REMOVAL OF HEAVY METALS FROM ELECTROPLATING WASTEWATER USING BACTERIA

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Pollution of water bodies by industrial discharges containing toxic chemicals are one of the major areas of concern globally. Heavy metal ions cause human health and ecological risk because they usually form compounds that can be poisonous, carcinogenic or mutagenic even in small concentrations. Heavy metals used for electroplating are copper, chromium, nickel, lead, cadmium, tin, zinc, brass or combinations of them. Platers immerse objects into a series of chemical baths in order to improve their surface conditions. Electroplating wastewater is typically from washing, rinsing and batch dumps and is usually at a very low pH of 2-5 and contains soluble forms of the various metals. Many conventional methods for treating wastewater containing metals have some technical constrain. The purposes of the study were to screen, isolate, and identify bacteria resistant to heavy metals, to be used in metals bioaccumulation studies. Isolation of single colonies of bacteria was conducted using series of dilution and spread plate method. The selected isolates were partially identified using biochemical tests and molecular technique by isolating the genomic DNA and amplification using Polymerase Chain Reaction (PCR). Optimization studies were performed to determine the optimum growth condition of the bacteria. Minimum inhibitory concentrations (MIC) of each isolate were determined in Luria Bertani (LB) Agar medium with metals chromium, copper, cadmium and lead concentrations from 50-200 mg/L. The bacteria were tested in the presence of individual metals for their growth studies. Bioaccumulation experiments were also performed with the living biomasses of *Bacillus sp.* and *Ochrobactrum sp.* under different pH (5, 7, and 9) and temperature (27 ºC, 32ºC and 37 ºC) with biomass free solution used as control. The environmental factors such as pH and temperature affected the bioaccumulation capacity tremendously. Scanning Electron Microscopy (SEM) and Energy disperse X-ray (EDX) were used to examine the bacterium cells before and after exposure to metal ions. Results from the present study shows that twenty one (21) bacterial single colonies were screened and five isolates resistant to cadmium, chromium, copper and lead were chosen after numerous round of culture and were named MH1, MH4, MH6, MH15 and MH21. Results from the optimization shows that 37°C was the optimum temperature for the growth of the bacteria and pH 7.0 was also the optimum pH for their growth. The examination of the bacteria using 16s rRNA gene sequencing analysis shows ten main taxonomic lineages. The bacteria MH15 and MH6 were identified as *Bacillus sp.* and *Ochrobactrum sp.*. The selected bacteria responded positively to the medium supplemented with up to 200 mg/L of metals by showing an extended lag phase. Growth studies of the bacteria show that they are able to survive the increasing concentrations of heavy metals. The results from the bioaccumulation experiments shows that the biomass of *Ochrobactrum sp*. shows better bioaccumulation capacity to Cu^{2+} ion up to 79.9%. While, chromium was removed more efficiently by living cells of *Bacillus sp.* than *Ochrobactrum sp.* biomass. The maximum chromium removal by *bacillus sp*. was 49.7 %. There was significant difference ($P \leq 0.05$) between temperature, pH and time in copper removal studies. Based on the results, the selected bacterial strains performed differently under different environmental conditions. Therefore, they could be used for heavy metals removal in different environments where the pH and temperature is closer to their optimum conditions. SEM and EDX of metals treated and untreated results show that there were visible changes in the bacterial cells morphology before and after bioaccumulation studies, revealing that the metals were accumulated on the cells of the bacteria.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENYINGKIRAN LOGAM BERAT DARIPADA AIR SISA ELEKTROPLATING MENGGUNAKAN BAKTERIA

Oleh

MOHAMMED UMAR MUSTAPHA

Jun 2016

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Pencemaran badan air oleh buangan industri yang mengandungi kimia toksik merupakan salah satu bidang utama yang diberi secara global dalam dunia kini. Ion logam berat boleh menyebabkan risiko pada kesihatan manusia dan ekologi kerana ion tersebut biasanya membentuk sebatian yang beracun, karsinogenik atau mutagenik walaupun pada konsentrasi yang kecil. Logam berat yang digunakan untuk penyaduran adalah tembaga, kromium, nikel, plumbum, kadmium, timah, zink, tembaga atau gabungan daripada mereka. Platers melibatkan objek ke dalam satu siri mandian kimia supaya dapat memperbaiki keadaan permukaan mereka. Penyaduran air sisa biasanya dari basuhan, bilasan dan tempat pembuangan sampah kelompok dan biasanya pada pH yang sangat rendah 2-5 dan mengandungi pelbagai bentuk larut daripada pelbagai logam. Kebanyakan kaedah konvensional bagi merawat air sisa yang mengandungi logam adalah kos yang tidak efektif dan tidak berkesan. Tujuan kajian ini adalah untuk menyaring, mengasingkan, dan mengenal pasti ketahanan bakteria terhadap logam berat, yang digunakan dalam kajian pengumpulan logam. Pemencilan koloni tunggal bakteria telah dijalankan menggunakan beberapa siri kaedah pencairan dan piring penyebaran. Pencilan terpilih kemudiannya dikenal pasti menggunakan ujian biokimia dan teknik molekular dengan menmencilkan DNA genomik dan amplifikasi menggunakan Tindalibalas Rantai Polimerase (PCR). Kajian pengoptimuman telah dilaksanakan bagi menentukan keadaan pertumbuhan optimum bakteria. kepehatan rencatan minima (MIC) bagi setiap pencilan telah ditentukan dalam media agar Bertani Luria (LB) dengan logam, kromium, kuprum, kadmium dan plumbum berkepekatan dari 50- 200mg/L. Bakteria tersebut telah diuji dengan kehadiran logam tunggal bagi menghaji tumbesarannya. Eksperimen pegumpulan telah dijalankan dengan biojisim hidup *Bacillus sp.* dan *Ochrobactrum sp.* di bawah pH (5, 7, dan 9) dan suhu (27 ºC, 32ºC dan 37 ºC) yang berbeza dengan larutan bebas biojisim yang digunakan sebagai kawalan. Faktor persekitaran, seperti pH dan suhu sangat mempengaruhi kapasiti bioakumulasi. Mikroskopi Elektron Penyaringan (SEM) dan X-ray Sebar Tenaga (EDX) telah digunakan bagi meneliti sel bakterium sebelum dan selepas pendedahan kepada ion logam. Dapatan kajian ini menunjukkan bahawa dua puluh satu (21) koloni tunggal bakteria telah disaring dan lima pencilan yang tahan pada kadmium, kromium, kuprum dan plumbum telah dipilih selepas pelbagai pusingan kultur dan telah dinamakan sebagai MH1, MH4, MH6, MH15 dan MH21. Dapatan dari pengoptimuman menunjukkan bahawa 37ºC merupakan suhu optimum bagi pembesaran bakteria, dan pH 7.0 merupakan pH optimum bagi tumbesarannya mereka. Pemeriksaan bakteria menggunakan analisis urutan gen 16s rRNA menunjukkan sepuluh barisan taksonomi utama. Bakteria MH15 dan MH6 telah dikenal pasti sebagai *Bacillus sp.* dan *Ochrobactrum sp.* Bakteria terpilih telah memberi tindakan positif pada medium yang disuplemen sehingga ke 200 mg/L logam yang menunjukkan fasa lag yang diperluas. Kajian pembesaran bakteria menunjukkan bahawa ianya boleh teruskan hidup lama pada peningkatan konsentrasi logam berat. Dapatan eksperimen bioakumulasi menunjukkan bahawa biojisim *Ochrobactrum sp.* memperlihatkan kapasiti bioakumulasi yang lebih baik pada Cu ion sehingga 79.9%. Sebaliknya, Cr telah disingkirkan dengan lebih efisien oleh sel hidup *Bacillus sp.* berbanding dengan biojisim *Ochrobactrum sp*. Penyingkiran kromium yang maksimum oleh *Bacillus sp.* adalah 49.7 %. Ianya terdapat perbezaan yang signifikan ($P \leq 0.05$) antara suhu, pH dan masa dalam kajian penyingkiran kuprum. Berdasarkan dapatan kajian, strain bakteria terpilih menonjolkan prestasi yang berbeza di bawah keadaan persekitaran yang berbeza. Oleh sebab itu, bakteria tersebut dapat digunakan bagi penyingkiran logam berat dalam persekitaran yang berbeza, iaitu pH dan suhu yang hampir pada kondisi optimanya mereka. Mikroskopi Elektron Penyaringan dan X-ray Sebaran Tenaga logam yang dirawat dan yang tidak dirawat menunjukkan bahawa terdapat perubahan yang ketara dalam morfologi sel bakteria sebelum dan selepas kajian biopengumpulan, menunjukkan bahawa logam terkumpul di permukaan sel bakteria.

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
ml	Millimeter
sec	Second
°	Degree
≥	Greater than or equal to
I	One
II	Two
+	Plus
-	Minus
%	Per cent
Abs	Absorbance
Cd(II)	Cadmium
$CdCl_2$	Cadmium chloride
$K_2Cr_2O_7$	Potassium dichromate
Cu(II)	Copper
dH2O	Distilled water
$CuCl_2$	Copper chloride
dNTP	Deoxynucleotide triphosphate
i.e.	For example
LB	Luria broth
MgCl2	Magnesium chloride
mg/L	Milligrams per liter
mg/mL	Milligrams per milliliter
MIC	Minimum Inhibitory Concentration
NA	Nutrient agar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
nm	Nanometers
Pb(II)	Lead
Pb(NO3)2	Lead nitrate
PCR	Polymerase chain reaction
rpm	Revolutions per minute
Tris	Tris hydroxymethylaminoethane
hrs	Hours
pH	Hydrogen Ion Concentration
µl	Microliter
OD	Optical Density
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
PCR	Polymerase Chain Reaction
BLAST	Basic Local Alignment Search Tool

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Heavy metal ions are chemical substance of environmental concern because of their toxicities and poisoning ability. Metals have a high relative density usually above 5.0 grams per cubic centimeter. Metal ions penetrate the water bodies through natural and anthropogenic sources. Examples of heavy metals are; mercury (Hg), cadmium (Cd), arsenic (Ar), chromium (Cr), lead (Pb), copper (Cu), zinc (Zn), nickel (Ni), and iron (Fe) (Wang *et al.*, 2006).

Heavy metal ions were divided into two groups according to physiological consideration as needed by living organisms in trace concentrations, but hazardous at higher concentrations like Mn, Co, Fe, and Zn and as non-essential or toxic with no biological role like Cd, Hg, Cr and Pb (Naja and Volesky, 2009; Valls and de Lorenzo, 2002). Metals in water bodies were often dispersed as metal species that are soluble in water, or suspended forms, colloids and sedimentary phases. Heavy metals were well known for their toxic effects metals like Cd, Ar and Hg have displayed their toxicity effect on living organisms. Chronic exposure to these metals may lead to permanent damage of organelles (Akhtar *et al.*, 2004).

However, several metal ions are useful to living organism in small concentrations, but become poisonous at higher concentration (Pattanapipitpaisal *et al.*, 2002). Unlike organic pollutants, heavy metals are not removable by the natural process of decomposition (Peng *et al.*, 2009). Heavy metals can be very toxic and they are a non-renewable resource and difficult to remove. Therefore, the accumulation ability of microbes may be exploited to minimize, clean up heavy metals from contaminated environments (Malekzadeh *et al.*, 2002). The presence of metals in water is of great interest due to their known toxic effects on the receiving water bodies and environment (Wang, 2007). Thus, the recovery of metal ions from wastewater became a vital environmental issue as the resulted effluents ended in our precious water bodies and farmlands which might affects the food chain, and poses threat to our ecosystems services (Yap *et al.*, 2004). Reclaiming heavy metals downstream of their source will minimize the hazard that they pose to the environment and humans.

Different methods were already being used to decontaminate the environment from the adverse effect of heavy metals, but yet most of the methods used were expensive. The method include; ion exchange, adsorption, chemical precipitation like hydroxides, sulfides as well as electrolytic reduction, solvent extraction and membrane filtration like Nano-filtration and reverse osmosis (Llanos *et al.*, 2010).

Removal heavy metals using reverse osmosis (RO) method are promising, nevertheless limitations like the need for pretreatment, producing huge volumetric sludge and membrane fouling are some of the limitations (Malamis *et al.*, 2012). In addition, the method involves high operating pressure and requires more energy. Also electrolytic processes are another method of treating metal-bearing effluents however, they are economical only for highly concentrated solutions and therefore the need to replace with a biological method with are cheap and efficient of treating metal-bearing effluents (Vijayaraghavan and Yun, 2008a).

Bioaccumulation using bacterial biomass is one of the most vital processes of advanced wastewater treatment, which decreases toxic substances left in wastewater. Microorganisms are richly present in almost everywhere. Microbes are organisms that are capable of tolerating unfavorable circumstances, and these properties evolved for the past millions of years (Hrynkiewicz and Baum, 2014a). Many literatures were reported using different bacterial species in metals removal (Bar *et al.*, 2007; Okeke, 2008; Srivastava *et al.*, 2008).

1.2 Problem Statement

Recent advances in urbanization and industrialization has significantly influenced the degradation of aquatic environment via discharge of toxic industrial wastewaters and domestic discharges (Minamisawa *et al.*, 2004). Heavy metals are harmful even at lower concentrations. Poor discharge of wastes containing metals result in long-term effects to human health and the water environment. Many literatures reported the severe ecological and human health related cases due to contamination of chromium, lead, arsenic, cadmium and copper in water and sediments of many marine environments (Alsop and Wood, 2011; Joutey *et al.*, 2015; Naja and Volesky, 2009). There are many electroplating factories in Klang and Kuala Lampur areas, performing several forms of plating activities, namely copper, chromium plating, silver cadmium and zinc, many of these industrial unit do not have facilities for wastewater treatment (Lee and Low, 1980; Low, Lee, & Leo, 1995; Wong, Lee, Low, & Haron, 2003). Effluents containing metals ions like zinc, copper, lead, nickel, and chromium are directly discharged into drains (Ho et al., 2012). Several technologies were developed over the years to remediate heavy metals in contaminated environment, but some of the methods need high energy and high amount of capital as well as generation of toxic sludge or other waste byproducts which are difficult to dispose (Lovley and Coates, 1997).

Bioaccumulation using bacterial biomass is one of the efficient processes of advanced wastewater treatment, which decreases toxic substances left in wastewater. Microorganisms are abundantly present in almost everywhere. Some of these microorganisms have the capability of degrading heavy metals. These potential elements could be removed by microbial remediation under suitable condition such as neutral pH, ambient temperature as well as the presence of oxygen. Though, the application of this technology as a solution to metals contamination still has many challenges to overcome, especially for large scale industrial application, despite many studies involving bioaccumulation and biosorption processes, a comprehensive research dealing with different environmental conditions for removal of metals, which are more applicable to industry, are still limited. Therefore, the need of improved metals recovery system from the environment is very important to save aquatic fauna and flora as well as human life from harmful effect of heavy metals.

1.3 Scope of Study

The aim of the research was to find a improved way to overcome heavy metals pollution problem in the environment. The research was conducted by screening metal resistant bacterial strains, which were isolated from contaminated electroplating industry wastewater. The bacteria were optimized and enriched in the laboratory condition to enhance their metal removal capacity. The isolated microbes were also characterized by biochemical tests and molecular technique for their identification. Heavy metals removal by living cells of *Bacillus* sp and *Ochrobactrum* sp. were considered. The metals, Cu (II) and Cr (VI) were used as a tested heavy metals element for bioaccumulation study. These heavy metals were selected due to their excessively utilization in electroplating industries and exist in effluents from manufacturing processes (Kanani, 2004; Wong et al., 2003) Characterization of biosorbents was carried out using Scanning Electron Micrographs (SEM), Energy Dispersive X-ray Spectroscopy (EDX) respectively to study the morphology of the cells prior and after bioaccumulation.

1.4 Aims and Objectives

The main objectives of the work were to remove heavy metal from electroplating wastewater using bacteria. The following are the objectives of the research:

- To measure the physicochemical parameters and heavy metals content in wastewater
- To characterize and identify the bacteria using biochemical method and molecular techniques.
- To evaluate the growth and bioaccumulation potential of the bacterial in removing metal ions in waste water.

1.5 Significance of the Study

The study was conducted to remove heavy metals from polluted wastewater which causes environmental degradation. Heavy metal causes health and environmental risk because it tends to bio accumulate in our bodies via ingestion and inhalation (Tchounwou *et al.*, 2012). The study examines the bioaccumulation capacity of bacterial strains isolated from contaminated environment to be used for heavy metal removal which may serve as an alternative to replace the previous methods of metal removal proved to be very expensive. This biological method of environmental control will provide cheap and easy method of wastewater treatment, especially for small scale industries that cannot afford water treatment plants. The method may provide a possible way out of metal removal from waste or natural waters. The study may provide an insight as well as information to fulfil Malaysia's obligations in maintaining the international Industrial effluent discharge limits and help in keeping up-to-date information about water quality management.

1.6 Thesis Organization

The thesis consist five chapters each chapter describes the sequence of the study.

Chapter 1 Shows the heavy metal pollution problem in Malaysia and current technologies employed to remove metal ions from contaminated wastewater. The chapter also describes the statement of problem, objectives of the study, significance and scope of the study as well as organization thesis.

Chapter 2 Shows an overview of related data based on microbial metal removal process, metal toxicity and bacterial community involves in metal removal as well as the mechanisms for heavy metals removal are discussed in details.

Chapter 3 Discusses the material and methods and experimental procedure employed in the study. The chapter moreover describes the analysis of sample and the characterization of biosorbents before and after treatment. Different equipment and chemicals used were also stated.

Chapter 4 Presents the results from the study and discussion covering heavy metals removal by living bacterial cells of *Bacillus* and *Ochrobactrum* sp. under different environmental conditions like pH and temperature. The optimization and growth kinetics of *Bacillus* sp. and *Ochrobactrum* sp in copper and chromium removal were presented in the chapter.

Chapter 5 Represent the overall conclusions based on the findings achieved from the results and discussions (Chapter 4). Recommendations for future study were also suggested in the chapter.

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APPENDICES

Appendix A

Reagents and Glass Used

Nutrient broth medium	(Merck, Germany)
Nutrient agar ready to pour	(Oxoid Ltd)
Peptone water	(Oxoid Ltd)
75% ethanol	(Sigma)
Primers	(1st Base Laboratories)
Barritt’s reagent	(Fisher scientific)
Kovac’s oxidase reagent	(Fisher scientific)
Mannitol salt agar	(Merck, Germany)
Buffer powder	(Merck, Germany)
MacCkonkey agar	(Merck, Germany)
Beef extract	(Sigma- Aldrich)
MRVP test reagent	(Sigma- Aldrich)
Peptone	(Fisher Scientific)
Gram stain kit set	(Sigma- Aldrich)
Copper (ii) chloride ($CuCl_2$)	(Merck, Germany)
Chromium ($K_2Cr_2O_7$)	(Merck, Germany)
Cadmium (ii) chloride ($CdCl_2$)	(Merck, Germany)

Glass and Equipment Used

Autoclave	(Tomy Vertical Autoclave)
Shaker incubator	(Protech Shaker Incubators SI-50)
Laminar air flow hood	(Labotech)
Atomic absorption spectroscopy	(Shimadzu 6800)
Spectrophotometer	(Spectronic)
Chiller	
Microscope	(Nikon Osaka japan)
Weighing balance	(Afcoseter-200a)
Water distillation unit	(Millipore)
Centrifuge	(Remi)

Apppendix B

Media preparation
Nutrient Agar:

Composition	**Quantity**
Peptone	5g
Yeast extracts	3g
Beef extract	2g
Sodium chloride	5g
Agar	20g
Distilled water	1000 ml

Nutrient Broth

Composition	**Quantity**
Peptone	5g
Yeast extract	3g
Beef extract	2g
Sodium chloride	5g
Agar	5g
Distilled water	1000 ml

MRVP Medium:

Composition	**Quantity**
Peptone	7g
Dextrose	5g
Dipotassium Phosphate	5g
Double distilled water	1000ml
pH	6.9

Citrate Agar:

Composition	**Quantity**
Sodium Chloride	5g
Sodium Citrate	2g
Ammonium Dihydrogen Phosphate	1g
Dipotassium Phosphate	1g
Magnesium Sulfate	0.2g
Bromothymol Blue	0.08g
Agar	15g
Double Distilled Water	1000ml
PH	6.9

Preparation of Kovac's Reagent:
Composition Quantity

Concentrated HCL	25ml
Amyl alcohol	75ml
Dimethyl amino benzene aldehyde	5g

Barritt's Reagent
Composition Quantity

Solution A	
Alpha-Naphthol	0.5ml
Ethanol absolute	95ml
Constant string of alpha-naphthol in ethanol solution	
Solution B	
Potassium hydroxide	40g
Creatine	0.3g
Distilled water	100ml

Simmons citrate agar
Composition Quantity

Ammonium dihydrogen phosphate	0.1g
Dipotassium phosphate	0.1g
Sodium chloride	0.5g
Sodium citrate	0.2g
Magnesium sulfate	0.02g
Agar	1.5g
Bromothymol blue	0.008g
Distilled water	100ml
PH	6.9

Starch agar
Composition Quantity

Peptone	5g
Beef extract	3g
Soluble starch	2g
Agar	15g
Distilled water	100ml
PH	7

Appendix C

LOCUS KT964694 947 bp rRNA linear ENV 28-SEP-2015
DEFINITION [Bacillus sp].MH6 strain 16S ribosomal RNA gene partial sequence
ACCESSION KT964694
SOURCE wastewater
AUTHORS Mohammed U.M, Halimoon,N. and Wan Johari,Wl.
TITLE Removal of Heavy metals from Industrial wastewater using Bacteria
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 975)
AUTHORS Halimoon,N. and Wan Johari,Wl.
TITLE Direct Submission
JOURNAL Submitted (28-SEP-2015) Department of Environmental sciences,

Unversity putra malaysia, seri serdang selangor, serdang,
##Assembly-Data-START##
Assembly Method: mega software v. 6.0
Sequencing Technology: Pacific Biosciences
##Assembly-Data-END##

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LOCUS KT879912 947 bp rRNA linear ENV 28-SEP-2015
DEFINITION [Bacillus sp]. MH15 strain 16S ribosomal RNA gene partial sequence
ACCESSION KT879912
SOURCE wastewater
ORGANISM Bacillus sp.
REFERENCE 1 (bases 1 to 947)
AUTHORS M.U Mustapha, Halimoon,N. and Wan Johari,Wl.
TITLE Removal of Heavy metals from Industrial wastewater using Bacteria
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 947)
TITLE Direct Submission
COMMENT Bankit Comment: ALT EMAIL:umardrc@gmail.com.

##Assembly-Data-START##
Assembly Method: mega software v. 6.0
Sequencing Technology: Pacific Biosciences
##Assembly-Data-END##

FEATURES Location/Qualifiers
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(A-B) The 16S rRNA sequence and accession number of isolate MH6 and MH15 as deposited in the GenBank database identified as *Brevundimonas sp.* and *Bacillus* sp. with accession numbers KT879912.

LIST OF PUBLICATIONS

M. U Mustapha and Normala Halimoon. Microorganisms and Biosorption of Heavy Metals in the environment: a review paper (Accepted for publication by microbial & biochemical technology Journal).

M. U Mustapha and Normala Halimoon Screening and isolation of heavy metal tolerant bacteria in industrial effluent a conference paper. Published by Procedia environmental sciences 30 (2015) 33 – 37.

M. U Mustapha, Normala Halimoon and wan lutfi W.J Identification of heavy metal resistant bacteria (MH1) *ochrobactrum sp*. isolated from industrial effluent Canadian Journal of pure and applied sciences Published.

M. U Mustapha, Normala Halimoon and wan lutfi W.J characterization of metal resistant bacteria isolated from contaminated wastewater (Accepted by American journal of applied sciences).

M.U Mustapha, Normala Halimoon, Wan Lutfi Wan Johari Removal of copper and chromium using living biomass of *Ochrobactrum sp.* MH-6 strain isolated from electroplating wastewater (under preparation)

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