ASSESSMENT OF HEAVY METALS REMOVAL USING BIOFLOCCULANT PRODUCED BY *Bacillus subtilis* UPMB10

By

WANG YANG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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ASSESSMENT OF HEAVY METALS REMOVAL USING BIOFLOCCULANT PRODUCED BY *Bacillus subtilis* UPMB10

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Chairman : Zufarzaana binti Zulkeflee, PhD
Faculty : Forestry and Environment

Due to urbanization, industrialization, and agricultural activities, heavy metal pollution has become one of the most significant environmental issues. Heavy metals are highly soluble in the aquatic environment, which makes it possible for them to bio-accumulate and magnify in living organisms, leading to health issues. Therefore, metal-contaminated wastewater must be treated before being released into the environment. Conventional methods for metal removal are expensive and generate secondary pollution. Bioflocculants have received more attention as they can be an alternative solution to remove heavy metals because of their safety and biodegradability. The heavy metal removal efficiency of bioflocculant produced by *Bacillus subtilis* UPMB10 for three metals, zinc (Zn), arsenic (As) and lead (Pb) in single and mixed synthetic metal solutions and ex-mining lake water collected from Kg. Gajah, Perak was studied. One-factor-at-a-time (OFAT) analysis was used to assess the influence of bioflocculant dosage, initial pH, and initial metal concentration on the efficiency of heavy metal removal. The concentration of metals before and after treatment was measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). The results showed that at 5 mg/L of metal concentration and pH 7, the removal efficiency of Zn and As significantly increased with an increment in bioflocculant dosage from 5% (v/v) to 20% (v/v) ($p \leq 0.05$). However, the removal efficiency of Pb significantly declined with increased bioflocculant dosages ($p \leq 0.05$). In the 5 mg/L of metal concentration with the bioflocculant dosages of 5% (v/v), heavy metals (Zn, As, Pb) showed the highest removal efficiency at pH 7 than at pH 4 and 9 with significant difference ($p \leq 0.05$). With bioflocculant dosages of 5% (v/v) at pH 7, the efficiency to remove heavy metals (Zn, As, and Pb) significantly increased as the heavy metal concentration increased from 1 to 60 mg/L ($p \leq 0.05$). Under all conditions, Zn and As were more effectively removed from mixed metal solution than from single metal solution. Conversely, Pb was more effectively removed from single metal solution. In this study, the highest removal efficiencies for Zn (66.40%) and As (45.45%) were found to occur in a mixed metal solution of 60 mg/L with a bioflocculant dosage of 5% (v/v) and pH 7. Meanwhile, the highest Pb removal efficiency (98.74%) was found in a single metal solution of 60 mg/L with a bioflocculant dosage of 5% (v/v) and pH 7. On the other hand, heavy metals (Zn, As, Pb) in the ex-mining lake water samples were effectively removed at 56.4%, 64.0%, and 78.0% with bioflocculant dosages of 20% (v/v), respectively. Overall, bioflocculant produced by *B. subtilis* UPMB10 has the potential to remove Zn, As, and Pb, and could be used as an effective bioflocculant agent for environmental remediation of heavy metals.

Keywords: Bacillus subtilis UPMB10, biopolymer, ex-mining lake, bioremediation, bioflocculant, trace metals

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

PENILAIAN PENYINGKIRAN LOGAM BERAT MENGGUNAKAN BIOFLOKULAN YANG DIHASILKAN OLEH *Bacillus subtilis* UPMB10

Oleh

WANG YANG

Februari 2023

Pengerusi : Zufarzaana binti Zulkeflee, PhD
Fakulti : Perhutanan dan Alam Sekitar

Oleh sebab urbanisasi, perindustrian, dan aktiviti pertanian, pencemaran logam berat telah menjadi salah satu isu alam sekitar yang paling signifikan. Logam berat sangat larut dalam persekitaran akuatik, yang menjadikan ia mampu untuk berbioakumulasi dan berbiomagnifikasi dalam organisma hidup, menyebabkan masalah kesihatan. Oleh itu, air sisa tercemar logam mestilah dirawat sebelum dilepaskan ke persekitaran. Kaedah konvensional untuk penyingkiran logam adalah mahal dan menghasilkan pencemaran sekunder. Bioflokulan telah menerima lebih banyak perhatian kerana ia boleh menjadi penyelesaian alternatif untuk menyingkirkan logam berat kerana ianya selamat dan boleh terbiodegradasi. Kecekapan penyingkiran logam berat bioflokulan yang dihasilkan oleh *Bacillus subtilis* UPMB10 untuk tiga logam iaitu zink (Zn), arsenik (As), dan plumbum (Pb) dalam larutan logam sintetik tunggal dan campuran, serta air tasik bekas lombong yang diambil dari Kg. Gajah, Perak telah dikaji. Analisis satu faktor pada satu masa (OFAT) telah digunakan untuk menilai pengaruh dos bioflokulan, pH awal, dan kepekatan logam awal ke atas kecekapan penyingkiran logam berat. Kepekatan logam sebelum dan selepas rawatan diukur dengan menggunakan spektrometri penyerapan optik plasma berkembar (ICP-OES). Hasil kajian menunjukkan bahawa pada kepekatan logam 5 mg/L dan pH 7, kecekapan penyingkiran Zn dan As dengan signifikan meningkat dengan peningkatan dos bioflokulan dari 5% (v/v) hingga 20% (v/v) ($p \leq 0.05$). Walau bagaimanapun, kecekapan penghilangan Pb dengan signifikan menurun dengan peningkatan dos bioflokulan ($p \leq 0.05$). Pada kepekatan logam 5 mg/L dengan dos bioflokulan 5% (v/v), logam berat (Zn, As, Pb) menunjukkan kecekapan penyingkiran tertinggi pada pH 7 berbanding pH 4 dan 9 dengan perbezaan yang signifikan ($p \leq 0.05$). Dengan dos bioflokulan 5% (v/v) pada pH 7, kecekapan untuk menyingkirkan logam berat (Zn, As, dan Pb) dengan signifikan meningkat apabila kepekatan logam berat meningkat dari 1 hingga 60 mg/L ($p \leq 0.05$). Dalam semua keadaan, Zn dan As lebih berkesan disingkirkan dari larutan logam campuran daripada larutan logam tunggal.

Sebaliknya, Pb lebih berkesan dihilangkan dari larutan logam tunggal. Dalam kajian ini, kecekapan penyingkiran tertinggi bagi Zn (66.40%) dan As (45.45%) didapati berlaku dalam larutan logam campuran 60 mg/L dengan dos bioflokulan 5% (v/v) dan pH 7. Manakala, yang kecekapan penyingkiran tertinggi bagi Pb (98.74%) didapati dalam larutan logam tunggal 60 mg/L dengan dos bioflokulan 5% (v/v) dan pH 7. Sebaliknya pula, logam berat (Zn, As, Pb) dalam sampel air tasik bekas perlombongan telah disingkirkan secara berkesan pada 56.4%, 64.0%, dan 78.0% dengan dos bioflokulan sebanyak 20% (v/v). Secara keseluruhannya, bioflokulan yang dihasilkan oleh *B. subtilis* UPMB10 berpotensi untuk menyingkirkan Zn, As, dan Pb, dan boleh digunakan sebagai agen bioflokulan yang berkesan untuk remediasi alam sekitar bagi logam berat.

Kata kunci: Bacillus subtilis UPMB10, biopolimer, tasik bekas perlombongan, bioremediasi, bioflokulan, logam surih.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Zufarzaana binti Zulkeflee, PhD
Senior Lecturer
Faculty of Forestry and Environment
Universiti Putra Malaysia
(Chairman)

Looi Ley Juen, PhD
Senior Lecturer
Faculty of Forestry and Environment
Universiti Putra Malaysia
(Member)

Noor Hafizah Mohd Pushiri, PhD
Senior Lecturer
Faculty of Forestry and Environment
Universiti Putra Malaysia
(Member)

____
ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 May 2023

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Signature: ____________________________
Name of Chairman of Supervisory Committee: Dr. Zufarzaana binti Zulkeflee

Signature: ____________________________
Name of Member of Supervisory Committee: Dr. Looi Ley Juen

Signature: ____________________________
Name of Member of Supervisory Committee: Dr. Noor Hafizah Mohd Pushiri

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LIST OF ABBREVIATIONS

UPM	Universiti Putra Malaysia
Zn	Zinc
As	Arsenic
Pb	Lead
Hg	Mercury
Cu	Copper
Co	Cobalt
Cr	Chromium
Fe	Iron
Ni	Nickel
Mn	Manganese
K	Potassium
WHO	World Health Organization
PAC	Polyaluminium Chloride
PFS	Poly Ferric Sulphate
PAM	Polyacarylamide
EPS	Extracellular Polymeric Substances
NLWQS	National Lake Water Quality Criteria and Standards
NAHRIM	National Hydraulic Research Institute of Malaysia
PVDF	Polyvinylidene Fluoride
PP	Polypropylene
PE	Polyethylene
OFAT	One-Factor-At-A-Time Analysis
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry

OD	Optical Density
ANOVA	Analysis Of Variance
RPM	Revolution Per Minutes
^{o}C	degree Celsius
HCL	Hydrochloric
NaOH	Sodium Hydroxide
HNO_3	Nitric Acid
PPM	Parts Per Million
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
$CaCl_2$	Calcium Dichloride
COD	Chemical Oxygen Demand

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Heavy metals are naturally occurring elements with a high atomic weight that is greater than 4.5 times the density of water (Tchounwou et al., 2012). Heavy metals enter the aquatic ecosystem via natural processes, including atmospheric deposition, crustal weathering, rotting organisms, and hydrodynamic alteration (Elzwayie et al., 2017). Heavy metals are also regarded as trace elements since they are present in diverse environmental matrices at trace concentrations (Bolan et al., 2013). Some heavy metals, including zinc (Zn), arsenic (As), lead (Pb), chromium (Cr), cadmium (Cd), nickel (Ni), and mercury (Hg), can cause toxicity to many environmental organisms at a low level of exposure (<1 mg/L) (Bhat et al., 2019). A variety of heavy metals are dangerous to human health. Zn is an essential element for human health, but consuming too much zinc can cause gastrointestinal distress, including nausea and vomiting, and lead to damage to the liver, kidneys, and nervous system (Verma and Dwivedi, 2013; Zhang et al., 2012). As is a toxic element that can cause various cancers, skin lesions, and cardiovascular disease and also affect the nervous and respiratory systems (Fu and Wang, 2011; Renu et al., 2016). Pb is also a toxic element that can have severe and permanent health effects on humans, particularly children (Sankhla et al., 2016). Long-term exposure to Pb can elevate the risk of ischemic heart disease, stroke, and decrease fertility and renal function (Fu and Wang, 2011).

The issue of heavy metal contamination is increasingly causing concern worldwide, as it has been associated with rising ecological and public health problems (Tchounwou et al., 2012). Owing to industrial and economic growth, heavy metals are released into the aquatic system via anthropogenic activities such as mining, untreated wastewater, agriculture, and industrial plants, thus directly threatening human health and environment (Wardhani et al., 2017). Wagh et al. (2018) reported that in the Kadava River, India, concentrations of Pb, Ni, Cr, and Fe were higher than the standard of BIS drinking water quality because of agriculture, leaching of fertilisers and pesticides, and domestic waste into the aquifer system. Industrial effluents from local factories that have not been treated and contain higher levels of heavy metals have been discharged into the Wen-Rui Tang River in Wenzhou, China (Xia et al., 2018). The concentration of Zn, As, Pb, and Cr in the Wen-Rui Tang River was categorized as moderately contaminated to heavily contaminated based on the Sediment Quality Standard (Xia et al., 2018). In terms of mining, Malaysia was one of the world's largest tin producers, with Kinta Valley historically proving to be the most productive tin-producing district (Ashraf et al., 2010). Before the tin crisis of 1985, the tin mining industry contributed a giant socio-economic benefit to Malaysia (Ashraf et al., 2010). The mining area was changed into abandoned pools, lakes, and tin tailings after mining activity ceased (Hamzah et al., 2018). These ex-mining lakes are now usually converted into recreational areas, retention ponds, or irrigation. (Hamzah et al., 2018). For instance, the Kg. Gajah lake, which was formed by past mining activities, is now utilized for commercial aquaculture and agriculture, serving as a source of food and income for the local community (Hamzah et al., 2018). Unfortunately, ex-mining lakes are mostly polluted with heavy metal residues that have the potential to persist at elevated concentrations for centuries (Koki et al., 2017). The detrimental effect can be amplified in the food web through bioconcentration, bioaccumulation, and biomagnification processes due to the recalcitrant nature of heavy metals (Mandeng et al., 2019). Nevertheless, these ex-mining lakes could become an alternative available water resource to meet the demands of the residents after a good water treatment process. Hence, innovative and environmentally friendly water treatment methods should be further studied in order to guarantee the quality of ex-mining lake water as an alternative water resource for the public water supply.

The removal of heavy metals is no longer a new subject. Over the years, chemical precipitation, adsorption, membrane filtration, ion exchange, photocatalytic, and electrocoagulation have been predominant methods for eliminating heavy metals from water resources (Carolin et al., 2017; Joseph et al., 2019). All these methods have fundamental limitations such as poor selectivity, efficiency, incomplete removal, and the generation of large quantities of toxic sludge (Ayangbenro et al., 2017; Crini and Lichtfouse, 2019). Additionally, high costs in operation and maintenance and the requirement for workers' education make it difficult to implement in developing countries (Renu et al., 2016). The method of heavy metal removal used in water treatment needs to be cost-effective and high-efficiency, so flocculation is the preferred and most widely utilised method (Vidu et al., 2020). Flocculation is the process of adding polymers to a suspended system to produce flocs. These larger particle flocs can then be removed or separated by filtration or floatation (Fu and Wang, 2011). Furthermore, flocculation is a practical method for treating heavy metals in wastewater (Fu and Wang, 2011). Heavy metal ions and compounds dissolved on the surfaces of suspended solids or colloidal particles can be removed by flocculation (López-Maldonadoe et al., 2014). Common chemically synthesized flocculants including poly aluminum chloride (PAC), poly ferric sulphate (PFS), and polyacrylamide (PAM) are frequently applied in water treatment. However, chemical flocculants can cause toxicity and corrosivity, emit foul odors, and is pollution-intensive (Subramanian et al., 2009). For instance, aluminum in chemical flocculant may cause Alzheimer’s disease, cardiovascular disease, and carcinogenic (Chang et al., 2009), while acrylamide is a neurotoxin and carcinogen that affects different body systems such as genitourinary, gastrointestinal, reproductive, nervous, and immune systems (Bin-Jumah et al., 2021; Raffan and Halford, 2019). Biological flocculant, also known as bioflocculant, is a flocculating substance composed of special polymeric materials produced by microorganisms, which can make suspended solid particles, bacterial cells, and colloidal particles settle down in an aqueous environment (Zulkeflee et al., 2016).

Bioflocculation is an emerging new technique and is becoming a potentially feasible option for substituting chemical flocculants due to the nature of its producer, being biodegradable, and generally non-toxic (Ayangbenro et al., 2019). Salehizadeh and Shojaosadati (2003) found that *Bacillus firmus* was successful in removing 98.3% of Pb^{2+}, 74.9% of Cu^{2+}, and 61.8% of Zn^{2+} from an aqueous solution. Another study reported that *Paenibacillus elgii* B69 produced a bioflocculant that could remove Al^{3+} (72%), Pb^{2+} (60%), Cu^{2+} (53%), and Co^{2+} (49%) from synthetic mixed metal solutions (Li et al., 2013). Bioflocculant produced by *Paenibacillus Validus* MP5 was reported by Rawat and Rai (2012) to eliminate 27% of Zn^{2+},16% of Ni^{2+},15% of Cd^{2+}, 9% of Cr^{6+}, and 7.5% of Pb^{2+} from synthetic mixed metal solutions. These studies found that the bioflocculant produced by bacteria has a high potential for heavy metal removal. However, due to the low metal removal efficiency of bioflocculant reported by previous research, further study is required to identify and explore new bioflocculant produced by bacteria in order to remove heavy metals efficiently in aqueous solution.

Bacillus subtilis UPMB10 was isolated from the root of the oil palm and is a variant of *Bacillus subtilis* UPMB13 (Kuan et al., 2016). Similar to its variant, *Bacillus subtilis* UPMB10 was assumed to be able to produce a high-performing bioflocculant. However, the removal of metals by UPMB10's bioflocculant has yet to be proven feasible. Therefore, this study attempted to explore the possibility and potential of removing heavy metals (Zn, As, and Pb) in both single and mixed metal solutions. The effectiveness of bioflocculant in removing heavy metals may be influenced by external conditions, such as bioflocculant dosage, pH, and initial metal concentration (Okaiyeto et al., 2016). By addressing these gaps, this study aimed to provide insights into the optimal conditions required for bioflocculant to remove heavy metals and to validate the effectiveness of bioflocculant in remediating real water samples, specifically ex-mining lake water. The information provided from this study contributed to a general understanding of the heavy metal removal efficiency of *B. subtilis* UPMB10's bioflocculant in both synthetic metal solutions and real water. Additionally, the study aimed to contribute to the knowledge of the optimal conditions required for efficient heavy metal removal using bioflocculant, which can help in the development of future studies and the implementation of this method in the industry.

1.2 Problem Statement

In recent years, the problem of heavy metal pollution resulting from increased industrial activity has raised widespread societal concern. Various human activities, including metal plating industries, tanneries, and mining operations, release heavy metal effluent into water sources, leading to an increase in the heavy metal concentration in natural water and a decline in environmental quality (Carolin et al., 2017; Wu et al., 2016). Heavy metal contamination in water, specifically Zinc (Zn), Arsenic (As), and Lead (Pb), poses a significant environmental concern due to its potential health hazards to both humans and the ecosystem (Ayangbenro and Babalola, 2017). Acute zinc poisoning caused by water pollution can lead to respiratory disorders, abdominal pain, dermatitis, vomiting, and anemia (Verma and Dwivedi, 2013; Zhang et al., 2012). Long-term ingestion of As-contaminated water by humans can result in cellular diseases such as skin, lung, bladder, and kidney cancer, poor appetite, and cardiovascular disease (Hughes et al., 2011; Tchounwou et al., 2012). The accumulation of Pb in vital organs can damage the nervous, blood, gastrointestinal, cardiovascular, and renal systems, causing symptoms such as dizziness, insomnia, anemia, weakened immunity, abdominal pain, and constipation (Jan et al., 2015; Tchounwou et al., 2012).

Currently, various methods have been employed for heavy metal removal, including chemical precipitation, ion exchange, flocculation, and membrane filtration (Vidu et al., 2020). Nevertheless, these methods have several drawbacks such as incomplete removal, high energy requirements, and the production of toxic sludge, making them unsustainable in the long term (Mahamadi and Nharingo, 2010). Bioflocculant as an eco-friendly, biodegradable and without secondary pollution method for heavy metal removal have shown promise (Ayangbenro et al., 2019). Although bioflocculant has advantages in treating heavy metals compared to conventional methods, the optimal conditions such as bioflocculant dosage, pH, and initial metal concentration for achieving maximum heavy metal removal efficiency are poorly understood. Since more than one heavy metal ion can exist in water, the removal ability of multiple heavy metal ions simultaneously using bioflocculant should also be examined. Furthermore, the bioflocculant was applied to real water samples to validate its effectiveness in real-world applications. In this study, ex-mining lake water was chosen as the water sample because ex-mining lakes have the potential to become a temporary source for human consumption and other domestic needs during increasing demand for finding an alternative water source (Koki et al., 2019). Therefore, this study aims to identify the optimal conditions (bioflocculant dosage, initial pH, and initial metal concentration) for removing heavy metals (Zn, As, and Pb) and the removal efficiencies of these metals in both single and mixed synthetic metal solutions under different conditions. Additionally, the study evaluated the effectiveness of the bioflocculant in remediating ex-mining lake water.

1.3 Significant of the Study

In response to the issue of secondary pollutant accumulation and health issues caused by chemical flocculants like poly aluminum chloride (PAC), poly ferric sulfate (PFS), and polyacrylamide (PAM) used for heavy metal removal, various attempts have been made to adopt a safer approach, including the use of bioflocculant (Ayangbenro et al., 2019; Yan et al., 2020). Bioflocculant is a biopolymer compound generated by a microorganism or its metabolites that has the benefits of being environmentally friendly and biodegradable (Li et al., 2020). Bioflocculants have been gaining popularity as promising replacement for chemical flocculants in water treatment. The goal of this research is to contribute to the effort of removing heavy metals from water by exploring the possibilities of using bioflocculant produced by *Bacillus subtilis* UPMB10. This involved testing the efficacy of the bioflocculant in removing heavy metals under different conditions, including single and mixed synthetic metal solutions. Furthermore, evaluating the metal removal efficiency of bioflocculant UPMB10 in ex-mining lake water can provide insights into its effectiveness in real-life scenarios. The findings of this study contributed to eco-friendly strategies for heavy metal removal. This can help safeguard water quality and facilitate sustainable development goals, improving access to clean water for communities.

1.4 Research Aim and Objective

This research aims to evaluate the removal efficiency of Zn, As, and Pb by bioflocculant produced by *B. subtilis* UPMB10. The specific objectives of this study are:

1. To optimize parameters (bioflocculant dosage, initial pH and initial metal concentrations) of heavy metals (Zn, As and Pb) removal using bioflocculant produced by *B. subtilis* UPMB10
2. To compare the removal of Zn, As and Pb in synthetic single and mixed solutions
3. To apply the optimum parameter of bioflocculant to remove metals from ex-mining lake water

1.5 Research Questions

In order to achieve the research objectives, several underlying questions are provided:

What is the optimal condition required to remove Zn, As and Pb using bioflocculant?

How does Zn, As and Pb removal efficiency using bioflocculant differ between synthetic single and mixed solutions?

How does the efficiency of Zn, As and Pb removal from ex-mining lake water using bioflocculant?

1.6 Thesis Organization

This thesis is divided into five (5) main chapters that provide the required information for a better understanding of the overall study. Following this chapter, the remaining chapters of the thesis are outlined below:

Chapter 2 lists the literature review in relation to this study done by previous researchers. Included within the chapter are heavy metal pollution, metal removal methods, bioflocculant and its application, and factors affecting the performance of bioflocculant in the removal of heavy metal.

Chapter 3 provides a detailed description of the materials and methods used in this study. The methods include the culturing of *Bacillus subtilis* UPMB10, Flocculation Assay, removal of heavy metals in synthetic metal solutions using bioflocculant produced by *Bacillus subtilis* UPMB10 by manipulating the bioflocculant dosage, initial pH, and initial metal concentration, and removing heavy metals in the ex-mining lake water.

Chapter 4 covers the results and research findings of this study. The relationship between the growth of *Bacillus subtilis* UPMB10 and the flocculating activity of its bioflocculant was discussed. Discussion on the factors (bioflocculant dosage, initial pH, and initial metal concentration) affecting the capability of heavy metal removal and actual applications of this bioflocculant in the ex-mining lake water using bioflocculant.

Chapter 5 gives a summary of this thesis as well as discusses the overall findings and limitations of this study and makes recommendations for future research.

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