.3 \times 10^2 \pm 3.3 \times 10^2$	0.3×10^2 - 6.1×10^2

Note: [a] POMFD wastewater treated with *L. oryzoides*.
[b] POMFD wastewater treated with *L. peploides*.
[c] POMFD wastewater treated with *P. stratiotes*.

TABLE 5. BACTERIAL SIMILARITY IDENTIFICATION FROM BLAST AND RDP II DATABASES FOR ISOLATED BACTERIAL DNA SAMPLES

	BLAST			RDP II		
Macrophytes root	Accession	Description	Maximum identification (%)	Accession	Bacteria similarity	S_ab score
P. stratiotes	HE981752.1	*Bacillus megaterium* partial 16S rRNA gene, isolate A8-1	100	S000330853	*Bacillus megaterium;* MO31; AY553118	1.000
P. stratiotes	AB680017.1	*Brevundimonas diminuta* gene for 16S rRNA, partial sequence, strain: NBRC 3140	100 99	S000413819	*Brevundimonas diminuta;* IFO 3140; D49422	0.963
L. peploides	EU931560.1	*Pseudomonas aeruginosa* ZFJ-5 16S ribosomal RNA gene, partia	100	S001169573	*Pseudomonas aeruginosa;* ZFJ-5; EU931560	0.991
L. peploides	FJ544319.1	*Pseudomonas mendocina* strain 81006 16S ribosomal RNA gene, partial sequence	100	S000702052	*Pseudomonas mendocina;* DQ641475	0.967
L. oryzoides	AY512827.1	*Acidovorax avenae* 16S ribosomal RNA gene, partial sequence	89	S000001712	*Acidovorax* sp. KSP2; AB076843	0.499
L. oryzoides	JX077093.1	*Bacillus cereus strain* -Y111 16S ribosomal RNA gene, partial sequence	99	S000006757	*Bacillus cereus;* AH 527; AF290555	0.993

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