BIOMASS PRODUCTION OF *Aureispira* sp. CCB-QB1 AND ITS APPLICATION AS A NOVEL BIOFLOCCULANT

NUR HASYIMAH BINTI ABDUL RAHMAN

UNIVERSITI SAINS MALAYSIA

2022

BIOMASS PRODUCTION OF *Aureispira* sp. CCB-QB1 AND ITS APPLICATION AS A NOVEL BIOFLOCCULANT

by

NUR HASYIMAH BINTI ABDUL RAHMAN

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

May 2022

ACKNOWLEDGEMENT

In the Name of Allah, the Most Gracious, the Most Merciful.

Alhamdulillah and thanks to Allah for the blessings given to me to accomplish my master study. First of all, I would like to express my special thanks to my main supervisor, Prof. Dr. Amirul Al-Ashraf Abdullah for his knowledge, suggestion and guidance throughout the completion of this project. From this research study, I have gained a lot of knowledge and valuable experiences. Besides that, I wish to convey my deepest gratitude to my co-supervisor, Dr. Go Furusawa for his supervision, support and help. My sincere thanks extended to Universiti Sains Malaysia (USM) especially Centre for Chemical Biology (CCB) for providing me with all the necessary laboratory equipments and facilities to complete my research study. I would like to express my deepest appreciation to School of Chemical Sciences for AAS analysis and School of Biological Sciences for allowing me to use bioreactor for the optimization study. Additionally, I wish to thank all staffs from CCB for their valuable help and support. My special gratitude also goes to all my lab mates and friends for their encouragement and continuous support to make this project completely successful. Last but not least, I would like to convey my heartfelt gratitude and deepest love to my parents and family members for their constant encouragement and endless moral support.

TABLE OF CONTENTS

APPENDICES

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
µl	Microliter
AAS	Atomic Absorption Spectroscopy
Ag	Silver
ANOVA	Analysis of Variance
Ar	Argon
ASW	Artificial seawater
ºC	Degree Celsius
Ca^{2+}	Calcium ions
$CaCl_2$	Calcium chloride
CCB-QB1	Centre for Chemical Biology-Queens Bay 1
CCD	Central Composite Design
Cd	Cadmium
Co	Cobalt
Cr	Chromium
Cu	Copper
df	Degrees of freedom

DW	Distilled water
EPS	Extracellular polysaccharides
F	Variance ratio
Fe	Iron
g	Gram
g/l	Grams per liter
h	Hour
H-ASWM	High-nutrient artificial seawater medium
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hg	Mercury
L	Liter
Mg^{2+}	Magnesium ion
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
Mn	Manganese

NaCl	Sodium chloride
NaOH	Sodium hydroxide
ND	Not detected
Ni	Nickel
nm	Nanometer
OAT	One-factor-at-a time
OD	Optical density
OD_{600}	Optical density at 600 nm
P	Probability
Pb	Lead
pH	Potential of hydrogen
ppm	Parts per million
RPM	Revolutions per minute
RSM	Response Surface Methodology
SD	Standard deviation
UV	Ultraviolet
vvm	Volume of air per unit of medium per unit of time
Zn	Zinc

3D	Three-dimensional
16S rRNA	16S ribosomal ribonucleic acid

PENGHASILAN BIOJISIM *Aureispira* sp. CCB-QB1 DAN APLIKASINYA SEBAGAI BIOFLOKULAN YANG UNIK

ABSTRAK

Bioflokulan penting untuk mengasingkan bahan pencemar, terutamanya zarah organik dan logam berat dari sisa air. Kajian sebelum ini menunjukkan bahawa *Aureispira* sp. CCB-QB1 menyebabkan penggumpalan sel apabila ada kehadiran ion kalsium. Ini menunjukkan bahawa sel bakteria ini mampu untuk menyerap bahan buangan dan mempunyai peluang yang besar untuk menjadi bioflokulan yang unik. Oleh itu, dalam kajian ini, kaedah pengkulturan dalam bioreaktor bagi *Aureispira* sp. CCB-QB1 dikaji. Kemudian, aktiviti bioserapan sel mati terhadap logam berat (Fe^{3+}dan Cu^{2+}) juga dikaji. Sel mati QB1 dihasilkan dengan merawat sel hidup menggunakan 0.5 % formaldehid. Sel-sel mati didapati menunjukkan aktiviti flokulasi yang kuat terhadap kaolin (kepekatan asal 0.2 %) dengan kehadiran 7 mM $CaCl_2$. Untuk pengkulturan dalam kelalang, 5 ml ampaian sel mati menunjukkan keseluruhan 96 % aktiviti flokulasi setelah digoncang dengan menggunakan kelajuan goncangan 150 dan 200 rpm. Selain itu, kedua-dua tripton dan pepton didapati sangat membantu untuk pertumbuhan sel QB1. Kehadiran pepton dalam sel kultur juga membantu dalam penggumpalan sel-sel QB1. Untuk pengkulturan dalam bioreaktor, keadaan kultur QB1 dioptimumkan dengan menggunakan Metodologi Permukaan Respons (RSM). Keadaan yang dioptimumkan adalah 3.39 % kepekatan garam laut, 0.9 % kepekatan pepton, dan tempoh pengeraman selama 29 jam. Tambahan pula, sel-sel mati QB1 menunjukkan kestabilan untuk mengekalkan aktiviti flokulasi yang tinggi (> 90%) untuk pelbagai pH (pH 2 hingga 9) dan suhu (10 ºC hingga 60 ºC) larutan kaolin. Untuk ujian bioserapan, sekitar 98 **%** Fe^{3+} berjaya diasingkan daripada setiap sampel 25, 50 dan 100 ppm setelah dieramkan selama 10 min. Sementara itu, sekitar 90 % Cu^{2+} berjaya diasingkan daripada kedua-dua sampel 25 dan 50 ppm setelah 20 min tempoh pengeraman. Semua hasil kajian diperoleh menunjukkan bahawa sel-sel mati QB1 berpotensi sebagai bioflokulan dan biosorben yang unik. Untuk kajian pada masa hadapan, rawatan sinar UV dicadangkan sebagai salah satu kaedah alternatif untuk menyediakan sel mati QB1 menggantikan formaldehid yang biasa digunakan.

BIOMASS PRODUCTION OF *Aureispira* sp. CCB-QB1 AND ITS APPLICATION AS A NOVEL BIOFLOCCULANT

ABSTRACT

Bioflocculants are important to remove pollutants, especially organic particles and heavy metals from wastewater. Previous study showed that *Aureispira* sp. CCB-QB1 demonstrated cell aggregation when calcium ions are available. This suggested that these bacterial cells are capable to adsorb the waste materials and have a great chance to be a novel bioflocculant. Therefore, in this research, the methods for cultivation of *Aureispira* sp. CCB-QB1 inside a bioreactor was studied. Then, the biosorption activity of the dead cells toward heavy metals (Fe^{3+} and Cu^{2+}) was tested. The QB1 dead cells were prepared by treating the living cells using 0.5 % of formaldehyde. The dead cells were discovered to show robust flocculating activity toward kaolin (initial concentration 0.2 %) in the presence of 7 mM $CaCl_{2.}$ For cultivation inside flasks, 5 ml of the dead cell suspension showed overall of 96 % of flocculating activity after shaken using both 150 and 200 rpm agitation speeds. Besides, both tryptone and peptone were found to highly supported the growth of QB1 cells. The presence of peptone in the cell culture also helps in the aggregation of the QB1 cells. For cultivation inside a bioreactor, QB1 culture conditions were optimized using Response Surface Methodology (RSM). The optimized conditions were 3.39 % of sea salt concentration, 0.9 % of peptone concentration, and 29 h of incubation period. Additionally, QB1 dead cells showed stability to maintain high flocculating activity (> 90 %) for wide range of pH (pH 2 to 9) and temperature (10 ºC to

60 ºC) of kaolin solution. For the biosorption test, about 98 % of Fe^{3+} was removed successfully from each of 25, 50 and 100 ppm samples after incubated for 10 min. Meanwhile, around 90 % of Cu^{2+} was removed successfully from both samples of 25 and 50 ppm after 20 min incubation periods. All the results obtained showed that QB1 dead cells are having the potential to be a novel bioflocculant and biosorbent. For future studies, the UV light treatment was proposed as an alternative way to prepare the QB1 dead cells replacing the commonly used formaldehyde.

CHAPTER 1

INTRODUCTION

1.1 Background of Research

Flocculation can be described as the agglomeration of suspended particles for producing larger floc useful in wastewater treatment, dredging, water purification, downstream processing, and other related industrial processes. In general, flocculating agents can be divided into three common groups: inorganic flocculants, organic synthetic flocculants, and naturally occurring flocculants (microbial flocculants or bioflocculants) (Sam et al., 2011). Generally, chemically synthesized flocculants are greatly used due to their high flocculating performance and low production cost. Nonetheless, their continuous application has brought negative effects on environment and human health, including causing cancer and Alzheimer's disease (Li et al., 2010). To overcome these problems, the chemical flocculants need to be replaced with biodegradable and harmless flocculants.

Recently, microbial flocculants or bioflocculants (naturally occurring flocculants) have drawn more focus as a biotechnological tool for recovering polluted water through bioflocculation, as they are environmentally friendly and safer (Liu et al., 2010). Bioflocculants can be explained as a kind of extracellular biodegradable polymer produced by microorganisms during their growth process. According to Zhao et al. (2012), algae, fungi, bacteria, and actinomycetes have been discovered to have the ability to produce extracellular biopolymers, including proteins and polysaccharides. However, naturally occurring bioflocculants usually show low flocculating efficiency and require a complicated preparation process that will be costly (Takagi and Kodowaki, 1985). Therefore, searching for new potential bioflocculants with an efficient flocculation process and lower production cost is the main research target nowadays (Nontembiso et al., 2011).

On the other hand, pollution by heavy metals is also a serious problem because of its toxic impact and accumulation formed in the food chain, which will eventually disturb the normal ecological system and lead to health problems (Iyer et al., 2005). Some common industries operating in leather tanning, ceramics, electroplating, wood preservation, and glass manufacturing will usually discharge high quantities of hazardous heavy metals to the environment without properly treating them (Tian et al., 2012). Since heavy metals continuously exist in nature and cannot be destructed, thus it is necessarily required to find for an environmentally friendly way to remove the toxic metal from polluting the surrounding environment (Sekhar et al., 2004). Previously, several physical and chemical conventional methods, including filtration, activated carbon adsorption, ion exchange, chemical precipitation, electrochemical treatment, and separation processes, have been applied for heavy metals removal from wastewater (Yan and Viraraghavan, 2001).

Nevertheless, these techniques (physical and chemical conventional methods) have some limitations, which are high operation cost, inefficient, and less practical to be used for natural environmental conditions (Yan and Viraraghavan, 2001). In the meantime, biosorption (microbial biomass) has attracted significant interest as an easy, economical, and more effective alternative method for heavy metal recovery. Biosorption can be described as the passive adsorption of harmful organic or inorganic substances by certain kinds of biomass (Hasan et al., 2010). Nowadays, biosorbent produced from bacteria, algae, fungi, and yeast have gained wide attention for the biosorption of metal ions. Vijayaraghavan and Prabu (2006) reported that several biosorbents such as *Aspergillus chrysogenum and Aspergillus ustus* from fungi biomasses were applied for the recovery of heavy metals. At the same time, in certain cases, the biosorption process required large physical force involving centrifugation or filtration to remove the toxic contaminants (Vijayaraghavan and Yun, 2008).

To sum up, bioflocculation can be described as the action of some bacteria and algae (bioflocculants) causing the clumping together (flocs) of fine and dispersed organic particles, resulting in faster and more complete settling of the organic solids in wastewater (Kumar et al., 2004). On the other hand, describing about biosorption, it is a physiochemical process that occurs naturally in certain biomass which allows it to passively concentrate and bind contaminants onto its cellular structure. In brief, biosorption can be defined as the ability of biological materials to accumulate heavy metals form wastewater through several pathways of uptake (Al-Garni et al., 2009). Both of these two processes are important as biotechnological tool in remediating contaminated wastewater and polluted environment (Desouky et al., 2008).

Before this, it was recorded that marine filamentous bacterium from the family *Saprospiraceae* was able to aggregate to capture its prey such as *Vibrio* sp. in seawater (Furusawa et al., 2003). Moreover, a new *Aureispira* strain, *Aureispira* sp. CCB-QB1 was identified to show the rapid formation of cell aggregation in the presence of calcium ions. The filamentous cell structure and the extracellular polysaccharides (EPS) production by this bacterial cell may help its cell aggregation (Furusawa et al., 2015a). Furthermore, *Aureispira* QB1 strain can be grown within a shorter incubation period compared to other known bioflocculants, in which the cell culture only needs around 29 hours of the incubation period. Based on the findings of this study, it was expected that *Aureispira* sp. CCB-QB1 has a great chance and potential for a novel bioflocculant and biosorbent, owing to its ability to form cell aggregation with an easier preparation method compared to the current available bacteria and fungi.

1.2 Research Objectives

The objectives of this research are:

1. To test the flocculating activity of *Aureispira* QB1 cells using kaolin solution.
2. To optimize the culture condition of *Aureispira* QB1 cells.
3. To study the biosorption activity of *Aureispira* QB1 on Fe^{3+} and $Cu^{2+.}$

CHAPTER 2

LITERATURE REVIEW

2.1 Flocculants

Water pollution happens due to human activities, including domestic, industrial, and agricultural sectors (Crini and Badot, 2007). Nowadays, people living in many areas are unable to access clean and safe water, especially for drinking purposes, due to human carelessness (Agunbiade et al., 2017). To overcome this problem, poisonous and contaminated materials in the wastewater should be well treated or removed before being release the wastewater into the environment (Prasertsan et al., 2006). There are diverse solid-liquid separation techniques that can be chosen to recover the suspended solid materials from wastewater, such as filtration, centrifugation, and flotation. However, due to high energy usage, these techniques are unable to be economically sustainable. In most industries, settling separation techniques are common methods applied. The overall process requires a longer time, and the separation is less efficient (Granados et al., 2012).

Due to this, the necessity for the use of flocculants for a variety of industrial processes starts to be gaining worldwide attention (Shih et al., 2001). For removing organic matter from wastewater, flocculation is generally applied because of the cost-effectiveness of this process (Liu et al., 2019). Flocculants or flocculating agents are macromolecule matter that is unique and can flocculate suspended particles, colloidal solids, and cells (Zhang, 2005). Flocculants can be divided into three groups which are inorganic flocculants (aluminium sulfate and polyaluminium chloride), organic synthetic flocculants (polyacrylamide derivatives, and polyethyleneimine), and naturally occurring flocculants (chitosan, sodium alginate and microbial flocculants) (Zhang et al., 2007).

Chemical flocculants, including the inorganic and organic flocculants, are usually used in sewage treatment, food production, fermentation industries, and drinking water purification (Shih et al., 2001). The study shows increasing needs for inorganic and organic flocculants for many wastewater processes due to their effective flocculation performance and low cost (Deng et al., 2003). Nevertheless, they are non-biodegradable and may cause health implications and several environmental consequences due to their toxicity. For example, acrylamide monomers of polyacrylamide are identified to be carcinogenic and neurotoxic (Ruden, 2004). Furthermore, aluminium which is the main component of polyaluminium chloride, has been found to contribute to Alzheimer's disease (Arezoo, 2002).

Due to the harmful impacts from the chemically synthesized flocculants usage, there is a need to find alternative flocculants which are environmentally friendly. In recent years, bioflocculants (naturally occurring flocculants or microbial flocculants) have received wide interest and been considered a promising substitute for the chemical flocculants because they are biodegradable and harmless to human and the environment (He et al., 2004). Thus, they have been applied in numerous industrial sectors related to detergents, textiles, and wastewater treatment (Kumar et al., 2004). Since there are concerns about human health and environmental safety, thus the usage of bioflocculants will start to gain interest in many sectors.

2.2 Bioflocculant

Bioflocculants are extracellular biodegradable polymers containing protein, cellulose, polysaccharide, lipid, nucleic acid, glycoprotein, and glycolipid generated by microbial cells (algae, bacteria, fungi, and actinomyces) during their growth (Zheng et al., 2008). Many microorganisms, for example, the *Klebsiella pneumoniae* (Nakata and Kurane, 1999), *Rhodococcus erythropolis* (Takeda et al., 1991; Kurane et al., 1994), *Citrobacter* (Ike et al., 2000), *Bacillus mucilaginosus* (Deng et al., 2003), *Bacillus licheniformis X14* (Li et al., 2009) and *Paecilomyces* (Hiroaki and Kiyoshi, 1985) were discovered as bioflocculant-producing bacteria. The nutrient compounds of the growth medium and conditions of the fermentation process have been identified to affect the production of bioflocculant (He et al., 2004).

Currently, the research on bioflocculants has attracted extensive attention. Bioflocculants are capable of eliminating suspended solids, and organic and inorganic particles by their flocculating activity and are discovered to enhance sludge settling in treatment systems. They also have been reported to be efficient in removing heavy metals and in decreasing the turbidity of various kinds of wastewater released from the industry (Gao et al., 2009; Lin and Harichund, 2011). These special advantages make the bioflocculant have great potential and suitable for providing better applications in downstream processing, wastewater treatment, and fermentation processes (Salehizadeh and Shojaosadati, 2001). Furthermore, recovering suspended solids from wastewater will not just reduce the number of contaminants released but also will help some of the food factories gain extra income since the recovered solids can be reused as feed additives for livestock (Deng et al., 2003).

Bioflocculants with various functional characteristics, especially those which are mainly polysaccharides, were discovered to be useful as lubricants, agents for film-forming, stabilizers, water retention agents, and friction reducers in numerous industries such as paper, pharmaceuticals, food, textiles, adhesives, detergents, and cosmetics products (Agunbiade et al., 2017). More importantly, bioflocculant have some benefits over the commonly used synthetic organic flocculants, which are non-toxic and safe for humans and ecosystems. Moreover, these naturally occurring flocculants are producible through fermentation processes, and the efficiency of their flocculating activity depends on the properties of the flocculants produced (Lian et al., 2008).

As described by Gao et al. (2006), the word flocculation refers to the process of aggregation of microorganism cells to make flocs, whereby suspended particles agglomerate to form a bigger floc in combined with other compounds available inside the fermentation medium. Louis Pasteur was the first person responsible for reporting the microorganism systems' flocculation (Salehizadeh and Shojaosadati, 2001). Since that, bioflocculation has been further analysed, and relationship between the cell aggregation and accumulation of extracellular bioflocculants was established (Takadi and Kodowaki, 1985). Meanwhile, researching the flocculating properties and mechanisms involved may assist us in having a better understanding of the functions of bioflocculants in actual applications, hence improving the effectiveness of the treatments (Lian et al., 2008).

In general, two primary mechanisms were suggested to be related to bioflocculants: neutralization of the charged particles and bridging of the particles, resulting in aggregation and settling (Li et al., 2009). Charge neutralization will take place when suspended solids and bioflocculant both have opposite charges. The adsorption of the bioflocculant will work on to reduce the surface charge density of the suspended particles. Later, the particles will start to approach towards each other, and consequently, the attractive forces will be more effective (Li et al., 2009). Meanwhile, the bridging process was discovered to play an important function in the flocculating activity of the bioflocculant ZS-7 produced by *Bacillus licheniformis* X14 and bioflocculant EPS SM9913 from *Pseudoalteromonas* sp. SM9913 (Li et al., 2008). When particles are attracted and adsorbed towards the bioflocculant chains, then bridging mechanisms will occurred. Numerous particles that are adsorbed onto the long molecular chain will be adsorbed by other flocculant chains at the same time as well. This causes three-dimensional flocs to be formed to settle down quickly (Li et al., 2009).

For the flocculation process, neutralization of the charged particles rarely happens since numerous microbial flocculants and suspended particles normally have negative charges. The flocculating mechanisms of bioflocculants are still not fully explored compared to the conventional methods involving the usage of chemical flocculants. Their mechanisms and flocculating performance are well developed and explained (He et al., 2010). To sum up, the high production cost and low flocculating ability are the reasons causing the industrial production of bioflocculants still not established (Gao et al., 2006).

2.3 Pollution by Heavy Metals

The non-biodegradable and highly toxic heavy metals are introduced into the environment mainly through various industrial sectors, fossil fuel burning, battery production, mining, and agricultural activities (Majumdar et al., 2008). Wastewater from industry highly contains many types of inorganic and organic contaminants in both soluble and insoluble forms, increasing the overall heavy metals contents (Kumar et al., 2008). Releasing the wastewater leads to the deposition of harmful heavy metals directly into the aquatic and terrestrial ecosystems (Senthilkumaar et al., 2000). Since heavy metals cannot be broken down into non-toxic forms, they accumulate or assimilated with water bodies and sediment, hence persist in the environment indefinitely. Heavy metals accumulated in the soil may reduce the soil's fertility and cause negative impacts on the microbial communities living in it (Dutton and Fisher, 2011).

In addition, the accumulation of heavy metals through the food chain may cause toxic effects on organisms and give rise to various health problems (Abdel-Baki et al., 2011). Some of the health risks resulting from the heavy metals exposure are damage to the internal organ, cancer, stunted growth, circulatory and nervous system impairment, and in an adverse case may cause death (Table 2.1). Some heavy metals that need to be a concern for including iron (Fe), zinc (Zn), manganese (Mn), chromium (Cr), copper (Cu), cadmium (Cd), lead (Pb), and mercury (Hg) as they are all dangerous even at low concentrations (Akpor and Muchiem, 2010). The toxicity level of each heavy metal is decided based on the quantity accessible to organisms, dosage being taken in, the way and period of exposure (Mani and Kumar, 2014).

Table 2.1 Sources and effects of heavy metals to life forms (Ayangbenro and Babalola, 2017).

Metal	Source	Effects on Human
Antimony	Coal combustion, mining, smelting, soil erosion, volcanic eruption	Cancer, conjunctivitis, dermatitis, and liver diseases.
Arsenic	Atmospheric deposition, mining, pesticides, rock sedimentation, smelting.	Brain damage, cardiovascular and respiratory disorder.
Beryllium	Coal and oil combustion, volcanic dust.	Allergic reactions, heart disease and cancer
Cadmium	Fertilizer, mining, pesticide, plastic, refining, welding.	Bone disease, coughing, headache and hypertension.
Chromium	Dyeing, electroplating, paints production, steel fabrication, tanning, textile	Bronchopneumonia, chronic bronchitis and irritation of the skin.
Copper	Copper polishing, mining, paint, plating, printing operations.	Abdominal pain, anemia and diarrhea and metabolic disorders.
Mercury	Batteries, coal combustion, geothermal activities, mining, paint industries, paper industry, volcanic eruption, weathering of rocks.	Blindness, deafness, kidney problem and loss of memory.
Lead	Coal combustion, electroplating, manufacturing of batteries, mining, paint, pigments.	Anorexia, chronic nephropathy, high blood pressure, hyperactivity and insomnia.

The highest concentration of several heavy metals that are permitted in water is 0.01, 0.05, 0.01, 0.015, 0.002, and 0.05 mg/L for Ar, Cd, Cr, Pb, Hg, and Ag, respectively as recorded by the Comprehensive Environmental Response Compensation and Liability Act (CERCLA), USA (Chaturvedi et al., 2015). The South and Southeast Asian countries, such as Peninsular Malaysia, Vietnam, India, Thailand, Philippines, Indonesia, Bangladesh, and Pakistan have paid much attention to contamination of agricultural soils and crops by heavy metals due to their potential effects on human health and long-term sustainability of food production in the contaminated areas (Luo et al., 2009). Furthermore, as been stated by the Asian standards for heavy metals, the typical range for soil are 135–270, 250–500, and 300–600 mg/kg for Cu, Pb, and Zn, respectively (Nagajyoti et al., 2010 and Jamali et al., 2007). Significantly, heavy metals recovery required important consideration, especially for the treatment based on the volume, metals concentration, sources of wastewaters, and salinity. It was reported that the conventional technologies for the removal of heavy metals from wastewater, such as chemical oxidation or reduction, precipitation, ion exchange, coagulation, reverse osmosis, flocculation, membrane processes, adsorption on activated carbon, and electrochemical treatment, have limitations and several disadvantages (Ahn et al., 2009; Patrón-Prado et al., 2010).

Some of these methods are very expensive, and inefficient, especially when the heavy metal concentration is less than 100 ppm, and incapable of achieving the treatment aims (Yan and Viraraghavan, 2001). For example, the precipitation method which is applied in electroplating industries brings no any benefit, but leads to the loss of important resources that will be disposed of at the landfills. Although the membrane separation processes are having potential for commercial applications, unfortunately, the implementation of high-pressure membrane operations such as reverse osmosis is restricted because it requires high pressure and has low water permeability. The activated carbon adsorption is a well-known method for heavy metal removal. However, the expensive cost of activated carbons is limiting this approach (Muthukrishnan and Guha, 2006).

To summarise, the conventional techniques for heavy metal removal have many disadvantages and lead to some problems such as the generation of harmful sludge that require proper discard, high energy usage, partial removal of metal, and cost limitation (Hawari and Mulligan, 2006; Zafar et al., 2007). Therefore, finding a new cost-effective approach for the removal and detoxification of harmful heavy metals from wastewater is important since heavy metals are a critical concern to humans and the environment (Volesky and Naja, 2007). This search has directed focus to the usage of biological resources (Javanbakht et al., 2011) for heavy metal removal, known as biosorption (Al-Garni et al., 2009). Biosorption has been suggested as an effective, easy, and harmless method of toxic-metal removal from wastewater (Javanbakht et al., 2011).

`

2.4 Biosorption

2.4.1 Introduction to Biosorption

The common technique utilized by microorganisms to continue to survive in heavy metals polluted environments is biosorption (Gadd, 2000). The biosorption method can be described as efficient sequestration of either organic or inorganic contaminants by a specific kind of biomass. The biosorption process is more selective and efficient even for low concentrations of heavy metals (Hasan et al., 2010). The main benefits of biosorption are the cost-effectiveness and relatively higher capability of this process for the metal removal from the solutions (Javanbakht et al., 2011). During the last two decades, algae, bacteria and fungi have been used successfully as biosorbents for heavy metals removal. In addition, biosorbents can be simply regenerated for numerous usages (microorganisms can be easily culture and reproduce) and they do not produce harmful sludge by-product since they are biodegradable (Diniz et al., 2008).

Both living and dead cells can be used as biosorbents for heavy metal removal from wastewater. However, there are distinct advantages in using either living or dead biomass (Gupta and Rastogi, 2008). The main advantages of living biomass are they can be self-renewing resulting in an increase of cell mass enabling biosorption of more heavy metal ions. However, in practical operations, living cell as biosorbents does not always qualify for heavy metal removal and recovery from toxic industrial wastewater (Gadd and White 1992). Therefore, the use of dead biomass can avoid the problem of toxicity of heavy metals toward living cells. Moreover, the biosorption process involving dead biomass is often faster as only cell surface-based binding, rather than active transport into cell, occurs (Matheickal and Yu, 1999). Dead biomass is the easy and non-destructive recovery of adsorbed metal ions, which allows regeneration of biosorbent for reuse. In contrast, metal ions accumulated inside living cells through a cellular ion transportation system are often recovered only when the cell is destroyed (Schiewer and Volesky, 2000). Prior to practical implementation, it is necessary to consider to choose either the naturally available many kinds of biomass or biomass wastes from the industry (mainly from processing plants or fermentation process) to be applied as biosorbents for removal of heavy metal (Mata et al., 2008). Reusing the biomass for heavy metals removal can be extra earnings for the industries that currently throw away the biomass as a waste. Furthermore, biomass use for biosorption process can be cultured using easy fermentation steps and in cheaper growth medium (Wang and Chen, 2009).

Generally, the biosorption mechanisms can be classified into two types which are metabolism-dependent and metabolism-independent mechanisms. Biosorption can be categorised as intracellular accumulation, extracellular precipitation, and cell surface adsorption based on where heavy metal is removed from solution. Intracellular accumulation happens due to the movement and transport of the heavy metal through the cell membrane. This transport system is dependent on the metabolism of the cell. In addition, this kind of biosorption can only happen with living cells and is usually related to the microorganism's active defense system (Ahalya et al., 2003). On the other hand, the metabolism-independent biosorption involving the usage of dead materials happens due to physicochemical interaction linking the metal and functional groups located on the cell surface of the microorganisms. This kind of biosorption (involving the usage of dead materials) usually is able to be reversed and relatively rapid. Several types of mechanisms that are independent of the cell metabolism are chemical sorption, ion exchange, and physical adsorption. (Ahluwalia and Goyal, 2007).

2.4.2 Parameters Influencing Biosorption

The Biosorption of heavy metals is a complicated process that can be influenced by some factors. Several aspects that can significantly affect the mechanism of metal biosorption are the state of the biomass, either living or non-living, chemical characteristics of the metal solution, types of the biomaterials, and surrounding conditions (Das et al., 2008). Environmental conditions, including temperature, pH and components of the wastewater, types of microorganisms, the concentration of biosorbent, metal oxidation state, the mechanism for the removal of metal, and presence of both the inorganic and organic compounds may also give effects to the metal-binding ability of specific microorganisms (Gadd, 2001). Since binding sites of metal will be changing according to a certain pH level, hence it is important to determine which functional groups are responsible for the specific metal binding in order to easily identify the mechanisms involved (functional groups will help in the identification of the mechanism involving specific biosorption process). The configuration of the microorganism’s cell wall and biosorption sites play important roles in maintaining the stability of the microorganism-metal complex in a solution (Arjoon et al., 2013). Therefore, a complete investigation of the microbial cells and their functional groups is required to help in understanding the factors influencing the mechanism of interaction between the biomass and metal involved (Kumar et al., 2010).

2.4.3 Microbial Biomass as Biosorbent

Some microorganisms such as algae, bacteria, yeast, and fungi have been discovered to have the ability to actively accumulate and remove heavy metal ions in both living and dead biomass. Applications of biosorption processes at a large scale have demonstrated that the usage of dead biomass is more practical than the living material since dead cells do not require any nutrients for living and maintenance (Park et al., 2010). When the living biomass binds with the metal ions, its performance depends on both the nutrient supply and the age of the cell. Living cells also have the risk of cell death due to the toxic effect of the heavy metals (Yan and Viraraghavan, 2000). In order to accomplish the aim of biosorption, the living microorganisms selected should be able to develop resistance against the metal ions when in contact with the heavy metal pollutant. The selected microorganisms may be originally from the contaminated area or another environment and brought to the polluted places such as mining sites, smelting sites, and several industries operating based on heavy metals (Sharma et al., 2000). Besides that, there will be no hazardous effect on the non-living microorganisms, and the biosorbents can be kept for a longer time without any harmful impact on their activity (Zhou, 1999). Extra knowledge and understanding of the microbial's metabolic pathways are important as they can help to increase the stability and survival rates of the microorganisms (Gavrilescu, 2004). To choose the most suitable microbial biomass as biosorbent, conditions of the surrounding and pre-treatment needed for heavy metals removal are required to be studied first. The most crucial factor is that the biosorbent chosen must have a high sorption ability (Romera et al., 2007)**.**

2.4.4 Mechanisms of Heavy Metal Uptake by Microorganisms

The organisms responsible for heavy metal removal usually will have tolerance for absorption of several heavy metals even at high concentrations. Above all, they should have the ability to change the hazardous pollutants to non-toxic forms (transformational abilities). They must be able to keep the contained metal that will remain inside the microorganisms (Mosa et al., 2016). Biosorption is independent of the metabolic cycle. The microorganism's cellular structure has the ability to trap heavy metal ions and directly absorb them into the binding sites located at the cell wall (Malik, 2004). The metal constituents available at the cell surface will determine the quantity of the metal sorbed. The mechanisms of biosorption include a few processes, which are ion exchange, precipitation, electrostatic interaction, surface complexation, and redox process (Yang et al., 2015).

The advantages of these processes (as stated above) are fast reactions and can achieve equilibrium in several minutes. The non-living cells, living biomass, and even the fragments of cells and tissues can perform the biosorption as passive uptake through surface complexation directly into the cell wall (Javanbakht et al., 2014). Apart from that, another method involves the heavy metal ions passing through the membrane cell into the cytoplasm by the metabolic cycle of the cell. This technique is called active uptake or bioaccumulation. Bioaccumulation is carried out by living biomass, and this process depends on numerous chemical, physical and biological mechanisms, including various intracellular and extracellular processes (Fomina and Gadd, 2014).

The main factors that are important for the mechanisms of metal uptake by a different type of biosorbents include the microorganism's cell surface, metal ions interchange, and complex formations at the active chemical sites of the cell surface. The cell wall of microorganisms is mainly composed of polysaccharides that allow the exchange of bivalent metal ions and counter ions of the polysaccharides. Examples of the biosorption that happens due to chemical ion exchange are the biosorption of Pb (II) and Cd (II) using A. *rubescens* and L. *scrobiculatus* biomasses (Anayurt et al., 2009). The existence of anionic structures causes the cellular surface of the microbes to contain a negative charge that allows them to bind with the metal cations. Some of the anionic groups responsible for metal adsorption are the amine, ester, alcohol, thiol, hydroxyl, phosphoryl, thioether, carboxyl, sulfhydryl, and sulfonate groups (Gavrilescu, 2004).

To help in assessing the uptake of heavy metals by different microbes, the cell wall structures that differ among various microorganisms should be analysed. For Gram-positive bacteria, the peptidoglycan layer, which consists of glutamic acid, teichoic acid, meso-diaminopimelic acid, alanine, and polymer of glycerol, are all the active sites required for the metal-binding processes. In contrast, the active sites for the Gram-negative bacteria include the glycoproteins, lipoproteins, enzymes, phospholipids, and lipopolysaccharides (Lesmana et al., 2009; Gupta et al., 2015). Additionally, bacteria and fungi were also discovered to develop specific resistance mechanisms to tolerate the toxicity effects of heavy metals. Specific heavy metals will be attached to numerous ligands on their cellular surfaces due to the ionic characteristic; then the necessary metals will be displaced from their respective binding sites (Valls et al., 2000). When metal is attracted and bound with cell ligands, microorganisms will take it up and transform the harmful metal from one oxidation state to another and directly reduce its toxicity (Chaturvedi et al., 2015). For example, archaea and eubacteria have the ability to oxidize Fe (II), Mn (II), Co (III) or reduce the concentrated Fe (III), Mn (IV), and Co (II) to less toxic form. Moreover, several bacterial species such as *Bacillus* and *Pseudomonas* were discovered to reduce Cr (VI) to Cr (III) and Hg (II) to Hg (Rajkumar et al., 2012).

As described by Gavrilescu (2004), the bacteria cellular walls are identified as polyelectrolyte that connects with metal ions through several ways, such as extracellular precipitations, van der Waals forces, covalent bond, and redox interactions, to retain the electro-neutrality. Sorption may take place on the surface following either one of these two reversible pathways, which are physical adsorption or electrostatic adsorption. Physical adsorption is one of the common mechanisms of metal uptake, and van der Waals' forces will help it to occur (Won et al., 2008). The physical adsorption is a rapid process because of the non-specific attraction forces, while the rapid electrostatic adsorption involves the attraction forces between charged species and the adsorbing phase (Sahmoune and Louhab, 2010).

For instance, the biosorption process of *Rhizopus arrhizus* for removing zinc, nickel, lead, and copper happen by physical adsorption (Fourest and Roux, 1992). Kuyucak and Volesky (1988) described that the biosorption of several heavy metals such as uranium, zinc, copper, cobalt, and cadmium using non-living biomasses (dead cells) including bacteria, fungi, yeasts, and algae usually takes place by electrostatic interactions between the metal ions and the cell wall of the microorganisms. For example, the copper biosorption using *Zoogloea ramigera* (bacterium) and *Chlorella vulgaris* (alga) involves the electrostatic interactions (Ahalya et al., 2003). All of these mechanisms involved are important to determine the success of the biosorption process (Brown et al., 2000).

2.5 Problems of Commonly Use Bioflocculants and Biosorbents

2.5.1 Current Bioflocculants

Although the naturally occurring bioflocculant has received wide attention as a safe and biodegradable substitute for the chemical flocculants, its low flocculating ability in the application and large dosage demand have become the main challenges in the bioflocculant establishment for wastewater treatment system (Deng et al., 2003). In general, bioflocculants have complex structures, and different types of bacteria produce bioflocculant with different characteristics (Nakamura et al., 1976). As reported by Khalil and Aly (2001), a study conducted on the characteristics of a bioflocculant produced by *Bacillus mucilaginosus* described that starch may be converted to a flocculant by a series of chemical processes however the flocculating activity is normally dissatisfying.

Besides, the high expenses due to expensive substrates usage for the production of bioflocculant become the obstacle to its industrial-scale application (Zhao et al., 2012). The complicated preparation process involving two-stage fermentation, precipitation process, and drying process together with the large amount of carbon or nitrogen sources needed in the culture medium, led to the expensive production cost for bioflocculants application. Carbon and nitrogen sources are important to support the growth of bacteria that is responsible for producing bioflocculants. It has been well recorded that manipulating any of these sources highly affects the bacterial growth and production of bioflocculant (Sheng et al., 2006). Thus, one of the alternatives to overcome this problem and help to reduce the production cost is using a cheaper substrate for the industrial production of bioflocculant (Liu and Cheng, 2010). Recently, various wastes such as potato starch wastewater (Guo et al., 2015), corn stover (Wang et al., 2013), and other wastes produced by the canning factories are alternatively reused as cheaper carbon sources (Tong et al., 1999).

Additionally, the effects of several factors, such as, the temperature of the culture medium and initial pH, on the production of bioflocculant by microorganisms need to be studied first to determine the cost-optimal culturing medium (Gomaa, 2012). Normally, the total costs for bioflocculants production and application will be identified based on the prices of raw materials and the flocculation outcome. Therefore, to utilize bioflocculants extensively for industrial purposes, it is necessary to find various microorganisms capable of producing bioflocculant that have strong flocculating activity and cheaper production costs (Gao et al., 2006).

2.5.2 Usage of Common Biosorbents and Obstacles to their Application

Biosorption was preferred as an alternative method to replace the conventional physical-chemical process for the removal of heavy metals because it is more economical and environmentally friendly. Biosorbents prepared from many kinds of fungi, algae, bacteria, and yeast have attracted interest as potential candidates for the biosorption of metal ions (Dwivedi et al., 2012).

Wang and Chen (2009) reported that both the living and dead fungi were usually utilized as biosorbents for heavy metal pollution. These microorganisms were chosen as they are abundance, may save cost, release the low amount of sludge and easily regenerate (Vijayaraghavan and Yun, 2008). Fungi biomasses become the potential scavengers of metal ions due to the present of carboxyl with a negative charge and phosphate groups of the cell wall components (Varshney et al., 2010). Recently, new fungi biomasses, which are *Aspergillus chrysogenum* and *Aspergillus ustus*, were selected to prepare biosorbents for metals removal (Lodeiro et al., 2006). Numerous researchers have used various fungal biomasses origin from *Rhizopus oryzae*, *Rhizopus arrhizus*, *Penicillium*, *Aspergillus niger*, and *Aspergillus oryzae* earlier for the removal of metal ions from wastewater samples (Fourest and Volesky, 1996).

Nevertheless, the longer incubation period was needed for harvesting fungal mycelium, which is around 5 to 7 days. Therefore, incubation period becomes one of the impediments to fungal application as a biosorbent. (Alothman et al., 2019). Similarly, many studies reported that a long incubation time was needed to treat heavy metal pollution from mixed solutions (Azzam and Tawfik, 2015). In some cases, centrifugation