ARTICLE

Copper-resistant, biofilm-forming bacteria for potential use in rehabilitation of copper-contaminated wastewater

Opulencia, R. B.*[1], Bambase, M. E. Jr.[2], Perdigon, K. M. D.[1], Demafelis, R. B.[2], Llamado, A. A.[1], Franco, R. A. G.[1], Mogul, R. T.[1], and Raymundo, A. K.[1]

[1]Microbiology Division, Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños, College, Laguna
[2]Department of Chemical Engineering, College of Engineering and Agro-Industrial Technology, University of the Philippines Los Baños, College, Laguna

Abstract—Ninety bacterial isolates from copper-contaminated wastewater from a local semiconductor company were screened for resistance to varying concentrations of copper. Of the copper-resistant isolates, 49 produced biofilm at varying thickness on microtiter plate using Tryptic Soy Broth as substrate. Isolate T21 could tolerate up to 125 ppm copper and was provisionally identified as *Bacillus megaterium* based on comparative analysis of 16S rRNA sequences. Isolate T21 was immobilized as biofilm on a 30-liter fixed-bed upflow reactor using polyvinyl chloride corrugated pipe and Tryptic Soy Broth combined with alcohol distillery slop (75:25 v/v) as carrier and growth medium, respectively. When used to treat copper-containing wastewater effluent from a local semiconductor company, the immobilized biofilm reduced copper to as much as 65.95%, showing potential to clean up copper-contaminated wastewater.

Keywords—biofilm, bioremediation, copper, metal resistance, *Bacillus megaterium*

INTRODUCTION

Copper is an important engineering metal that functions as a conductor of heat and electricity, a building material, and a constituent of various metal alloys in several industries that include electric motors, electronics, and architecture. However, copper, at high levels, is toxic posing some serious threats to man, plants and animals. The excessive amounts of copper in fresh water resources and aquatic ecosystem damage the osmo-regulatory mechanism of fresh water animals (Lee et al., 2010) and cause mutagenesis in humans (Shawabkeh et al., 2004). It has known deleterious effects on soil biota and on many plant species (Lamb et al., 2012). The levels of copper in various segments of the environment, including air, water, soil and biosphere, have increased due to emissions from various industries such as mining, smelting, electroplating and electrolysis, and natural sources (Wang and Che, 2009; Dwivedi and Vankar, 2014). Copper contamination of water is contributed by copper discharged into effluents of various municipal and industrial wastes such as solid wastes from mines, mine water, water treatment plant discharge, bled electrolyte from electro-refining plant, etc. (Bhatia, 2002).

Various techniques have been employed to treat wastewaters with toxic metals, including adsorption, chemical precipitation, ion-exchange, membrane filtration, coagulation–flocculation, flotation, electrocoagulation, and adsorption (Bilal et al., 2013). However, these conventional methods have become either expensive, inefficient or require the use of aggressive conditions if wastewater or water are to be treated to low concentrations such as the permissible fraction of mg/l or μg/l (Schiewer and Volesky, 2000; Chojnacka, 2010).

Bioadsorption is one of the available efficient processes for heavy metal removal at low concentrations. It is a simple physicochemical process resembling conventional adsorption or ion exchange but the sorbent is of biological origin - bacterial, fungal, plant or animal -, which can bind the soluble chemicals to its cellular surfaces through surface complexation and precipitation, physical adsorption or ion exchange (Chojnacka, 2010). Inexpensive material, speed and regeneration of bioadsorbents are advantages of this method (Demirbas, 2008). Recent development in the field of environmental microbiology have focused on the use of microbial-based potential biosorbents such as yeast, bacteria and fungi (Pradhan and Rai, 2001; Liu et al., 2004; Tunali et al., 2006). Biosorption can be carried out either by immobilized biomass or in suspension but the former improves mechanical strength, rigidity, porosity and the overall metal ions removal capacity, as well as life time of the biosorbent (Rangsayatorn et al., 2004).

In nature, microorganisms are commonly found in close association with surfaces and interfaces as multicellular aggregates called biofilms (Lazar and Chifiriuc, 2010). Biofilms can be formed by single bacterial species or a consortium of many species of bacteria, fungi, and protozoa in environments where there is sufficient nutrient flow and surface attachment. These attached cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS), which is a polymeric conglomeration generally composed of extracellular DNA, proteins, and polysaccharides (Lear and Lewis, 2012).

The main objective of this study was to isolate a copper-resistant, biofilm-forming bacterium that could be immobilized as biofilm in a bioreactor to clean up copper-contaminated wastewater.

MATERIALS AND METHODS

Isolation and Purification of Copper-Resistant Microorganisms

One hundred ml of each of "raw" and "treated" wastewater samples collected from a local semiconductor company were centrifuged at 5000 rpm for 5 min. The "raw" wastewater was the water used to wash the electronic board after the etching process to remove residual copper. The "treated" wastewater was the raw wastewater that had gone through ion exchange to reduce copper. Each of the resulting pellet was dissolved in 1 ml of the respective wastewater sample. One

***Corresponding Author**
Email Address: rbopulencia@up.edu.ph
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tenth ml of each suspension was spread plated onto duplicate plates of Nutrient Agar (NA), NA + 1 ppm Cu, NA + 10 ppm Cu, and NA + 20 ppm Cu. The plates were incubated at 37°C until growth was observed, typically 3 days. Well-isolated colonies were streaked for isolation on NA + 20 ppm Cu. Isolated colonies from these plates were re-streaked for isolation on NA + 20 ppm Cu until a pure colony was obtained as determined by microscopic observation of Gram-stained samples.

Screening for Copper Resistance

Purified isolates were grown on Tryptic Soy Agar (TSA) + 20 ppm Cu, and TSA + 90 ppm Cu. Isolates that grew on 20 ppm Cu were inoculated onto separate TSA plates with 50, 70, or 100 ppm Cu. Isolates that grew on 90 ppm Cu were inoculated onto separate TSA with Cu up to 500 ppm.

Screening for Biofilm Formation using Tryptic Soy Broth

All isolates that grew at copper concentrations between 50 to 500 ppm were screened for biofilm formation as previously described (O'Toole and Kolter, 1998). Each isolate was inoculated in 5 ml Tryptic Soy Broth (TSB) and incubated for 24 h. One-tenth ml of 0.5 OD_{600nm} of the isolates was inoculated to 9.9 ml TSB. One hundred μL was inoculated onto each of 3 wells of sterile microtiter plates, which were then incubated at 37°C for 5 days. Fresh medium was added when necessary to prevent drying of isolates. Planktonic bacteria were removed by briskly shaking the microtiter plates in an autoclavable bag. The microtiter plates were washed with sterile distilled water and then vigorously shaken to remove the liquid. The wells were then stained for 10 min at ambient temperature with 125 μL of 0.1% v/v crystal violet solution (in distilled water). Excess crystal violet solution was removed, then the plates were washed with sterile distilled water until the liquid was devoid of crystal violet pigment. The plate was inverted, tapped vigorously on paper towels, and air-dried. The ability to form biofilm was scored visually by comparing the thickness of the biofilm formed by the positive control, *Pseudomonas aeruginosa*, one of the model organisms for biofilm studies (Spoering and Lewis, 2001). When the biofilm was as thick as that produced by *P. aeruginosa*, the test organism was judged a strong biofilm former under tested conditions. *P. aeruginosa* was obtained from a colleague, Prof. Noel G. Sabino, who isolated and identified said isolate. The negative control included the culture medium only.

Isolation of Copper-resistant, Biofilm-forming Microorganisms Isolation of Genomic DNA

The total genomic DNA of the isolates were extracted using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, California, USA) by following the manufacturer's instructions. Eluted DNA was analyzed by agarose gel electrophoresis. Five µl of the eluted DNA and 2 µl 2X loading dye (New England Biolabs Inc., Massachusetts, USA) were loaded into the wells of a 1% (w/v) agarose gel prepared in 0.5X TAE buffer (20 mM Tris-acetate, pH 8.0, 0.5 mM EDTA). A 100 bp DNA molecular weight marker (New England Biolabs Inc., Massachusetts, USA) was also loaded into the gel. Electrophoresis was performed in 0.5x TAE buffer at 100 volts using Mupid® submarine electrophoresis system (Advance Corporation, Tokyo, Japan) for 20-30 min until the tracking dye had run about two-thirds of the gel length. DNA was visualized under UV trans-illumination using Gel Doc™ XR+ System (Bio- Rad Laboratories Inc., California, USA) after staining with ethidium bromide (5 µg ml^{-1}).

PCR Amplification of 16S rRNA

Amplification of the 16S rRNA was performed in a 50 µl reaction volume that contained 1X Taq Master Mix (Vivantis Technologies, USA), 1.5 mM $MgCl_2$, 0.3 µM of each primer [8F: (5'AGAGTTTGATCCTGGCTCAG; Liu et al., 1997) and 1525R (5'AAGGAGGTGATCCAGCC; Embley, 1991)] and 3 µl of template DNA. The thermal cycling program used was as follows: 3 min initial denaturation at 95°C, followed by 35 cycles of 40 sec denaturation at 94°C, 50 sec primer annealing at 52°C, 1 min extension at 72°C, and a final 10 min extension at 72°C. A negative control using sterile distilled water instead of template DNA was included in the amplification process. The PCR product was examined by electrophoresis as previously described.

Sequencing

The PCR products were sent to Macrogen, Inc., (Seoul, South Korea) for purification and normal automatic sequencing. Both strands of the DNA molecule were sequenced using the universal primers 8f and 1525r (Lane, 1991).

Sequence Analysis

Bioinformatics tools were used to analyze resulting sequences from Macrogen, Inc. Forward and reverse sequences were aligned using ClustalW2 (Larkin et al., 2007) to obtain overlapping sequences into consensus sequence. During the process of alignment, individual bases were verified by comparison with the fluorescent signal for each sequence in chromatogram. Subsequently, overhang sequences and ambiguous nucleotides were excluded using ChromasPro (ChromasPro ver. 1.3; www.technelysium.com .au. chromas.html). The consensus sequence was used as query sequence to search for similar sequences in GenBank using Basic Local Alignment Tool (BLAST) program (Altschul et al., 1990).

To reveal the general taxonomic placement of the unknown isolate, the most similar reference sequences were selected for the subsequent phylogenetic analysis. The reference sequences with 97-100% similarity were considered for the multiple sequence alignment in ClustalW found in MEGA 6 software. Test for the best-fit substitution model was performed in MEGA 6 software to select for the most suitable model for phylogenetic tree analysis (Posada and Crandall, 2001). Thus, maximum likelihood was used to infer phylogenetic tree using Kimura 2-parameter with gamma distribution as the suitable substitution model. The robustness of the resulting tree was evaluated by bootstrap analyses based on 1,000 reiterations.

Screening for Cheaper Growth Medium and Substrate for Biofilm Formation

Isolate T21 and *Pseudomonas aeruginosa*, the control organism, were each inoculated in 5 ml TSB and incubated at ambient temperature for 24 h. One-tenth ml of 0.5 OD_{600nm} of each of the isolate was inoculated into 9.9 ml of each of the following media combinations: TSB, 75% TSB + 25% slops, 50% TSB + 50% slops, 25% TSB + 75% slops, and slops only. The slops were obtained from an ethanol production plant in Batangas, and were the residual fraction of the fermentation mixture after distilling off the ethanol using sugarcane as the raw material.

All media combinations were prepared in 0 ppm and 10 ppm copper concentrations. After 24 h incubation, 1 ml was taken from each of the media and was transferred to 9 ml of 0.85% NaCl. Successive ten-fold dilution series was prepared up to 10^{-5} dilution. One-tenth ml of each of the dilution was plated on duplicate TSA plates. CFU ml^{-1} was calculated after 24 h of incubation. Biofilm formation of each of isolate T21 and *Pseudomonas aeruginosa* was also determined on these different media combinations as described above.

Construction of a 30-L Bioreactor

A 30-L upflow fixed-bed column reactor with recirculation from a separate culture vessel was constructed (Figure 1). The reactor was designed and fabricated at the University of the Philippines Los Baños. The main body of the reactor was made of plexiglass. The dish bottom and top cover of the column, ball valves, and other connection fittings were made of stainless steel. Perforated support and restrainer plates were installed inside the column to contain the biofilm carriers during operation. Ball valves were placed at the top and bottom of the column to regulate the flow. A 1.0-HP regular water pump was used for recirculation from the culture vessel to the column reactor. Prior to use, the biofilm reactor, culture vessel, and all accessory components were disinfected with 5.25% (v/v) hypochlorite solution. Aeration was provided in the culture vessel by bubbling air at the bottom using an air pump.

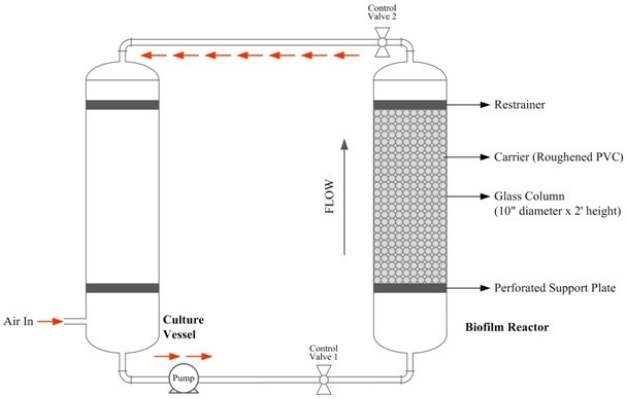

Figure 1. Schematic diagram of the biofilm reactor with culture vessel.

Immobilization of the Biofilm on the Bioreactor

Progressive inoculation of the organism was performed to achieve 27 L of suspended culture of isolate T21 starting with a pure culture of the organism in tube slants. Isolate T21 was cultivated in TSB media solution except in the final inoculation stage when 25% by volume of slops was added to the media. The final population of T21 was 4.75 x 10^8 CFU ml^{-1}. Incubation period for every stage of inoculation was fixed at 24 h. Corrugated PVC pipes with a nominal diameter of 1/2" were used as biofilm carrier. The PVC pipes were cut into 5-10 mm in length to minimize the void volume when packed inside the reactor, and roughened to create a grated surface to improve the attachment of the biofilm.

At the start of recirculation, about 31.5 L of mixed medium (75:25 v/v TSB:slops) were added into the system to fill with isolate T21 cells. The contents of the culture vessel were drained at the bottom and pumped into the bioreactor in an upflow direction to completely submerge the carrier. The overflow from the bioreactor would go back into the culture vessel. Recirculation rate was fixed at 0.172 L/min. Medium was replenished every 2 days by withdrawing 20 L of spent media and replacing with the same volume of medium. The biofilm was allowed to develop for 10 days.

Scanning Electron Microscopy

The resulting biofilm on PVC support was sent for analysis using scanning electron microscopy at the National Institute of Molecular Biology and Biotechnology at University of the Philippines Los Baños. Briefly, PVC pipes were cut open and first fixed in solution of 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer pH 6.8 then with 1% osmium tetroxide solution, dehydrated in ethanol from 50% to 100% alcohol and incubated in alcohol-isoamyl acetate mixtures with volume proportion of 50%-50% and 0-100%. Samples were dried

with carbon dioxide to a critical point, mounted on aluminum stubs, sputter coated with gold-palladium (JEOL JFC-1100, Japan), and observed on scanning electron microscope (Inspect S50, Netherland) operated at an accelerating voltage of 5kV.

Assessment of the Efficiency of the Biofilm Bioreactor in Removing Copper from Wastewater Discharge

After 10 days, copper was introduced into the system by including copper sulfate ($CuSO_4$) in the medium used for replenishment. In another run, wastewater effluent from a local semiconductor company was added. The copper concentration of the semiconductor wastewater was diluted from 35,000 ppm to about 4 ppm. Upon addition of copper or wastewater effluent, no further medium replenishment was done to avoid lowering copper concentration.

The first sample was withdrawn from the biofilm reactor after 3 times the hydraulic retention time (HRT) elapsed. For the given flow rate, the estimated HRT for the bioreactor was 3.68 h. Samples were withdrawn daily for 6 days for residual copper analysis using atomic absorption spectroscopy (AAS) at Soils Science Division, College of Agriculture, UPLB.

RESULTS

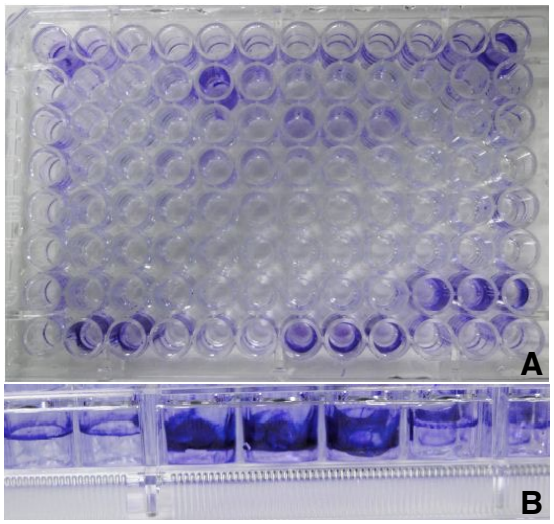

Figure 2. Biofilm formation on microtiter plate. **A**: top view; **B**: side view.

Ninety isolates from 'treated' wastewater sample were randomly selected and screened for resistance to varying concentrations of copper (50 to 500 ppm Cu). The highest concentration of copper that any of the isolates could tolerate was 250 ppm. Forty-nine of the isolates were capable of producing biofilm at varying thickness on microtiter plate using TSB as substrate (for representative plate, refer to Figure 2). The purple stain along the walls of the plate indicates the extent of biofilm formed by the organism. The intensity of the color of the dye is proportional to the thickness of the biofilm.

Several of the isolates, selected based on high tolerance to copper and strong formation of biofilm, have been provisionally identified as *Pseudomonas aeruginosa*, *Bacillus megaterium*, *Ochrobactrum* sp., *Brucella* sp., *Pseudochrobactrum* sp. and *Bacillus* sp. based on comparative analysis of 16S rRNA sequences. *Pseudomonas aeruginosa*, a well-documented biofilm former, is an opportunistic pathogen, which can infect the pulmonary tract, urinary tract, burns, wounds, and can also cause other blood infections (Iglewski, 1996). *Ochrobactrum* spp. are increasingly recognized as emerging pathogens. Most *Ochrobactrum* infections occur in immunocompromised patients (Apisarnthanarak et al., 2005) or associated with contaminated allografts (Chang et al., 1996) but *Ochrobactrum* can also cause infections in healthy humans (Galanakis et al., 2002). The close relatives of the genus *Pseudochrobactrum* are *Brucella* and *Ochrobactrum* (Kämpfer et al., 2006). The genus *Brucella* is a serious intracellular pathogen that causes brucellosis in animals and humans. Infections with *Brucella* spp. are highly infective and chronic, and difficult to produce vaccine (Taguchi et al., 2015). The isolate closely matched to *Bacillus* species grew very slowly. There is no known report on the potential pathogenicity of *B. megaterium*. Isolate T21, which is resistant to 125 ppm copper and identified as *B. megaterium* (Figure 3), was thus used in succeeding activities. The taxonomic placement was determined using 1164 bp of the 1466 bp expected 16S rRNA PCR product.

To further reduce the cost of the proposed process of utilizing isolate T21 in treatment of copper contamination, the commercially available growth medium TSB was tested in various combinations with distillery slops, an alcohol distillery waste, as growth substrate for biofilm production. Table 1 shows that the combination of 75% TSB and 25% slops can support growth of T21 closely similar to pure TSB. After 5 days of incubation, biofilm formation was also evident in the microtiter plate (Figure 4). The intensity of the color of the dye is proportional to the thickness of the biofilm as interpreted in Table 1, using *Pseudomonas aeruginosa* as positive control. Hence, the combination of 75% TSB and 25% slops was further used in our succeeding experiments with isolate T21.

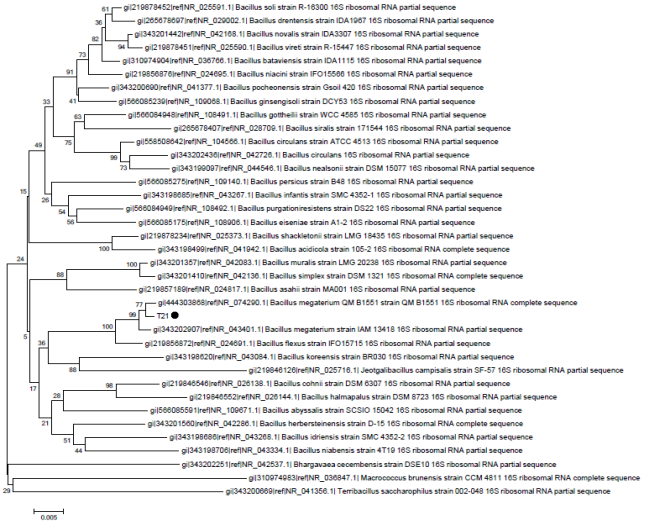

Figure 3. Phylogenetic tree of the 16S rRNA genes of isolate T21 and reference strains.

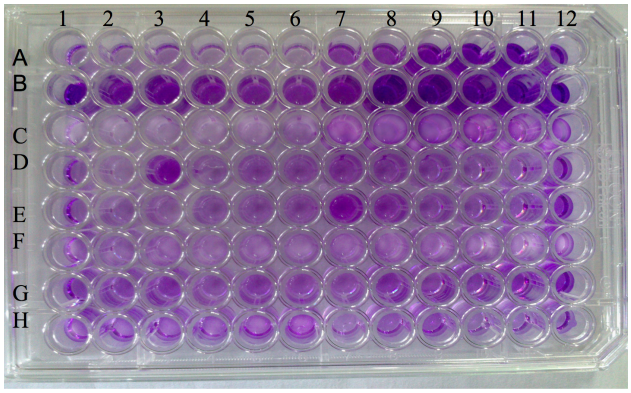

Figure 4. Biofilm formation of isolate T21 and *Pseudomonas aeruginosa* on different media combinations. In rows A to C, cells in lanes 1-6 and lanes 7-12 were grown in TSB and 75%TSB + 25% slops, respectively. In rows D to F, cells in lanes 1-6 and lanes 7-12 were grown in 50% TSB + 50% slops and 25% TSB + 75% slops, respectively. The medium used for all lanes in rows G and H was purely slops. The media in lanes 1-3 and 7-9 did not contain copper but lanes 4-6 and 10-12 contained copper at 10 ppm. Rows A and D were inoculated with T21, B and E with *Pseudomonas aeruginosa*, while rows C, F and H contained no microbial cells. In row G, lanes 1-6 were inoculated with T21 while lanes 7-12 were inoculated with *P. aeruginosa*.

Table 1. Standard plate count (CFU ml^{-1}) and biofilm formation[a] of bacterial isolates using different substrates supplemented with varying concentrations of copper and incubated at 37ºC for 5 days.

Substrate	*Pseudomonas aeruginosa*				Isolate T21			
	Copper (ppm)							
	0		10		0		10	
	CFU ml^{-1}	Biofilm	CFU ml^{-1}	Biofilm	CFU ml^{-1}	Biofilm	CFU ml^{-1}	Biofilm
Tryptic Soy Broth (TSB)	$6.1x10^8$	+++	$9.0x10^7$	+++	$1.3x10^7$	++	$1.0x10^7$	+
75% TSB + 25% alcohol distillery slops	$1.5x10^8$	++,+++,+++	$9.9x10^7$	+++	$5.9x10^6$	++,++,+++	$3.3x10^6$	+++
50% TSB + 50% alcohol distillery slops	<100	-,-,+	<100	+,+,-	<100	+,+,++	<100	+
25% TSB + 75% alcohol distillery slops	<100	+,+,-	<100	-,-,+ -,-,+	<100	+	<100	-,-,+
alcohol distillery slops	<100	+,-,-	<100	+,-,-	<100	+,-,-	<100	+,-,-

[a] Results were observed from triplicate. If the result was reproducible, a single entry of the result was presented. Otherwise, result from each of the triplicate was presented in the table.

+++ = strong former
++ = moderate former
+ = weak former
- = no biofilm formation

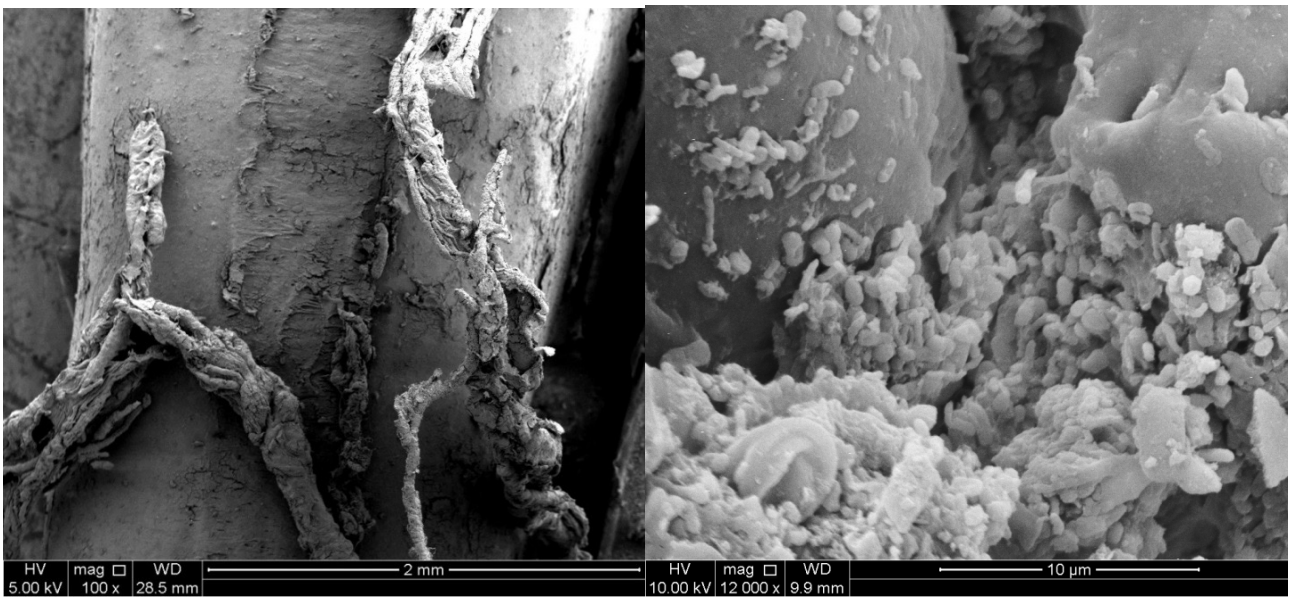

Figure 5. Scanning electron micrographs of biofilms on PVC of isolate T21 in the bioreactor using tryptic soy broth:distillery slops in a 75:25 ratio. **A**. Biofilms attached on a PVC with width ranging from 0.07 mm to 0.37 mm under 100x magnification. **B**. Detailed structure of the biofilm showing the matrix.

SEM showed biofilm attached on PVC pipes with width from 0.07 mm to 0.37 mm (Figure 5). Assessment of efficiency of the system showed reduction in copper concentration for all the bench-scale runs using aqueous copper solution and diluted actual semiconductor wastewater to as much as 65.95% of the copper in wastewater (Table 2).

Table 2. Residual copper content of samples after treatment in the bioreactor with biofilm of isolate T21.

Day	Residual Copper Content (ppm)[a]	
	Cu Solution	Diluted Wastewater
0	78.30 ± 1.32	4.20 ± 0.01
1	73.29 ± 4.84	2.31 ± 0.00
2	68.18 ± 6.77	1.73 ± 0.00
3	64.67 ± 0.19	1.65 ± 0.00
4	38.18 ± 19.49	1.46 ± 0.00
5	38.58 ± 8.69	1.49 ± 0.00

[a]Average values and standard deviations represent repeated measurements.

DISCUSSION

Heavy metal-resistant bacteria are preferred for bioremediation as they remain viable when challenged by heavy metal-contaminated wastes. Previous studies have shown that compared to dead cells, living cells are more effective in removing heavy metals as demonstrated by *Escherichia coli* and *Bacillus* sp. in removing cadmium and copper, respectively (Chelliah et al., 2008; Rani et al., 2010), and by living cells of *Pseudomonas putida* CZ, which showed significantly higher binding capacity to copper and zinc than its nonliving cells (Xin et al., 2005). Cell viability, hence, resistance to heavy metal, is required if the mechanism of biosorption depends not only on the physico-chemical interaction between the metal and the functional groups on the cell surface but also on the physiological activities of the cell. At certain concentrations, some heavy metals may be taken up inside the cell and used as cofactor of enzymes (Vest et al., 2013). The mechanism of biosorption by isolate T21 was not determined in this study. However, several reports indicate that *B. subtilis* sequesters heavy metal both extracellularly and intracellularly (Bai et al., 2014; Pan et al., 2007) while a strain of *B. megaterium* absorbs arsenic at its cell wall (Miyatake and Hayashi, 2009). Resistance to heavy metal is particularly necessary if the working microorganism is employed in a continuous bioreactor as used in this study.

The microorganism selected is not only copper-resistant but also biofilm-forming for possible treatment of copper contamination. Several groups in biomass such as structural polysaccharides, amino and phosphate groups of nucleic acids, amide, amine, sulfhydryl and carboxyl groups in the proteins (Ahluwalia and Goyal, 2007) could attract and sequester different heavy metals (Pradhan et al., 2007). The EPS in biofilm can greatly contribute to metal ions adsorption because of polysaccharides and proteins it mainly contains that often carry carboxyl and phosphate groups (Pal and Paul, 2008). The presence of EPS corresponds to an increase in the number and type of functional groups on the surface of *Hymenobacter aerophilus* that is reflected by the increased metal adsorption relative to that for EPS-free cells (Baker et al., 2010). Investigations on the proton and metal binding behavior of isolated EPS found that EPS exhibited great ability to complex heavy metals, the mechanisms of which may include proton exchange, global electric field or micro-precipitation of metals (Guibaud et al., 2009; Fang et al., 2010).

Hohapatra et al. (1993) reported that resting cells of a strain of *B. megaterium* can remove up to 39.1 mg of Cu per g of cell dry weight. Other bacteria, pure or in consortia, have shown better Cu removal than that reported here when conditions such as pH, contact time, and Cu concentration have been optimized or when employed in conjunction with another biosorption material. For instance, the copper-resistant bacterium *Stenotrophomonas maltophilia* PD2 can reduce 90% Cu under optimized conditions (Ghosh and Das Saha, 2013). The anaerobic, sulfate reducing bacteria (SRB) + iron (Fe^0) system exhibited removal ratio of Cu^{2+} at above 95% at all influent Cu^{2+} concentrations (Bai et al., 2013). Almost total removal of Cu was achieved with *B. megaterium* in combination with activated carbon up to a Cu concentration of 100 mg per liter (Hohapatra et al., 1993). Several parameters such as pH, contact time and temperature in the system described in this study may still be optimized for maximum absorption of Cu by isolate T21.

Polyvinyl chloride (PVC) is one of the most manufactured plastics and has been used extensively in water pipe systems due to its low cost, durability and inability to corrode. In this study, PVC was used as biofilm carrier as it has been proven to efficiently support biofilm formation (Zhao et al., 2011) comparable to other materials such as polyethylene (Rożej et al., 2015) and stainless steel (Zacheus et al., 2000). The PVC employed in this study was corrugated since grated surfaces increase surface roughness, which facilitates cell attachment (Qureshi et. al., 2001, Zhao et. al., 2011), hence, enhance biofilm formation.

Thus, this study designed an inexpensive and effective system with a potential to treat copper-contaminated wastewater by utilizing an easily cultivable bacterium that can form biofilm on a cheap carrier, PVC, and growth substrate composed of distillery waste product. The process may be incorporated as a part of hybrid or integrated installations for wastewater treatment.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

RB Opulencia is the project leader and main author of the paper but all authors contributed to the writing of the paper. AK Raymundo conceptualized the

framework of the project. RB Opulencia, AA Llamado and AK Raymundo supervised the microbiology portion of the study. ME Bambase Jr. and RB Demafelis supervised the engineering aspect of the study. KMD Perdigon, RAG Franco and RT Mogul served as research assistants and performed portions of the project.

REFERENCES

Ahluwalia, S.S., Goyal, D., (2006). Microbial and plant derived biomass for removal of heavy metals from wastewater. *Bioresource Technology,* 98, 2243-2257

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology,* 215, 403– 410

Apisarnthanarak, A., Kiratisin, P., Mundy, L.M. (2005). Evaluation of *Ochrobactrum intermedium* bacteremia in a patient with bladder cancer. *Diagnostic Microbiology and Infectious Disease*, 53,153–155

Bai, H., Kang, Y., Quan, H., Han, Y., Sun, J., Feng, Y. (2013). Bioremediation of copper-containing wastewater by sulfate reducing bacteria coupled with iron. *Journal of Environmental Management*, 129, 350-356

Bai, J., Yang, X., Du, R., Chen, Y., Wang, S., Qui, R. (2014). Biosorption mechanisms involved in immobilization of soil Pb by *Bacillus subtilis* DBM in a multi-metal-contaminated soil. *Journal of Environmental Sciences*, 26, 2056-64

Baker, M.G., Lalonde, S.V., Konhauser, K.O., Foght, J.M. (2010). Role of extracellular polymeric substances in the surface chemical reactivity of *Hymenobacter aerophilus*, a psychrotolerant bacterium. *Applied and Environmental Microbiology,* 76, 102-109

Bhatia, S. C. (2002). *Handbook of Industrial Pollution Control in Chemical Processes and Allied Industries (Vol II)*. Publishers and Distributor, New Delhi

Bilal, M., Shah, J. A., Ashfaq, T., Gardazi, S. M. H., Tahir, A. A., Pervez, A., Haroon, H., Mahmood, Q. (2013). Waste biomass adsorbents for copper removal from industrial wastewater- a review. *Journal of Hazardous Materials*, 263, 322-333

Chang, H.J., Christenson, J.C., Pavia, A.T., Bobrin, B.D., Bland, L.A., Carson, L.A., Arduino, M.J., Verma, P., Aguero, S.M., Carroll, K., Jenkins, E., Daly, J.A. , Woods, M.L., Jarvis, W.R. (1996). *Ochrobactrum anthropi* meningitis in pediatric pericardial allograft transplant recipients. *The Journal of Infectious Diseases*, 173, 656–660

Chelliah, E.R., Sundaresan, S., Govindan, S.S., (2008). Adaptive and cross resistance to cadmium (II) and zinc (II) by *Pseudomonas aeruginosa* BC15. *Biologica*, 63, 461-465

Chojnacka, K. (2010). Biosorption and bioaccumulation--the prospects for practical applications. *Environmental International*, 36, 299-307

Demirbas, A. (2008). Heavy metal adsorption onto agro-based waste materials: a review. *Journal of Hazardous Materials*, 157, 220-229

Dwivedi, A.K., Vankar, P.S. (2014). Source identification study of heavy metal contamination in the industrial hub of Unnao, India. *Environmental Monitoring and Assessment*, 186, 3531-3539.

Embley, T. M. (1991). The linear PCR reaction: a simple and robust method for sequencing amplified rRNA genes. *Letters in Applied Microbiology*, 13, 171–174

Fang, L.C., Huang, Q.Y., Wei, X., Liang, W., Rong, X.M., Chen, W.L., Cai, P. (2010). Microcalorimetric and potentiometric titration studies on the adsorption of copper by extracellular polymeric substances (EPS), minerals and their composites. *Bioresource Technology*, 101, 5774–5779

Galanakis, E., Bitsori, M., Samonis, G., Christidou, A., Georgiladakis, A., Sbyrakis, S., Tselentis, Y. (2002). *Ochrobactrum anthropi* bacteraemia in immunocompetent children. *Scandinavian Journal of Infectious Disease*, 34, 800–80

Ghosh, A., Das Saha, P. (2013). Optimization of copper bioremediation by *Stenotrophomonas maltophilia* PD2. *Journal of Environmental Chemical Engineering*, 1, 159-163

Guibaud, G., Hullebusch, E.V., Bordas, F., D'Abzac, P., Joussein, E. (2009). Sorption of Cd(II) and Pb(II) by exopolymeric substances (EPS) extracted from activated sludges and pure bacterial strains: modeling of the metal/ligand ratio effect and role of the mineral fraction. *Bioresource Technology*, 100, 2959–2968

Hohapatra, S. P., Siebel, M. A., Alaerts, G. J. (1993). Effects of *Bacillus megaterium* on removal of copper from aqueous solutions by activated carbon. *Environmental Science and Engineering and Toxicology*, 28, 615-629

Iglewski, B.H. (1996). *Pseudomonas*. In: *Baron's Medical Microbiology*, eds. Baron, S. *et al*. 4th ed. University of Texas Medical Branch.

Kämpfer, P., Rosselló-Mora, R., Scholz, H., Welinder-Olsen, C., Busse, H.-J. (2006). Description of *Pseudochrobactrum* gen. nov., with the two species *Pseudochrobactrum saccharolyticum* sp. nov. and *Pseudochrobactrum saccharolyticum* sp. nov. *International Journal of Systematic and Evolutionary Microbiology,* 56, 1823–1829

Lamb, D.T., Naidu, R., Ming, H., Megharaj, M. (2012). Copper phytotoxicity in native and agronomical plant species. *Ecotoxicology and Environmental Safety*, 85, 23–29

Lane, D. J. (1991). 16S/23S rRNA sequencing. In: *Nucleic Acid Techniques in Bacterial Systematics,* eds. Stackebrandt, E., Goodfellow, M. Wiley, New York.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, 23, 2947-2948

Lazar, V., Chifiriuc, M. C. (2010). Architecture and physiology of microbial biofilms. *Roumanian Archives of Microbiology and Immunology,* 69, 95-107

Lear, G., Lewis, G. D. (eds). (2012). *Microbial Biofilms: Current Research and Applications*. Caister Academic Press

Lee, J.A., Marsden, I.D., Glover, C.N. (2010). The influence of salinity on copper accumulation and its toxic effects in estuarine animals with differing osmoregulatory strategies. *Aquatic Toxicology*, 99, 65–72

Liu, H., Chen, Y., Lan, Y., Cheng, Y. (2004). Biosorption of Zn(II) and Cu(II) by the indigenous *Thiobacillus thiooxidans*. *Chemical Engineering Journal*, 97,195–201

Liu, W. T., Marsh, T. L., Cheng, H. Forney, L. J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied Environmental Microbiology*, 63, 4516–4522

Miyatake, M., Hayashi, S. (2009). Characteristics of arsenic removal from aqueous solution by *Bacillus megaterium* strain UM-123. *Journal of Environmental Biotechnology*, 9, 23-129

O'Toole, G.A., Kolter, R. (1998). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular Microbiology*, 3, 449-61

Pal, A., Paul, A.K. (2008). Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. *Indian Journal of Microbiology*, 48, 49-64

Pan, J., Liu, R., Tang, H. (2007). Surface reaction of *Bacillus cereus* biomass and its biosorption for lead and copper ions. *Journal of Environmental Sciences*, 19, 403–408

Posada, D., Crandall, K. A. (2002). The effect of recombination on the accuracy of phylogeny reconstruction. *Journal of Molecular Evolution*, 54, 396-402.

Pradhan, S., Rai, L.C. (2001). Biotechnological potential of *Microcystis* sp. in Cu, Zn and Cd biosorption from single and multimetallic systems. *Biometals,* 14, 67–74

Pradhan, S., Singh, S., Rai, L.C. (2007). Characterization of various functional groups present in the capsule of *Microcystis* and study of their role in biosorption of Fe, Ni and Cr. *Bioresource Technology*, 98, 595–601

Qureshi, F. M., Badar, U., Ahmed, N. (2001). Biosorption of copper by a bacterial biofilm on a flexible polyvinyl chloride conduit. *Applied Environmental Microbiology*, 67, 4349-4352

Rangsayatorn, N., Pokethitiyook, P., Upatham, E.S., Lanza, G.R. (2004). Cadmium biosorption by cells of *Spirulina platensis* TISTR 8217 immobilized in alginate and silica gel. *Environment International*, 30, 57-63

Rani, M. J., Hemambika, B., Hemapriya, J., Kannan, V. R. (2010). Comparative assessment of heavy metal removal by immobilized and dead bacterial cells: a biosorption approach. *African Journal of Environmental Science and Technology*, 4, 77-83

Rożej, A., Agnieszka, C., Beata, K., Kowalski, D. (2015). Structure and diversity of microbial biofilms on different pipe materials of a model drinking water distribution systems. *World Journal of Microbiology and Biotechnology*, 31, 37-47

Shawabkeh, R., Al-Harahsheh, A., Al-Otoom, A. (2004). Copper and zinc sorption by treated oil shale ash. *Separation and Purification Technology*, 40, 251–257

Schiewer, S., Volesky, B. (2000). Biosorption Processes for Heavy Metal Removal. In: *Environmental Microbe-Metal Interactions*. ASM Press, Washington D.C.

Spoering, A. L., Lewis, K. (2001). Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *Journal of Bacteriology*, 183, 6746-6751

Taguchi, Y., Imaoka, K., Kataoka, M., Uda, A., Nakatsu, D., Horii-Okazaki, S., Kunishige, R., Kano, F., Murata, M. (2015). Yip1A, a novel host factor for the activation of the IRE1 pathway of the unfolded protein response during *Brucella* infection. *PLOS Pathogens*. 11(3): e1004747. doi:10.1371/journal.ppat.1004747

Tunali, S., Cabuk, A., Akar, T. (2006). Removal of lead and copper ions from aqueous solutions by bacterial strain isolated from soil. *Chemical Engineering Journal*, 115, 203–211

Vest, K. E., Hashemi, H. F., Cobine, P. A. (2013). *The Copper Metallome in Prokaryotic Cells*. In: *Metallomics and the Cell*. *Metal Ions in Life Sciences 12*, ed. Banci, L. Springer Science + Business Media Dordrecht 2013

Wang, J., Chen, C. (2009). Biosorbents for heavy metals removal and their future. *Biotechnological Advances*, 27, 195–226

Xin, C. C., Yuan P. W., Qi L., Ji Y. S., Wei X. W., Ying X. C. (2005) Biosorption of copper(II) and zinc(II) from aqueous solution by *Pseudomonas putida* CZ1. *Colloids and Surfaces B: Biointerfaces,* 46, 101–107

Zacheus, O. M., Iivanainen, E. K., Nissinen, T. K., Lehtola, M. J., Martikainen, P. J. 2000. Bacterial biofilm formation on polyvinyl chloride, polyethylene and stainless steel exposed to ozonated water. *Water Research*, 34, 63-70

Zhao, G., Ye, L., Huang, Y., Yang, D., Li, L., Xu, G., Lei, Y. (2011). In vitro Model of Bacterial Biofilm Formation on Polyvinyl Chloride Biomaterial. *Cell Biochemistry and Biophysics*, 61, 371-376