

**REMOVAL OF LEAD IONS FROM AQUEOUS SOLUTION ONTO  
BIOMASS OF *BACILLUS SUBTILIS*.**

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**A project report submitted in partial fulfilment of the  
requirements for the award of Bachelor of Engineering  
(Hons.) Chemical Engineering**

**Faculty of Engineering and Science  
Universiti Tunku Abdul Rahman**

**April 2011**

## DECLARATION

I hereby declare that this project report is based on my original work except for citations and quotations which have been duly acknowledged. I also declare that it has not been previously and concurrently submitted for any other degree or award at UTAR or other institutions.

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**APPROVAL FOR SUBMISSION**

I certify that this project report entitled **“REMOVAL OF HEAVY METAL IONS FROM AQUEOUS SOLUTIONS ONTO BIOMASS OF BACILLUS SUBTILIS”** was prepared by **LAU MIEW CHEONG** has met the required standard for submission in partial fulfilment of the requirements for the award of Bachelor of ENGINEERING (Hons.) CHEMICAL ENGINEERING at Universiti Tunku Abdul Rahman.

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Specially dedicated to  
my beloved family.

## ACKNOWLEDGEMENTS

I am very thankful to everyone who had supported me and contributed to my successful completion of this project effectively and moreover on time. First and foremost, I express my heartfelt gratitude to my supervisor, Dr. Gulnaziya Issabayeva for her constant encouragement, invaluable advices and guidance throughout our research work. I shall remain ever grateful to her for her care, concern and sincere interest in my welfare. I must mention that without her timely help in writing and correction, this thesis could not have been submitted in time. She has taken pain to go thought the project and make necessary correction as and when needed. I thank her for providing us very useful information and techniques required to complete our research.

Besides of that, I would like to thanks to Ms Chen Siew Kim for her assistance in providing useful information and techniques regarding the project.

In addition, I would like to thanks to my course-mate, Ms Cheong Hui Min who has been working together with me, discuss the faced problem with me, and support me with some guidance for write this thesis. I would also like to thanks to my parents who had being fully supportive and encouraging.

Last but not least, I would like to express my appreciation to Universiti Tunk Abdul Rahman (UTAR) for providing me a platform to gain extra knowledge and develop problem solving skills throughout the experimental process and report writing.

**REMOVAL OF HEAVY METAL IONS FROM AQUEOUS SOLUTIONS  
USING BIOMASS OF BACILLUS SUBTILIS.**

**ABSTRACT**

The biosorption characteristic of *Bacillus subtilis* bacteria biomass on Lead, Pb(II) were examined as a function of initial pH and metal ion concentration. Dried biomass of *Bacillus subtilis* was used in the study. The equilibrium data obtained were analysed using both Langmuir and Freundlich isotherm models. The model parameters were examined using non-linear regression analysis. The results showed that the equilibrium data fitted well to Freundlich models, respectively. The maximum adsorption happened were found to be 294.1176 mg/g at pH 6.

The results indicated that *Bacillus subtilis* is a suitable biosorbent for the removal of Pb(II) ions from aqueous solution.

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## LIST OF SYMBOLS / ABBREVIATIONS

$c_p$	specific heat capacity, J/(kg·K)
$h$	height, m
$K_d$	discharge coefficient
$M$	mass flow rate, kg/s
$P$	pressure, kPa
$P_b$	back pressure, kPa
$R$	mass flow rate ratio
$T$	temperature, K
$v$	specific volume, m <sup>3</sup>
$\alpha$	homogeneous void fraction
$\eta$	pressure ratio
$\rho$	density, kg/m <sup>3</sup>
$\omega$	compressible flow parameter
Ppm	parts per million
ID	inner diameter, m
MAP	maximum allowable pressure, kPa
MAWP	maximum allowable working pressure, kPa
OD	outer diameter, m
RV	relief valve



## CHAPTER 1

### INTRODUCTION

#### 1.1 Microorganisms' biomass bioadsorbents.

In the early 1980s, the capability of some microorganisms to accumulate metallic elements was witnessed by some scientists. Numerous research reports have been published from toxicological points of view, but scientists were concerned with the effects of metal on the metabolic activities of the microbial cell and the consequences of their accumulation in the food chain due to the active metabolism of living cells (Volesky , 1987).

The research on biomass-metal interactions is dated back to the 1960s. It was found that although the biomass cannot destroy metals, it can change their properties in sometimes amazing ways. Biological metal binding were found to be useful for biohydrometallurgy and biogeochemistry. In the 1970s the first description of bioaccumulation as a method of wastewater treatment was elaborated.

However, biosorption became as regular branch of science since the 1990s. A large contribution is made by Professor Bohumil Volesky from McGill University in Canada, who provided many theoretical basis of the process and also made first attempts to commercialize it.

However, further research has revealed that inactive/dead microbial biomass can passively bind metal ions via various physicochemical mechanisms. Along with this new finding, research on biosorption became active, with numerous biosorbents

of different origins being proposed for the removal of metals/dyes. Biosorption does not depend only on the type or chemical composition of the biomass, but also on the external physicochemical factors and solution chemistry. There are mechanisms responsible for biosorption, which may be one or combination of ion exchange, complexation, coordination, adsorption, electrostatic interaction, chelation and microprecipitation (Volesky & Schiewer, 1999).

## 1.2 Objectives

The aim of this project is to remove heavy metal ions from aqueous solutions by using the dry biomass of *Bacillus subtilis* (bacteria). The heavy metal of interest (sorbate) in this study is lead ions, Pb(II)

Generally, the main objective is to investigate the biosorption capacity of bacterial biomass to remove lead metal ions from aqueous solutions.

Specific objectives:

1. Determine adsorption capacity of dry biomass of *Bacillus subtilis*.
2. Evaluate the effect of pH on the adsorption process.
3. Determine the kinetics of the adsorption of lead ions on *Bacillus subtilis*.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Heavy metals in the environment

Heavy metals are on the forefront of academic and regulatory concern, since millions of gallons of water containing toxic heavy metals are generated annually from several metal processing industries and discharged into the environment. Metals discharged into water bodies are not biodegraded but undergo chemical or microbial transformations, creating serious impact on the environment and public health (Volesky & Holan, 1995).

Some of the heavy metals are dangerous to health or to the environment (e.g. mercury, cadmium, lead, chromium), some may cause corrosion (e.g. zinc, lead), some are harmful in other ways such like arsenic may pollute catalysts. There are thirteen elements listed as the highest concern heavy metals which are arsenic, cadmium, cobalt, chromium, copper, mercury, manganese, nickel, lead, tin, and thallium.

Heavy metals are defined as metals with a specific weight that is usually more than  $5.0 \text{ g/cm}^3$ . Of the 90 naturally occurring elements, 21 are non-metals, 16 are light-metals and the remaining 53 (with As included) are heavy-metals. Most heavy metals are transition elements with incompletely filled 'd' orbitals. These 'd' orbitals provide heavy-metal cations with the ability to form complex compounds which may or may not be redox-active. Thus, heavy metal cations play an important role as trace elements in sophisticated biochemical reactions (Nies, 1999). However,



if the heavy metal cations exist in the environment in abundance in a mobilized form, then the trace elements impose significant effects on the ecology contrary their important roles in the biochemical reactions.

The toxicity of heavy metals occurs even in low concentrations of about 1.0-100 mg/L. Thus, cleanup processes have been widely applied for the removal of heavy metal ions. The conventional technologies exist such as electro-winning, electro-coagulation, ion-exchange and reverse osmosis. However, the technologies are either not effective for the low metal concentrations or not economically feasible because the resins used for the ion-exchange and reverse osmosis are very expensive.

### **2.1.1 Lead**

Lead is a type of trace element listed in the periodical table. However, a trace element is considered essential if it meets the following criteria: it is present in all healthy tissues of living things; its withdrawal from the body induces, reproducibly, the same physiological and structural abnormalities regardless of the species studied; its addition either reverses or prevents these abnormalities; the abnormalities induced by its deficiency are always accompanied by pertinent, significant biochemical changes and these biochemical changes can be prevented or cured when the deficiency is corrected (Allinson, 1978).

However, lead is the most common of the heavy metals elements. It is a soft, malleable metal which is included in group of heavy metals. It has a lustrous silver-blue appearance when freshly cut, but darkens to a dull greyish colour when exposed to moist air (Buzzle.com, 2000). The average molecular weight of lead is 207.2 g/mol and has a low melting point of 327°C. Lead has been commonly used for thousands of years because it is widespread and most importantly, it resists corrosion.

There are some uses of lead for example, lead can be used as pure metal, alloyed with other metals, or as a chemical compound. The uses of lead are as follows:

- Major component of lead acid battery which is commonly used in car battery.
- As projectiles for fishing sinkers and firearms due to its low melting point and cost.
- Commonly used in polyvinyl chloride (PVC) plastic as a stabiliser that covers electrical cords.
- The base metal used for organ pipes and is mixed with variable amounts of tin to control the tone of the pipe.
- Commonly used in soldering and as electrodes in the process of electrolysis.

The harmful effects of lead relate to damage to nervous system, circulatory system, blood forming system, reproductive system, gastrointestinal tract and kidney. Lead enters the organism through inhalation by breathing, ingestion, swallowing, or absorption through the skin. The greatest danger from lead comes from its tendency to accumulate in the human organism while the central nervous system is the most sensitive to the effects of lead. (Jeanne, 1998)

## **2.2 Conventional wastewater treatment of processes**

There are a few conventional treatment methods which are widely applied through the past decades to clean up the hazardous toxic metals from the environment. The few typically used are precipitation, electrochemical cells, ion-exchange method, reverse osmosis as well as biological method.

Each of the conventional treatment has its own advantages and disadvantages.

**Table 2.1 The comparison between treatment technologies.**

Technology	Concentration dependence	pH	Suspended solids	Effluent Concentration (mg/l)	Regeneration	Sludge
Biosorption Hydroxide	Yes	Yes	Yes	<1	Yes	No
Precipitation Sulfide	No	No	Yes	2-5	No	Yes
Precipitation Ion	No	No	Yes	<1	No	Yes
Exchange	Yes	Some	No	<1	Yes	Yes
Evaporation Reverse	Yes	Yes	Yes	1-5	---	No
Osmosis	No	Some	No	1-5	No	No
Adsorption	Yes	Some	Yes	1-5	Yes	No

The typical conventional treatment methods for metal removal are shown in Table 2.1 including precipitation, ion-exchange method, electrochemical cells, reverse osmosis, and biological methods. As can be seen, the conventionally used methods are rather not efficient because most of the methods can only be applied to effluent concentrations higher than 1 mg/l. Thus, the biosorption technology is an outstanding method compared to the conventional cleanup processes due to the effluent concentration flexibility. Beside of this, biomass can be easily obtained from fermentation industries, for example, *Saccharomyces cerevisiae* which is widely used in food and beverage production is proven to be effective in removing heavy metal ions from wastewater (Long & Chen, 2006). In other words, biosorption is an economical technology and effective process in heavy metal ions removal as compared to ion-exchange due to the highly priced resins and also the production of sludge.

Biosorption is a process where the heavy metal ions or sorbates interact with biological materials are bound to cellular surfaces in the process called biosorption or become accumulated inside the cells via bioaccumulation (Chojnacka, 2010). In the natural environment, human being is not able to take control over these processes, but an application in the industrial practice under controlled operation conditions can be utilized to serve the purpose of protecting the well-being of environment from hazardous heavy metals. Biosorption and bioaccumulation can occur unintentionally in virtually all biological wastewater treatment processes but necessary research was carried out in the past decades to obtain the best biosorbents for cost-effectives applications for environmental cleanup (Volesky, 2007).

### **2.3 Biosorption and bioaccumulation processes.**

Bioaccumulation is the cultivation of an organism in the presence of pollutants which are to be removed (Chojnacka, 2010). There are four commonly pathways by which microbes accumulate heavy metals; binding to the cell surface, intracellular accumulation, extra-cellular precipitation and lastly, volatilization. Biosorption and bioaccumulation are mainly used for the removal of metal cations from the solutions. Both of the biosorption and bioaccumulation are able to remove unwanted sorbates, e.g. heavy metal ions from aqueous solutions. However the major difference lies in the type of biomass used. Biosorption utilizes dead biomass whereas bioaccumulation only happens on living cells.

Table 2.2 shows the major differences between biosorption and bioaccumulation in terms of cost, pH, temperature, maintenance/storage, selectivity, versatility, degree of metal uptake, rate of uptake, toxicant affinity, regeneration and reuse, and toxicant recovery.

#### **Table 2.2 Differences between biosorption and bioaccumulation**

(Source: Vijayaraghavan & Yun, 2008).

Features	Biosorption	Bioaccumulation
Cost	Usually low. Most biosorbents used were industrial, agricultural and other type of waste biomass.	Usually high. The process involves living cells and; hence, cell maintenance is cost prone.
pH	The solution pH strongly influences the uptake capacity of biomass. However, the process can be operated under a wide range of pH conditions.	In addition to uptake, the living cells themselves are strongly affected under extreme pH conditions.
Temperature	Since the biomass is inactive, temperature does not influence the process. In fact, several investigators reported	Temperature severely affects the process.
Maintenance/ Storage	Easy to store and use	External metabolic energy is needed for maintenance of the culture.
Selectivity	Poor. However, selectivity can be improved by modification/processing of Biomass	Better than biosorption
Versatility	Reasonably good. The binding sites can accommodate a variety of ions	Not very flexible. Prone to be affected by high metal/salt conditions.
Degree of uptake	Very high. Some biomass are reported to accommodate an amount of toxicant nearly as high as their dry weight	Because living cells are sensitive to high toxicant concentration, uptake is usually low.
Rate of uptake	Usually rapid. Most biosorption mechanisms are rapid.	Usually slower than biosorption. Since intracellular accumulation is time consuming.
Toxicant affinity	High under favourable conditions.	Depends on the toxicity of the pollutant.
Regeneration and reuse	High possibility of biosorbent regeneration, with possible reuse over a number of cycles.	Since most toxicants are intracellularly accumulated, the chances are very limited.
Toxicant recovery	With proper selection of elutant, toxicant recovery is possible. In many instances, acidic or alkaline solutions proved an efficient medium to recover toxicants.	Even if possible, the biomass cannot be utilized for next cycle.

Both of the biosorption and bioaccumulation can be defined as the ability of biological materials to accumulate heavy metals from wastewater through metabolically mediated or physico-chemical pathways of uptake, depending on the condition of biosorbent e.g. living or non-living biomass (Ahalya, et.al.,2003). Bioaccumulation mechanism happens when the biosorbent used is of living cells whereas biosorption happens when non-living biomass is used.

It can be seen from Table 2.3 that biosorption is a simple physicochemical process with similar concept of conventional adsorption or ion exchange and it is a metabolically-passive process. As oppose, to bioaccumulation process which goes further. First stage is biosorption and then the subsequent stage occurs, related to transport of pollutant (mainly via energy-consuming active transport systems) inside the cells and eventually the metal concentration within the cell increases.

**Table 2.3 Comparison of biosorption and bioaccumulation processes.**

<b>Biosorption</b>	<b>Bioaccumulation</b>
Passive process	Active process
Biomass is not alive	Biomass is alive
Metals are bound with cellular surface	Metals are bound with cellular surface and interior
Adsorption	Absorption
Reversible process	Partially reversible process
Nutrients are not required	Nutrients are required
Single-stage process	Double-stage process
The rate is quick	The rate is slow
Not controlled by metabolism	Controlled by metabolism
No danger of toxic effect	Danger of toxic effects caused by contaminants
No cellular growth	Cellular growth occurs
Intermediate equilibrium concentration of metal ions	Very low equilibrium concentration of metal ions

In bioaccumulation more binding sites for the pollutants are available and lower residual concentrations can be reached. If biosorption and bioaccumulation processes are to be performed under laboratory conditions, the biomass should be suspended in the solution containing a sorbate at the first stage. Then the separation of pollutant-laden biomass after a couple of hours and the equilibrium would be reached. If heterotrophic organisms (bacteria or fungi) are intended to be used, organic carbon source should be supplied to wastewater (Chojnacka, 2010).

In bioaccumulation it is possible to reach lower residual concentration of sorbate because a cell offers binding sites on the surface and inside the cell. In bioaccumulation a part of the sorbate is transported into the cell releasing binding sites present on the surface, so additional amount of sorbate can be bound there according to the course of the equilibrium biosorption dependence.

## **2.4 Biosorption**

Biosorption is the result of the search for new nature based technologies that involve the removal of toxic metals from wastewater. It is a process that utilizes inexpensive dead biomass to sequester toxic heavy metals and is particularly useful for the removal of contaminants from industrial effluents (Volesky, 2007). Biosorption, as the name suggests, the prefix 'bio' means that the sorbent is of biological origin, and it is a surface biological matrix. Thus, the sources of biosorbents are abundant naturally and/or adsorbents can be prepared from waste biomass of algae, fungi or bacteria.

Biosorption process involves a solid phase (sorbent or biosorbent; biological material) and a liquid phase (solvent, normally water) containing a dissolved species to be sorbed (sorbate, metal ions). According to Volesky (2007), biosorption happens due to the higher affinity of the sorbent for the sorbate species, thus, the latter is attracted and bound there by different mechanisms. A variety of uptake mechanisms are involved in biosorption process including adsorption and ion exchange. The

process continues until equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution. The degree of sorbent affinity for the sorbate determines its distribution between the solid and liquid phase.

There are numerous advantages of the biosorption process, including low operation costs (if low-cost sorbents are used), low quantity of sewage sludge disposed as compared to conventional cleanup methods. The process is simple in operation and very similar to conventional adsorption or ion-exchange, except that sorbent of biological origin is employed and cheaper in price compared to the resins used in ion-exchange process. The section below shows the advantages and disadvantages of biosorption in further details.

#### *Advantages of biosorption*

- Non-living cells are less sensitive to metal ion concentration (toxicity effects).
- Can be operated at ambient conditions of pH and temperature.
- Low operating cost.
- Volume of chemical or biological sludge can be minimized.
- Supply of nutrients is not required.
- Dead biomass can be procured from industrial sources as waste product from the fermentation processes.

#### *Disadvantages of biosorption*

- Biosorption can be a problem when metal interactive sites are occupied, metal desorption is necessary prior to further use.
- The potential for biological process improvement is limited because cells are not metabolizing. Because production of the adsorptive agent occurs during pre-growth, there is not biological control over characteristic of biosorbent. This will be particularly true if waste biomass from a fermentation unit is being utilized.
- There is no potential for biologically altering the metal valency state.



The complex structure of microorganisms implies that there are quite many ways for the metal to be taken up by the microbial cell. The biosorption mechanisms are various and are not fully understood. They may be classified according to various criteria, depending on the cell's metabolism, biosorption mechanisms can be divided into two categories which are metabolism dependent and non-metabolism dependent.

Transport of the metal across the cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism. This means that this kind of biosorption may take place only with living cells. It is often associated with an active defence system of the microorganisms, which "behave" actively in the presence of toxic metal.

Basically biosorption by living organisms comprises of two steps. Firstly, metabolism independent binding where the metals are bound to the cell walls and then metabolism dependent intracellular uptake, whereby metal ions are transported across the cell membrane. There are few mechanisms involved in the biosorption process, for example the physical adsorption, ion-exchange, complexation and precipitation. The section below contains the definitions of each mechanism and some relevant literatures review data .

#### *Physical adsorption*

In this category, physical adsorption takes place with the existence of van der Waals' forces. Kuyucak and Volesky (1988), hypothesized that uranium, cadmium, zinc, copper and cobalt biosorption by dead biomasses of algae, fungi and yeasts takes place through electrostatic interactions between the metal ions in solutions and cell walls of microbial cells. Electrostatic interactions have been demonstrated to be responsible for copper biosorption by bacterium *Zoogloea ramigera* and alga *Chlorella vulgaris* (Aksu & Kutsal, 1992) and for chromium biosorption by fungi *Ganoderma lucidum* and *Aspergillus niger* of the same place it here.

#### *Ion Exchange*

Cell walls of microorganisms contain polysaccharides and bivalent metal ions exchange with the counter ions of the polysaccharides. For example, the alginates of

marine algae occur as salts of  $K^+$ ,  $Na^+$ ,  $Ca^{2+}$ , and  $Mg^{2+}$ . These ions can exchange with counter ions such as  $CO_3^{2-}$ ,  $Cu^{2+}$ ,  $Cd^{2+}$ , and  $Zn^{2+}$  resulting in the biosorption uptake of heavy metals (Kuyucak & Volesky, 1988). The biosorption of copper by fungi *Ganoderma lucidium* and *Aspergillus niger* was also investigated by ion exchange mechanism (Muraleedharan & Venkobachar, 1988).

#### *Complexation*

The metal removal from solution may also take place by complex formation on the cell surface after the interaction between the metal and the active groups. Aksu et al. (1992) hypothesized that biosorption of copper by *Carlina vulgaris* and *Zoogloea ramigera* takes place through both adsorption and formation of coordination bonds between metals and amino and carboxyl groups of cell wall polysaccharides. Complexation was found to be the only mechanism responsible for calcium, magnesium, cadmium, zinc, copper and mercury accumulation by *Pseudomonas syringae* (Veglio & Beolchini, 1997). Microorganisms may also produce organic acids for example like citric, oxalic, gluonic, fumaric, lactic and malic acids, which may chelate toxic metals resulting in the formation of metallo-organic molecules. These organic acids help in the solubilisation of metal compounds and their leaching from their surfaces. Metals may be biosorbed or complexed by carboxyl groups found in microbial polysaccharides and other polymers.

#### *Precipitation*

Precipitation may be either dependent on the cellular metabolism or independent of it. In the former case, the metal removal from solution is often associated with active defence system of the microorganisms. They react in the presence of toxic metals producing compounds, which favour the precipitation process. In the case of precipitation not dependent on the cellular metabolism, it may be a consequence of the chemical interaction between the metal and the cell surface. The various biosorption mechanisms mentioned above can take place simultaneously.

## 2.5 Bacterial biosorbent

Strong biosorbent behaviour of certain types of microbial cells towards metal ions is a function of the chemical makeup of the microbial cells of which it consists. This aspect is particularly important when it comes to the process application, whereby new biosorbents respective “chemicals” are capable of sequestering a relatively large amount of the metals (Volesky, 1987). Some types of biosorbents could have broad range binding of the majority of heavy metals with no specific priority, while others can even be very specific for certain types of metals.

**Table 2.4 Removal of heavy metals and reported removal efficiencies for different microorganisms.**

Organism Used	Metals removed	System	Reported efficiency and application
<i>Pseudomonas fluoresces</i>	Pb, Zn	Immobilized on PVC & Packed in columns	Used in Hungarian chemical Company
<i>Pseudomonas aeruginosa</i>	Uranium, Plutonium	Immobilized in plasma treated Polypropylene	75-80% removal
<i>Citrobacter sp.</i>	Cd, Pb, Cu, U	Immobilized on PAG	80-90% removal
<i>Bacillus subtilis</i>	Pb, Zn, Cu, Ni, Cd,	Fixed Reactor	AMT-Biocclaim
<i>Saccharomyces sp.</i>	Hg, Ag, Au, Pd		Tm 98% removal
<i>Streptomyces sp.</i>	Uranium	Trpped in silica gel matrix	80-100% removal
<i>Viridochromogenes sp.</i>			
<i>Rhizopus arrhizus</i>	Cr, Fe, Cu, U	-----	50-60% removal
<i>Dead algae Barkley, 1991</i>	Hg	Sorption column	95uptake Alga SORBTM being used
<i>Sargassum natans</i>	Pb, Cd, Cr	-----	3times more efficient than ion exchange resin
<i>S. flutions</i>	Cu		80-90% removal
<i>Aspergillus niger, Penicillium chrysoginum</i>	Ag, Zn		---
<i>Aspergillus oryzae</i>	Cd	Immobilized reticulated foam	95% removal

The investigation of the efficacy of the metal uptake by the microbial biomass is essential for the industrial application of biosorption, as it gives information about the equilibrium of the process which is necessary for the design of the equipment and also the most effective biosorbents to be utilized in large-scale process.

## 2.6 Taxonomy of bacteria

Bacteria is a major group of unicellular living organisms belonging to the prokaryotes, which are ubiquitous in soil and water, and also are symbionts of other organisms (Vijayaraghavan. & Yun, 2008). The most common bacteria can be present in three basic shapes: spherical or ovoid (*Coccus*), rod (*Bacillus*, with a cylindrical shape), spiral (*Spirillum*) and filamentous (*Sphaerotilus*). Although there is a great variety of shapes due to differences in genetics and habitat ecology. Bacteria vary in size as much as in shape. For many prokaryotes, the cells remain together in groups or clusters after division (pairs, chains, tetrads, clusters, etc.) (Wang & Chen, 2009).

*Eubacteria* is a prokaryotic form of life with a set of characters that unite its extraordinarily diverse taxon (description of the domain eubacteria). All known bacterial pathogens are *Eubacteria* and they have a relatively simple cell structure, which lack cell nuclei but possess cell walls (Salton, 1964). Cell size is an important characteristic for an organism. Small size of bacteria is very important because size affects a number of biological properties. Small size of bacteria ensures rapid metabolic processes.

Most of the bacteria size range from 0.2-2.0  $\mu\text{m}$  (micrometers) in diameter. A typical bacterial cell contains cell wall, cell membrane, cytoplasmic matrix consisting of several constituents, which are not membrane-enclosed: inclusion bodies, ribosome and the nucleoid with its genetic material (Wang & Chen, 2009).

Main functions of bacterial cell wall include:

- (1) The cell wall gives cell shape and protects it from osmotic lysis;
- (2) The wall can protect cell from toxic substances;
- (3) The cell wall offers the site of action for several antibiotics.
- (4) The cell wall is necessary for normal cell division.
- (5) The transport processes to/from cell.

### 2.6.1 Gram '+' and Gram '-' bacteria

Gram was a scientist who invented a technique called Gram staining by which bacteria can be colorized and divided into two groups based on the chemical, primarily the presence of high levels of peptidoglycan, and physical properties of their cell walls (William, 1997).

#### *Gram positive*

Gram positive bacteria are those bacteria having thick layer of peptidoglycan over inner cytoplasmic membrane and able to resist penetration by stains, therefore appear to be satin purple in colour.

#### *Gram negative*

Gram negative bacteria stain pink, and their peptidoglycan layer of the cell wall is much thinner as compared to Gram positive all and it is located between space of the outer and inner cytoplasmic membrane cell wall containing lipopolysaccharides. These bacteria appear to be pink to red in colour.

The bacterial cell wall provides structural integrity to the cell, but differs from that of all other organisms due to the presence of peptidoglycan (poly-N-acetylglucosamine and N-acetylmuramic acids), which is located immediately

outside of the cytoplasmic membrane (Rogers et. al., 1980). Peptidoglycan contains several different amino acids, three of which are D-glutamic acid, D-alanine, and mesodiaminopimelic acid that are not found in proteins (Wang & Chen, 2009). Peptidoglycan is responsible for the rigidity of the bacterial cell wall and determines the cell shape (Vikayaraghavan & Yun, 2008).

**Table 2.5 The chemical constituents in the cell walls and envelopes of Gram-positive and Gram-negative bacteria.**

<b>Gram-positive cell walls</b>	<b>Examples</b>
Peptidoglycan	All species
Polysaccharides	Streptococcus group A,B,C substances
Teichoic acids	
Ribitol	<i>Staphylococcus Aureus</i> <i>Bacillus subtilis</i> <i>Lactobacillus</i> spp.
Glycerol	<i>B.licheniformis</i> <i>M.lysodeikticus</i>
Teichuronic acids (aminogalacluronic or aminomannuronic acid polymers)	
Peptidoglycolipids (muramylpeptide-polysaccharide-mycolates)	<i>Corynebacterium</i> spp. <i>Mycobacterium</i> spp. <i>Nocardia</i> spp.
Glycolipids (“Waxes”) (polysaccharide-mycolates)	
<b>Gram-negative envelopes</b>	
LPS (Lipoteichoic acids)	All species
Lipoprotein	<i>E.coli</i> and many enteric bacteria <i>Pseudomonas aeruginosa</i>
Porins (major outer membrane proteins)	<i>E.coli Salmonella typhimurium</i>
Phospholipids and proteins	All species
Peptidoglycan	Almost all species.

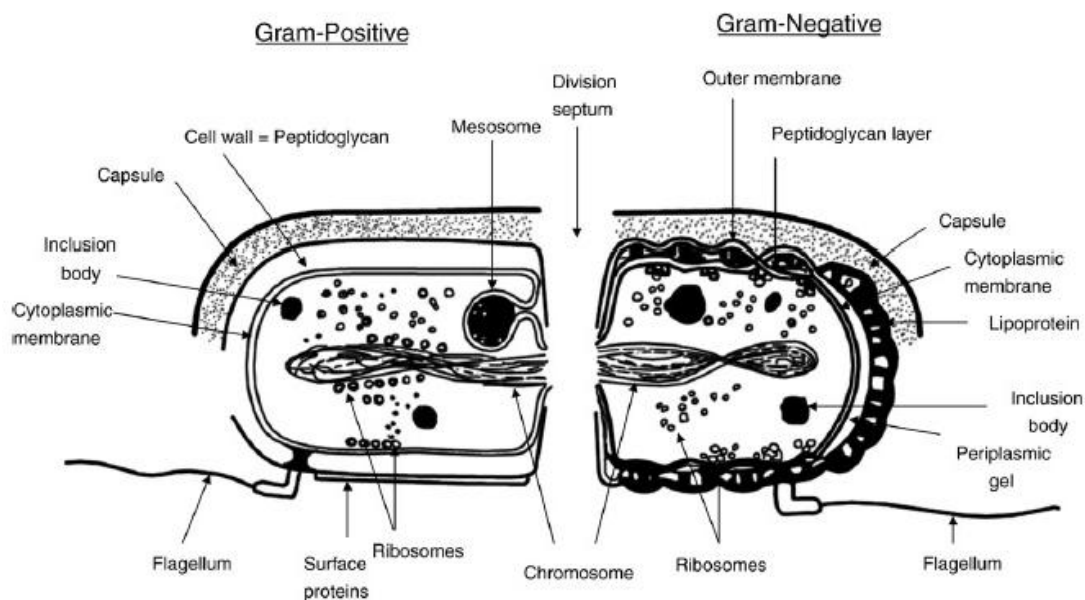


Figure 2.1 Structures of Gram-positive and Gram-negative bacteria.

Figure 2.1 is a schematic diagram showing the general structures of Gram-positive and Gram-negative bacteria. From the figure, the Gram-positive bacteria are comprised of a thick peptidoglycan layer connected by amino acid bridges. Polyalcohols are imbedded in the gram-positive cell wall, known as teichoic acids, which are lipid-covalently linked to lipids within the cytoplasmic membrane. The teichoic acids give the Gram-positive cell wall an overall negative charge, due to the presence of phosphodiester bonds between the teichoic acid monomers (Sonnenfeld, et. al., 1996). In general, 90% of the Gram-positive cell wall is comprised of peptidoglycan.

The Gram-negative bacteria can be differentiated from the Gram-positive's bacteria in several ways. Firstly, they are much thinner and composed of only 10-20% of peptidoglycan. Besides, the cell wall contains an outer membrane composed of phospholipids and lipopolysaccharides. The highly charged nature of lipopolysaccharides confers an overall negative charge on the Gram-negative cell wall (Vikayaraghavan & Yun, 2008).

## 2.7 *Bacillus subtilis*

In 1835, this organism originally was named as *Vibrio Subtilis*. In 1872, this organism was renamed *Bacillus subtilis*. There are some other names that are used for this strain of bacteria, which are *Bacillus uniflagellatus*, *Bacillus globigii*, and *Bacillus natto*. *Bacillus subtilis* bacteria were one of the first bacteria to be studied and was the first Gram-positive bacteria to be sequenced. The sequence was published in Nature Journal in November 1997 (William, 1997).

The genus *Bacillus* consists of a large number of diverse, rod-shaped Gram positive (or positive only in early stages of growth) bacteria that are motile by peritrichous flagella and are aerobic. According to Masud Hossain and Anantharaman (2006), *Bacillus subtilis* has a well-studied Gram positive wall and rod in- shape.

*Bacillus subtilis* grows in the mesophilic temperature range with optimal temperature between 25-35°C. Appropriate growth media is nutrient agar and nutrient broth. Stress and starvation are common in their environment, therefore, *Bacillus subtilis* has evolved a set of strategies that allows survival under such harsh conditions.

*Bacillus subtilis* is one of the most widely used bacteria for the production of enzymes and especially organic chemicals. Industrial applications include production of amylase, protease, inosine, ribosides and amino acids.

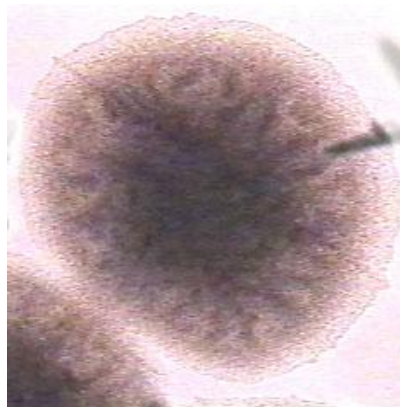


Figure 2.2 Sample image of *Bacillus subtilis* in a colony



*Bacillus subtilis* produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to the nutrients cycling (Earl et. al., 2008). *Bacillus subtilis* bacteria use their flagella for a swarming motility. This motility occurs on surfaces for example on agar plates, rather than in liquids. *Bacillus subtilis* are arranged in single colonies or chains. Cells arranged next to each other can only swarm together, not individually. These arrangements of cells are called 'rafts'. In order for *Bacillus subtilis* bacteria to swarm, the cells need to secrete a slime layer which includes surfactin, a surface-tension-reducing lipopeptide, as one of its components.

*Bacillus subtilis* has also been shown to produce a wide variety of antibacterial and antifungal compounds. It produces novel antibiotics such as *difficidin* and *oxydifficidin* that have activity against a wide spectrum of aerobic and anaerobic bacteria as well as more common antibiotics such as *bacitracin*, *bacilli* and *bacillomycin*. The use of *Bacillus subtilis* as biocontrol agent of fungal plant pathogens is being investigated because of the effects of antifungal compounds on *Monilinia fructicola*, *Aspergillus flavus* and *Aspergillus parasiticus* and *Rhizoctonia* (Bacillus subtilis Final Risk Assessment, 2011).

*Bacillus subtilis* contains catalase enzymes that responsible for the catalysis of the decomposition of hydrogen peroxide to water and oxygen, and superoxide dismutase, an enzyme that catalyzes the breakdown of superoxide into oxygen and hydrogen peroxide, respectively.

Historically, prior to the monographs by Smith in 1946 and 1952, *Bacillus subtilis* was a term given to all aerobic endospore-forming bacilli (Bacillus subtilis Final Risk Assessment, 1997). Most of the members of the genus, *Bacillus subtilis* have been considered strictly aerobic, this means that they require oxygen to grow and they cannot undergo fermentation. However, it can indeed grow in anaerobic conditions making them facultative aerobes.

The bacteria can make ATP (Adenosine triphosphate) in anaerobic conditions via butanediol fermentation as well as nitrate ammonification. *Bacillus subtilis* can

use nitrite as a terminal acceptor of electrons. *Bacillus subtilis* contains two unique nitrate reductases. One of it is used for nitrate nitrogen assimilation and the other one is used for nitrate respiration. Somehow, there is only one nitrite reductase that serves both purposes. Nitrate reductase reduces nitrate to nitrite in nitrate respiration, which is then reduced to ammonia by nitrite reductase.

The *Bacillus* spp. such as *subtilis*, *licheniformis* and *pumilus* are closely related and there has been difficulty distinguishing among the three species that historically were grouped together as the *subtilis*-group or *subtilis* spectrum (US Environmental Protection Agency, 1997). These three species clustered together (78%) in the ‘*subtilis*’ group in a numerical classification based on 118 unit characteristics of 368 strains of *Bacillus*.

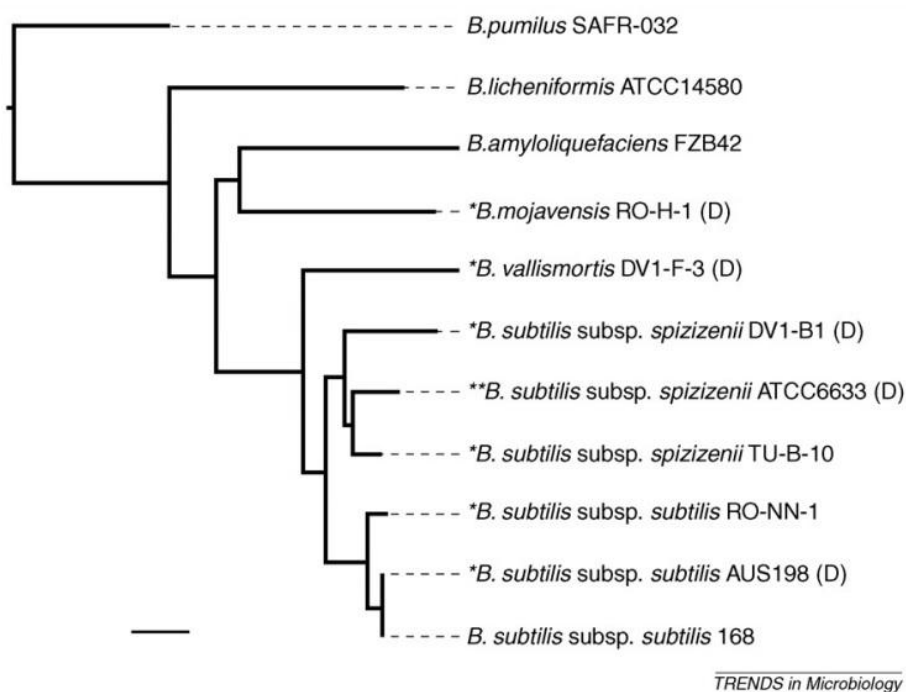


Figure 2.3 The relationship among sequenced strains of *B.subtilis*.

Figure 2.3 shows the neighbour-joining tree representing the relationship among sequenced strains of *B. subtilis* and its close relatives. According to Earl et al., (2008) this tree was generated from the alignment of partial *gyrA* sequences from each strain. (*gyrA* is an essential gene. Full name is gyrase subunit A)

However, this major cluster contained four subclusters that could be identified as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilis*, and *Bacillus amyloliquefaciens*. It is possible to differentiate *Bacillus subtilis* from *Bacillus licheniformis* and *Bacillus pumilis* by the use of pyrolysis-gas chromatography or by the use of API 20E and 50E system tests (Earl et. al., 2008).

*Bacillus subtilis* appears to have a low degree of virulence to humans. It does not produce significant quantities of extracellular enzymes or possess other virulence factors that would predispose it to cause infection. There are a number of reports where *Bacillus subtilis* has been isolated from human infections.

*Bacillus subtilis* is not a human pathogen, nor is it toxigenic like some other members of the genus. The virulence characteristics of the microorganism are low. The number of microorganisms challenging the individual must be very high or the immune status of the individual very low in order for infection with *Bacillus subtilis* to occur (Bacillus subtilis Final Risk Assessment, 2011).

### **2.7.1 Factors affecting biosorption**

The following factors are found to have effect on the biosorption process:

- Temperature seems not to influence the biosorption performances in the range of 20-35°C (Aksu & Kutsal, 1992).
- pH is the most important parameter in the biosorption process: it affects the solution chemistry of the metals, the activity of the functional groups in the biomass and the competition of metallic ions (Galun et al., 1987).
- Biomass concentration in solution influences the specific metal uptake. Usually for lower values of biomass concentrations there is an increase in the specific uptake.
- Biosorption is mainly used to treat wastewater where more than one type of metal ions present; the removal of one metal ion may be influenced by the

presence of other metal ions. For example: uranium uptake by biomass of bacteria, fungi and yeasts was not affected by the presence of manganese, cobalt, copper, cadmium, mercury and lead in solution (Sakaguchi & Nakajima, 1991).

According to Vijayaraghavan and Yun (2008), the cell wall of bacteria is the first component that comes into contact with metal ions/dyes, where the solutes can be deposited on the surface or within the cell wall structure. In such cases the mode of metal ions uptake is considered to be extracellular since biosorption process utilizes dead/inactive cells, thus the chemical functional groups of the cell wall play vital roles on a principle of ion-exchange. There are several functional groups are present on the bacterial cell wall, including the carboxyl, phosphonate, amine and hydroxyl groups (Doyle et.al., 1980).

Metal uptake by functional groups present in bacterial cell wall happens based on physical adsorption, ion exchange and chemical sorption, which is not dependent on the cells' metabolism. This functional groups act as metal binding groups and the process is relatively rapid and can be reversible (Kuyucak & Volesky, 1988). For example, the carboxyl groups which are negatively charged and abundantly available in bacterial cell wall actively involved in the binding of metal cations. Golab and Breitenbach (1995) indicated that the carboxyl groups of cell wall peptidoglycan of *Streptomyces pilosus* were responsible for the binding of copper, Cu(II).

## CHAPTER 3

### METHODOLOGY

This chapter contains information about with the cultivation of the bacteria *Bacillus subtilis* to obtain its biomass. Then the biomass is placed into a series of flasks with known concentrations of metal ions at different pH values ranging from 1 to 6. The final metal concentrations from the biosorption studies are measured using ICP-OES Optical Emission Spectrometer (Optima 7000DV; Perkin Elmer, Uberlingen, Germany).. Finally the results were analysed using adsorption isotherm models such like Langmuir and Freundlich. Meanwhile, the kinetic of the biosorption is quantified by the pseudo-first order and pseudo-second order equation.

#### 3.1 Preparation of biosorbent material

The nutrient broth for bacterial growth (Merck kGaA; Germany) is used for the culture medium of biosorbent. 8g of nutrient broth (Merck kGaA, Germany) is weighed by using weighing machine. Then the nutrient broth is dissolved in a bottle together with 1L of deionised water. The bottle is then covered with cotton and aluminium foil and autoclaved for 15 minutes at 121°C. The autoclaved nutrient broth is then cooled overnight to room temperature (27°C).

Bacteria strain of *Bacillus subtilis* was obtained from the Department of Sciences (UTAR). The cultivation is carried out using shake-flask method (Pauli & James, 1996). Bottle with the nutrient broth is placed into a laminar flow machine for the inoculation of agar based *B. subtilis* strain into the nutrient solution. 20-25 scopes

were scrubbed into the solution and covered by cotton and aluminium foil. The 1L bottle is placed on the Orbital shaker (SSL1; Stuart®) at 220 ppm, 27°C for 24~48 hours.

The cultivated bacteria in the scotch bottle is funnelled into centrifugal tubes and centrifuged at 10,000 rpm and 4 °C. The centrifuged bacteria were then scooped and placed on filter paper for drying purpose. The bacteria were dried after filtration, the filter paper (pore size 0.45µm) with the bacteria is placed in the oven (Beschickung-Loading Modell 100-800; Memmert) at 80°C overnight.

### **3.2 Preparation of stock solution**

0.1M stock solution is prepared by using lead (II) nitrate  $\text{Pb}(\text{NO}_3)_2$  (R & M marketing Essex U.K. with the  $M=331.20\text{g/mol.}$ ). For 500 ml of stock solution, 16.56 g of lead (II) nitrate powder needed to dissolve in 500 ml of deionized water. The amount of powder is calculated by using dilution equation.

### **3.3 Preparation of blank solution**

0.15M blank solution is prepared by using the Sodium Nitrate ( $\text{NaNO}_3$ ) (Merck kGaA Darmstadt Germany,  $\text{MW} = 84.99\text{g/mol.}$ ). To prepare 5L of Blank solution, 63.75g of sodium nitrate powder need to dissolve in 5L of deionized water. The amounts of powder require is calculate by using dilution equation.

### **3.4 pH measurement and adjustment.**

The pH of metal ion solution was measured using pH meter. Initial pH of each metal ion solution was adjusted to the required pH value by using either 0.1 M of HCl or 0.1 M of NaOH for pH 4 to 6, and 1M of HCl for pH 1 to 3. The initial pH of lead metal ion solution usually was in the range of pH4 to 5.

### 3.5 Metal uptake

Uptake of metal ions was calculated from a metal mass balance yielding:

$$q = \frac{V(C_i - C_f)}{m} \quad (3.1)$$

where,

$q$  = mg metal ions per g dry biosorbent.

$V$  = the reaction volume (l)

$C_i$  and  $C_f$  are the initial and residual metal concentrations (mg/l)

$m$  = amount of dry biosorbent (g).

The concentration of the metal ions was determined by using Optical Emission Spectrometer (Optima 7000DV; Perkin Elmer, Uberlingen, Germany).

The efficiency of heavy metal removal was calculated from the amount of metal ions adsorbed on the biosorbent and the amount of metal ions available in the synthetic solution as the following equation.

$$\text{Percentage removal} = \frac{\text{mg heavy metal ions removed}}{\text{mg heavy metal ions available}} \times 100 \quad (3.2)$$

### 3.6 Metal-tolerance levels

*Bacillus subtilis* absorption capacity was tested with different concentrations and pH value for the metals (lead). The experimental solutions were prepared by supplementing synthetic solution with the concentration of 5, 10, 15, 25, 35, 45, 55, 70, 85, 100, 150, 200, and 250 ppm for lead. All concentrations of the metals ion were obtained by dilution of the stock solution.

### 3.7 Equipment used



Figure 3.1 ICP-OES Optical Emission Spectrometer (Optima 7000DV; Perkin Elmer, Uberlingen, Germany).



Figure 3.2 Oven (Beschickung-Loading Modell 100-800; Memmert)





Figure 3.3 Orbital shaker (SSL1; Stuart®)

### 3.8 Batch isotherm study

To determine the metal biosorption capacity, batch biosorption experiments were conducted dry using *Bacillus subtilis* biomass. 0.1 g of biomass was suspended in solutions containing different initial metal ion concentrations in a series of flasks and shaken in an incubator shaker at 30°C and 225 rpm for 24 hours. The samples were collected and filtered by using filter paper (pore size 0.45 µm). The final concentrations of metal ions filtrates were determined using Optical Emission Spectrometer (Optima 7000DV; Perkin Elmer, Uberlingen, Germany).

#### 3.8.1 Langmuir isotherm model

Langmuir theoretically examined the adsorption of gases on solid surfaces and considered sorption as a chemical phenomenon, which show the Langmuir isotherm equation has a hyperbolic form:

$$Q_e = Q_{th}^e \frac{K_{eq} C_e}{1 + K_{eq} C_e} \quad (3.3)$$

Langmuir equation can be transform to the linearized form:

$$\frac{1}{Q_e} = \frac{1}{Q_{th}^e} + \frac{1}{(Q_{th}^e K_{eq}) C_e} \quad (3.4)$$

where,

$Q_e$  = the adsorption capacity by weight at equilibrium.

$Q_{th}^e$  = the theoretical maximum adsorption capacity by weight.

$K_{eq}$  = represent the equilibrium constant of adsorption reaction

$C_e$  = concentration of adsorbate at equilibrium.

(Langmuir, 1918)

Langmuir isotherm is also known as single-sorbate isotherm. This isotherm equation is most frequently applied in equilibrium study of biosorption. However, it should be realized that the Langmuir isotherm offers no insight into the mechanism aspects of biosorption (Liu & Liu, 2008). In the simplest case the following assumptions are valid:

- Fixed number of adsorption sites; at equilibrium, at any temperature and gas pressure a fraction of the surface sites  $\theta$  is occupied by adsorbed molecules, and the fraction  $1-\theta$  is free.
- All sorption sites are uniform (i.e. constant heat of adsorption)
- Only one sorbate
- One sorbate molecule reacts with one active site
- No interaction between sorbed species.

Assumption of a value for the surface area covered per molecule then could allow computation of the active specific surface area of the sorbent using Avogadro's number. However, the concept of "surface area" cannot be used in gel-like sorbents that most biosorbents may be.

### 3.8.2 Freundlich isotherm equation

Freundlich proposed an empirical isotherm equation. The general form of this model is:

$$Q_e = k_F C_e^{\frac{1}{n_F}} \quad (3.5)$$

This can be linearized by taking logarithm of both sides of the equation to give:

$$\log Q_e = \log k_F + \frac{1}{n_F} \log C_e \quad (3.6)$$

where,

$k_F$  and  $n_F$  are Freundlich constants.

(Freundlich, 1907)

As the Freundlich isotherm equation is exponential, it can only be reasonably applied in the low to intermediate concentration ranges. Similar to the Langmuir equation, Freundlich isotherm equation also have been widely employed in biosorption research (Liu & Liu, 2008).

### 3.9 Biosorption kinetics studies

Batch kinetic studies were carried out to determine the equilibrium time which is defined as the time needed to reach equilibrium. Batch kinetic studies were conducted at pH 1 to 6 range with the same concentrations of metal ions in the batch biosorption study. The initial concentration was adjusted to approximately 50 mg/L

while the reaction mixture pH was not controlled after the initial of batch kinetic experiments. 0.05 g of dried biomass was added to the solutions in 125mL Erlenmeyer flasks. The samples were placed on a shaker at 225 rpm. Samples were collected at every 20 min in the 1st hour, 30 min in the 2nd hour, and the hourly in the 48 hours period. Upon collection, samples were immediately filtered by using filter paper (pore size 0.45 $\mu$ m). The final pH values were measured and concentrations of the filtrates were then determined using ICP.

There are two kinetic models: pseudo-first- and second-order reactions. These two equations have been widely used to describe biosorption data obtained under non-equilibrium conditions. Almost all of the biosorption kinetic studies, both pseudo-first- and second- order kinetic equations have been commonly employed in parallel, and one is often claimed to be better than another according to marginal difference in correlation coefficient (Liu & Liu, 2008).

### 3.9.1 Pseudo-first-order equation

The pseudo-first-order kinetic equation also call as Lagergren (1898) equation is show at below.

$$\frac{dQ_t}{dt} = k_1'(Q_s - Q_t) \quad (3.7)$$

where,

$Q_t$  is the amount of adsorbate adsorbed at time t,

$Q_s$  is the value at equilibrium,

$k_1'$  is constant.

The pseudo-first-order Lagergren equation is indeed in line with the concept of linear driving force (Lagergren, 1898). After linearization, the equation is shown as.

$$\log(q_e - q_t) = \log(q_e) - \frac{k_1 t}{2.303} \quad (3.8)$$

### 3.9.2 Pseudo-second-order equation

The pseudo-second-order kinetic equation has been frequently employed to analyze biosorption data obtained from various experiments using different adsorbates and biosorbents (Liu & Liu, 2008).

$$\frac{dQ_t}{dt} = k'_2 (Q_e - Q_t)^2 \quad (3.9)$$

where,

$$k'_2 = \text{constant}$$

(Ho & McKay, 1999)

After linearized,

$$\frac{t}{q} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \quad (3.10)$$

Where  $q$  and  $q_e$  are the amount of metal adsorption per unit weight of biosorbent (mg/l) at time  $t$ , and at equilibrium respectively, and  $k_1$  and  $k_2$  are the adsorption rate constants.

## CHAPTER 4

### RESULTS AND DISCUSSIONS

This chapter presents the results of the experiments conducted using different initial lead ions concentrations and pH of synthetic solutions. The performance of the biosorbent in the metal ions uptake was evaluated using two isotherm models which are Langmuir and Freundlich. Also the kinetic studies based on pseudo first-order and pseudo second-order reaction models have been carried out. Evaluation of the effect of initial pH and the contact time on the percentage of lead uptake is also presented.

#### **4.1 Batch biosorption isotherm studies**

The biosorption of the metal ions adsorption on the surface of bacterial cell wall is typically represented by the Langmuir and Freundlich isotherm models. The batch adsorption experimental data were fit to the Langmuir and Freundlich linearized equations described in section 3.8 (page 28). Biosorption process availing batch technique indicates the interaction between metal ions and biosorbent.

##### **4.1.1 Freundlich isotherm**

The Freundlich isotherm is an empirical model that relates the adsorption intensity of the sorbent to the biosorbent. The isotherm is adopted to describe reversible adsorption and is not restricted to monolayer formation.

A plot of  $\log Q_e$  versus  $\log C_e$  gives a straight line with slope  $\frac{1}{n}$  and intercept  $\log k_F$ . The value of  $k_F$  and  $n_F$  along with the linear regression co-efficient ( $R^2$ ) was observed from Freundlich isotherm model.

Figure 4.1 shows adsorption isotherms obtained based on Freundlich model for the solutions of different pH. The comparison of the isotherms (Freundlich, 1907).

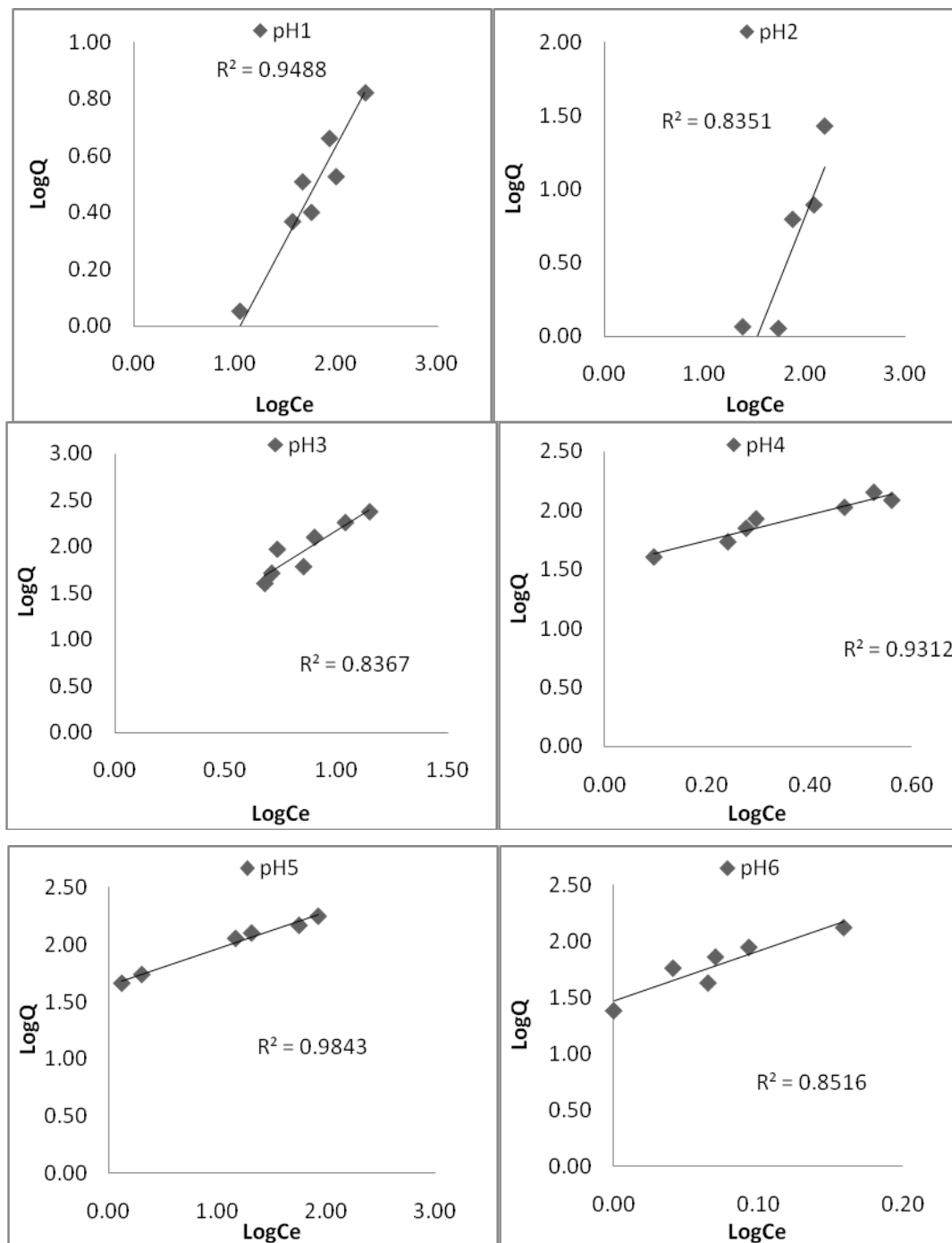


Figure 4.1 Freundlich isotherms for *Bacillus subtilis* at different pHs.

Table 4.1. shows the regression coefficients obtained for Pb(II) adsorption for Freundlich model. Higher values of the correlation coefficients indicate good fit of the model equation. Greater values of  $n_F$  indicate the affinity of biosorbent to investigated metals and imply strong binding of metal ions.

**Table 4.1** Freundlich isotherm parameters for lead adsorption on *Bacillus subtilis*.

pH	Freundlich constant		
	$K_F$	$n_F$	$R^2$
1	n/a	1.4988	0.9488
2	n/a	0.5864	0.8351
3	n/a	0.6698	0.8367
4	0.4211	0.9088	0.9312
5	0.4996	3.1368	0.9843
6	0.3803	0.2248	0.8516

Footnote: n/a = not available.

#### 4.1.2 Langmuir isotherms

Figure 4.2 shows linear plots of Langmuir adsorption isotherms obtained at different pH of lead solutions (Langmuir, 1918).

The  $Q_{th}^e$  and  $K_{eq}$  are estimated by plotting  $1/Q_e$  against  $1/C_e$ . The model simulations along with experimental observations for lead adsorption with the experimental values of  $Q_{th}^e$  and  $K_{eq}$  along with the linear regression co-efficient ( $R^2$ ) are given in

Table 4.2. The data show that the constant value (K) for pH 4 to pH 6 are in the range of 0.23 to 0.33. pH 6 are observed to have highest  $Q_{th}^e$  compared to other pHs



solution. This shown that the *Bacillus subtilis* prefers pH range closer to its natural environment rather than acidic one.

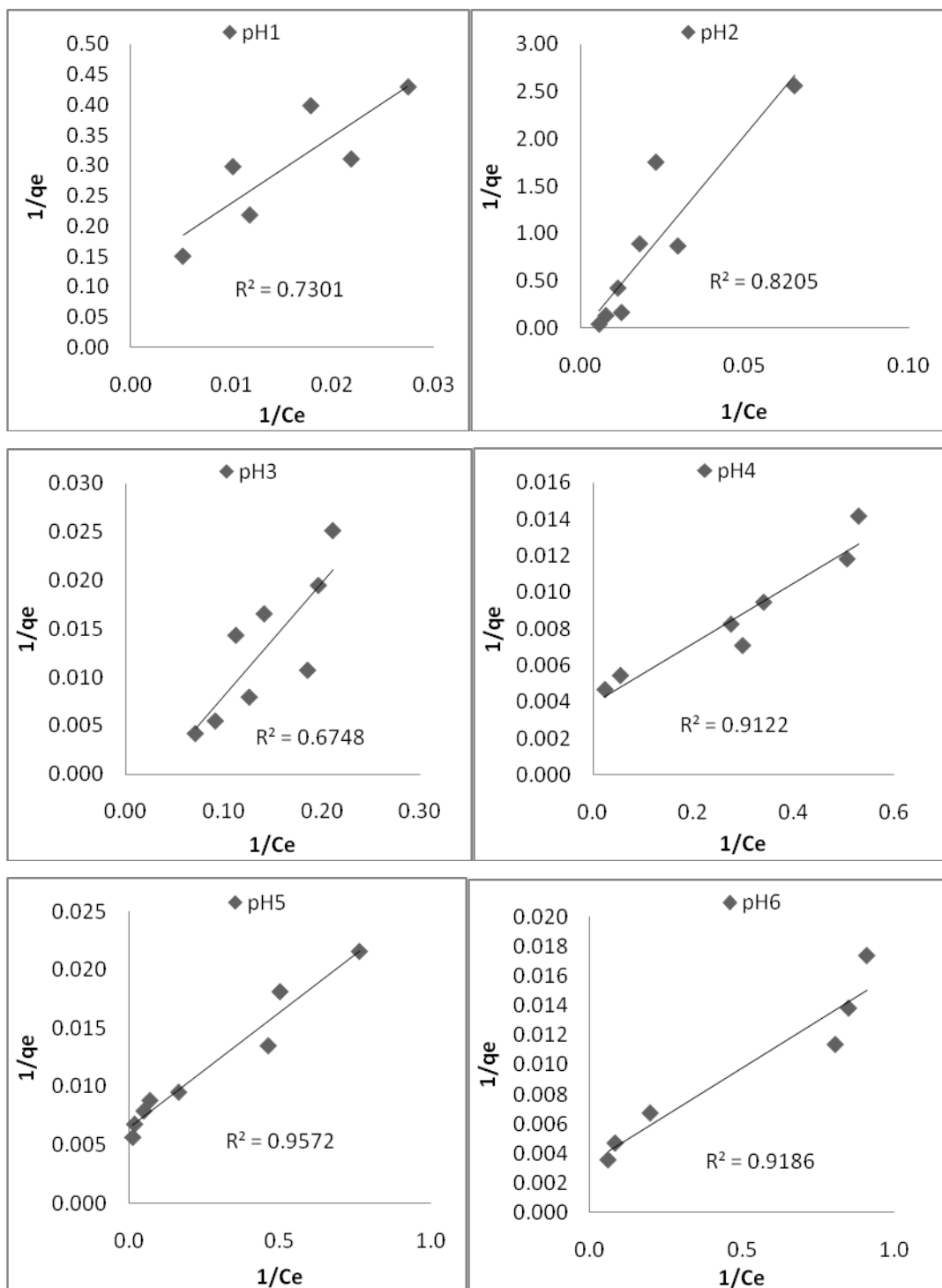


Figure 4.2 Langmuir isotherm models for *Bacillus subtilis* different pHs

**Table 4.2** Langmuir isotherm for lead adsorption on *Bacillus subtilis*.

pH	Langmuir constant		
	$Q_{th}^e$ (mg/g)	$K_{eq}$	$R^2$
1	7.74	0.01	0.73
2	n/a	n/a	0.82
3	n/a	n/a	0.67
4	256.41	0.24	0.91
5	266.25	0.32	0.96
6	294.12	0.27	0.92

Comparison of the two models, Langmuir and Freundlich, shows that Freundlich model exhibits better fit. Therefore, biosorption process in this study may be interpreted as multilayer adsorption.

#### 4.2 Biosorption kinetics study

The evaluation of batch adsorption kinetics is required for the design of industrial scale reactors. Kinetic models were employed to analyze the adsorption rates of lead. The experimental batch biosorption kinetics data were modelled using pseudo first order and pseudo second order reaction equations.

The pseudo first order and pseudo second order rate constants  $k_1$ ,  $k_2$  and  $q_e$  were estimated from the model including the corresponding correlation coefficients, respectively. Figure 4.3 shows change in natural log of lead removal ( $q_e/q_t$ ) versus time. Analysis of the plotted data does not provide sufficient information to establish any trend behaviour. Based on this it is concluded that first-order reaction model is not suitable for the obtained experimental data.

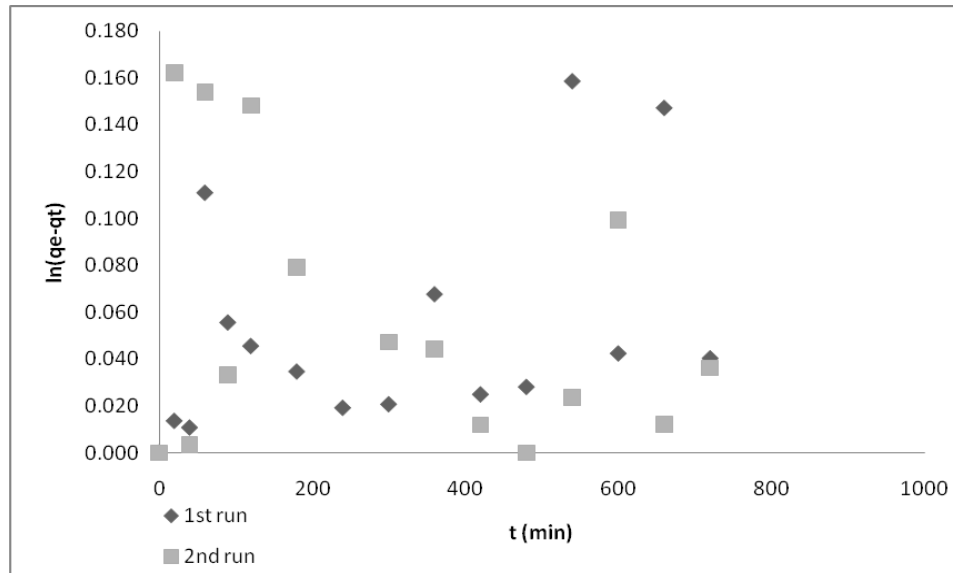


Figure 4.3 Pseudo first reaction model for Pb adsorption at pH 6.

Figure 4.4 shows a plot of time versus  $t/q_t$  for lead removal by biomass of *Bacillus subtilis*. A clear straight line for the obtained data indicates a good fit into the model. Therefore, it is concluded that kinetics of lead adsorption on *Bacillus subtilis* biomass follow the pseudo-second order reaction model. Based on the model, values of  $Q_e$ ,  $k_2$  and  $R^2$  were determined, which are 97.09, 0.0013 and 0.9994, respectively.

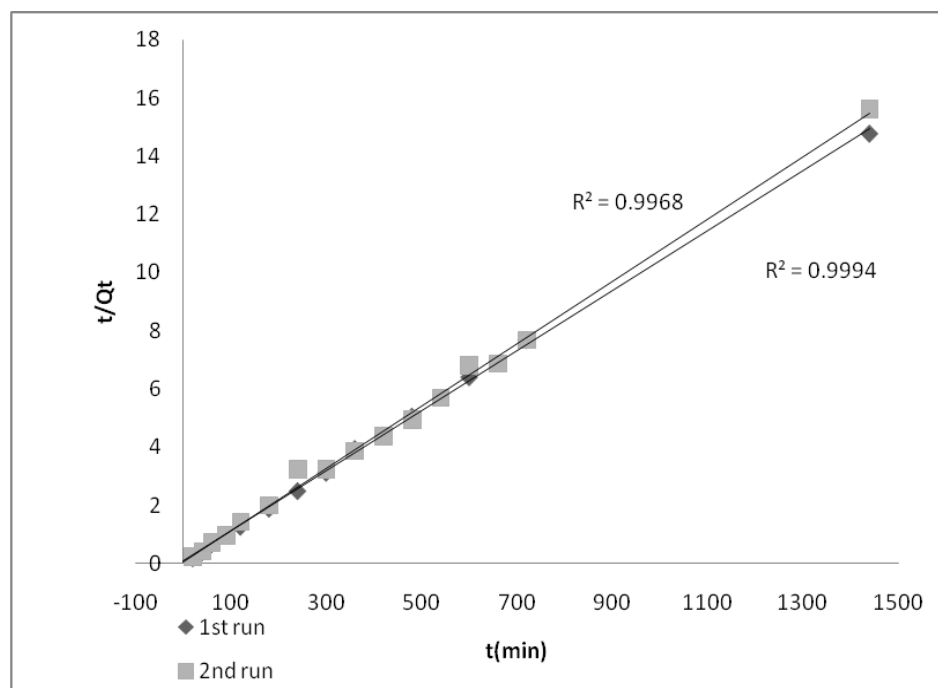


Figure 4.4 Pseudo second reaction order model for Pb adsorption at pH 6.

In this investigation it is concluded that the pseudo second order kinetics match satisfactorily with the experimental data. This shows that the biosorption process in this study may be interpreted as chemisorptions process (Ho & McKay, 1999).

### 4.3 Effect of initial pH on Pb(II) biosorption

In these batch biosorption experiments, the influence of pH on Pb(II) ions biosorption was studied using synthetic solutions. Figure 4.5. shows percentage of Pb(II) ions removal by bacterial biomass over a range of tested pHs. The effect of pH in batch system was studied by varying the solution pH from 1 to 6. At low pH values, Pb(II) ions uptake was observed to be at the lower levels. As the pH increased to 6, the amount of Pb(II) ions uptake increased from 5% to 91.6%.

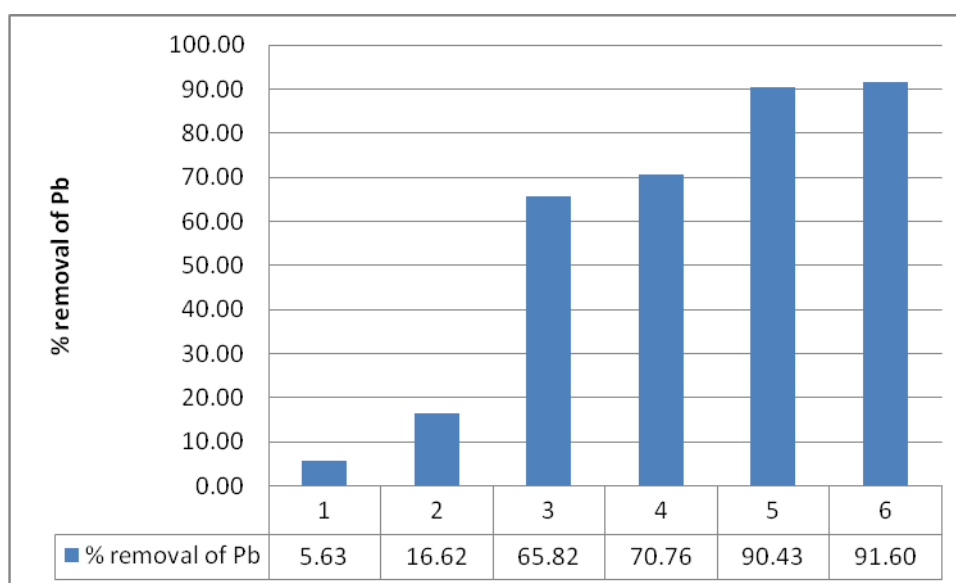


Figure 4.5 Effect of pH on the biosorption of Pb on *Bacillus subtilis*

Figure 4.5 very clearly shows that *Bacillus* biomass showed high percentage removal of Pb (91.6%) at pH 6 as compared to low percentage removal of Pb(II) ions (5.6%) at pH 1. Biosorption of heavy metals usually leads to the acidification of aqueous solutions. So, when more acidic solutions are used it may rescue the inhibition of the biosorption process.

The removal of metal as a function of pH at the constant initial concentration of lead of 50 ppm is shown in Figure 4.5. Removal rate increases from pH 2 to pH 3 and it increases even more at pH 4 and pH 5. The maximum removal rate was achieved for pH 5 and pH 6 system.

It is known that at very low pH higher concentration of protons result in their predominant adsorption at the essentially negatively charged binding sites on bacterial cell wall surface leaving metal ions in the solution. At higher pH hydroxyl groups dominate in solution and complex metal cations, preventing attachment to ligands on cell wall surface. The results found in this study corroborate with other previous works (Shankar & et al, 2007), which also reported low metal removal at very low pH and maximum removal rate at neutral pH range.

#### **4.4 Effect of initial lead ions concentration on biosorption capacity**

As a rule, increasing the initial metal concentration in the solution results in an increase of the biosorption capacity because the initial metal concentration provides a driving force to overcome mass transfer resistance between the biosorbent and biosorption medium (Wang & et al., 2006). The effect of initial metal ion concentration on the biosorption capacity of *Bacillus subtilis* was studied at different pH from pH 1 to pH 6. These experiments were carried out using synthetic solutions containing different lead concentrations in a range of 5 to 200 mg/l. The amount of metal ions adsorbed per unit mass of bacterial biosorbent *Bacillus subtilis* initially increased over the increase of the initial lead concentration. As lead concentration increased further, a saturation level of the adsorbent surface was observed in the investigated solution. Such observation was based on the insignificant changes in

removal of lead from the solution when lead concentration increased above 120 mg/L.

As can be seen from Figure 4.6, the maximum biosorption capacities of *Bacillus subtilis* biomass to remove Pb(II) ions were determined at pH 6 and 200 mg/l initial concentration. The metal uptake in pH 3 to pH 6 solutions increased due to the increase of the initial Pb(II) concentration. But in pH 1 and pH 2 systems the metal uptake remained almost the same although the initial concentration of Pb(II) was higher. The availability of adsorption significantly decreases as the initial concentration increases infinite number of adsorption sites (Fosso-Kankeu & et. al., 2009).

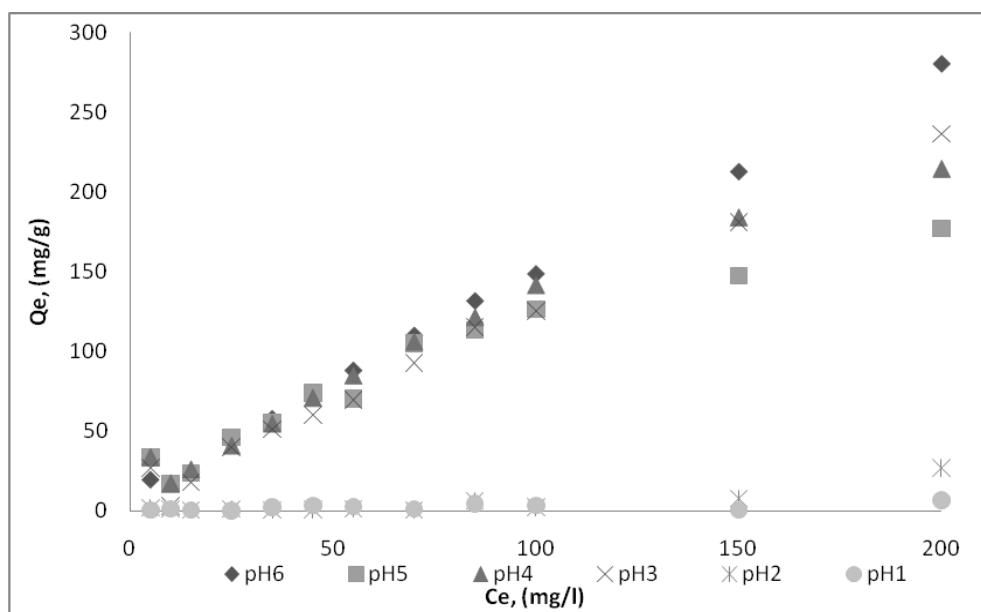


Figure 4.6 Metal uptake at different initial concentrations of lead

#### 4.5 Effect of contact time on the biosorption

The effect of contact time on the biosorption of Pb(II) at 50 mg/l concentration onto *Bacillus subtilis* biomass was investigated at constant temperature (27°C). It was determined that rapid adsorption of Pb(II) ions took place in the first 20 and 40 min, as shown in Figure 4.6.

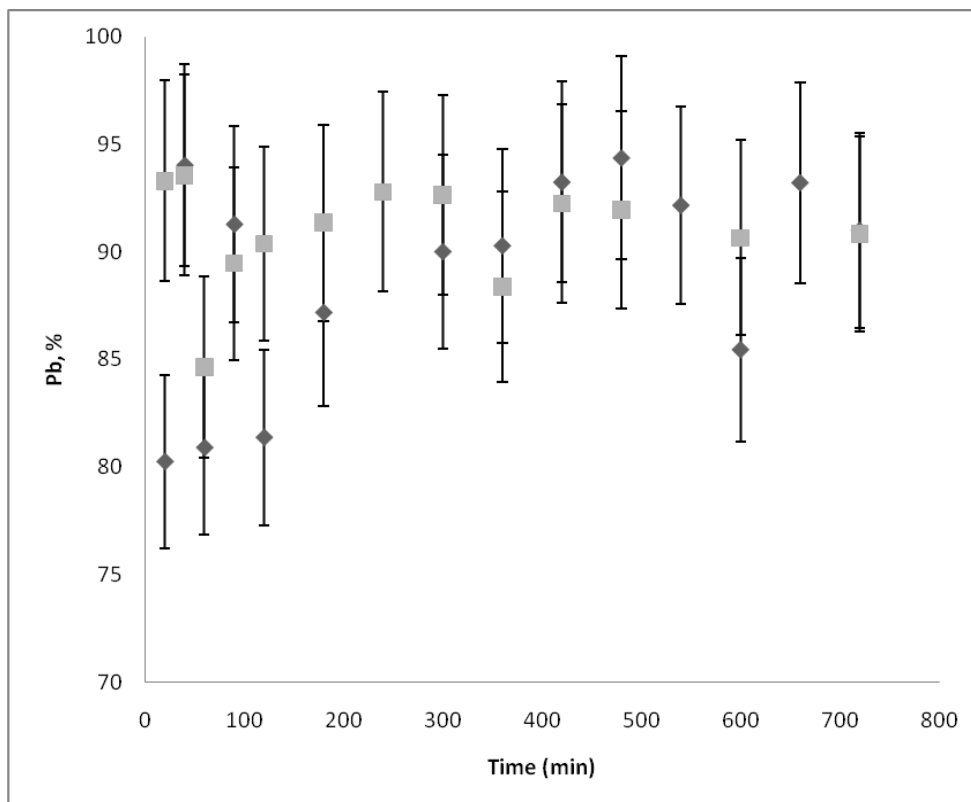


Figure 4.7 Effect of contact time on Pb(II) biosorption (bars represent 5% error).

The uptake of lead ions did not significantly change over the extended contact time. Rapid initial uptake of metal ions may be an important parameter for a practical application of biosorption in industrial wastewater treatment. Biosorption is metabolism-independent, it would be expected to be a rapid process. Usually, free cell microbial biosorption is comprised of two phases which are a very fast initial uptake in 30~60 min period, followed by a slower attainment of equilibrium within next 2 to 3 hour (Vijayaraghavan & Yun, 2008).

## CHAPTER 5

### CONCLUSIONS AND RECOMMENDATIONS

#### 5.1 Conclusions

The results demonstrate that bacterial isolate *Bacillus subtilis* may be used as inexpensive, effective and easily cultivable biosorbent for the removal of Pb(II) ions from aqueous solutions. The biosorption process of metal ions on *Bacillus subtilis* was found to be dependent on experimental condition such as the initial pH, initial metal ion concentration and contact time. The adsorption equilibrium data fitted well to Freundlich model for Pb(II) ions in the studied concentration range. This shows that the biosorption of *Bacillus subtilis* is multilayer adsorption. The maximum uptake of metal is 294.12 mg/g.

For evaluation of the effect of pH on the adsorption process, it was shown that pH 5 and 6 showed the best percentage removal of lead, more than 90%. As compared with pH 1 to 4, whereby pH 3 and 4 showed only nearly 70% removal of lead; and at pH 1 and 2 the uptake was not more than 20% of lead. This shown that lead uptake is more efficient under basic pH range than in the acid pH range of solutions.

For determination of the adsorption process kinetics in the removal of lead ions on *Bacillus subtilis* biomass, it is observed that the biosorption of *Bacillus subtilis* corresponded well to the pseudo second order reaction model and therefore the process is considered to follow a chemisorption mechanisms.



Additionally, it is observed that the exposure time for the biosorption of *Bacillus subtilis* can reach the optimum percentage of lead removal very quickly between initial 20 to 40min.

## 5.2 Recommendations

When bacteria are cultivated, it is preferred to use the rolling method to cultivate bacteria compare to use the orbital shaker. This is because *Bacillus subtilis* is aerobic bacteria and requires oxygen for its survival, growth and reproduction. A 1 liter bottle has a cylindrical shape, where by using orbital shaker, the oxygen hardly reaches the bottom part. This may cause the bacteria growth to be immature and incomplete results in that less bacteria obtained from the cultivation.

Another technique that can be considered for cultivate ion of the bacteria might be a setup of an oxygen bubble inlet that allows oxygen to enter from the bottom of the bottle. This will increase the oxygen contact time with the bacteria and increase the growth of bacteria so that the bacteria growth is mature.

Centrifuge technique to separate out the bacteria *Bacillus subtilis* is easier and faster comparing to the use of the filter paper. This is because when filter paper is used the bacteria biomass blocks the filter paper pores and the filtration process therefore take much longer time..

After the biosorption test is done, the sample solution is taken for the ICP-OES to get the concentration of heavy metal ions content in the solution. It is recommended do not keep the sample solution more than 1 day for ICP analysis. This is because the sample solution obtained by filtration may contain some of the bacteria and samples older than 1 day may lack of the accuracy of measurement.

## REFERENCES

- Ahalya, N., T., Kanamadi, R. (2003). Biosorption of heavy metals. *Res. J. Chem. Environ.* 71-78.
- Aksu, Y. S., Kutsal, T. (1992). The biosorption of copper (II) by *C. vulgaris* and *Zramigera*. *Environ Technol.* 579-586.
- Allinson, R. (1978). Journal of Parenter Enteral Nutr. *Plasma Trace Elements During Total Parenteral Nutrition* 2: 35-40.
- Bacillus subtilis* Final Risk Assessment. (2011), February 1. Retrieved March 10, 2011, from USEPA, Biotechnology Program under the Toxic Substances Control Act (TSCA): [http://epa.gov/biotech\\_rule/pubs/fra/fra009.htm](http://epa.gov/biotech_rule/pubs/fra/fra009.htm)
- Bacillus subtilis*. (n.d.). Retrieved August 25, 2010, from The Lab rat: HYPERLINK "<http://www.thelabrat.com/restriction/sources/Bacillussubtilis.shtml>"  
<http://www.thelabrat.com/restriction/sources/Bacillussubtilis.shtml>
- Bacillus subtilis- Microbe Wiki*. (n.d.). Retrieved July 20, 2010, from Microbe Wiki: HYPERLINK "[http://microbewiki.kenyon.edu/index.php/Bacillus\\_subtilis](http://microbewiki.kenyon.edu/index.php/Bacillus_subtilis)"  
[http://microbewiki.kenyon.edu/index.php/Bacillus\\_subtilis](http://microbewiki.kenyon.edu/index.php/Bacillus_subtilis)
- Bergey, D. H., Holt, J. G., Krieg, N. R., Sneath, P. H. (1994). *Bergey's Manual of Determinative Bacteriology* (9th ed.). Lippincott Williams & Wilkins.
- Buzzle.com. (2000). *Uses of Lead*. Retrieved January 27, 2011, from Buzzle.com: <http://www.buzzle.com/articles/uses-of-lead.html>
- Chojnacka, K. (2010). Biosorption and bioaccumulation - the prospects for practical application. *Environment International* (36) 299-307.
- Chojnacka, K. (2010). *Biosorption and bioaccumulation in practice*. Nova Science Publishers.
- Chojnacka, K. (2010). Environment International. *Biosorption and bioaccumulation - the prospects for practical applications* , 36: 299-307.
- Doyle, R., Matthews, T., Streips, U. (1980). Chemical basis for selectivity of metal ions by the *Bacillus subtilis* cell wall. *J Bacteriol* 471-480.

- Earl, M., Losick, R., Kolter, R. (2008). Ecology and genomics of *Bacillus subtilis*. *Cell Press* 269-275.
- Freundlich, H.M.F. Over the adsorption in solution. *Journal of Physical Chemistry*, 1906, vol. 57A, p. 385-470.
- Fosso-Kankeu, E., & et al. (2009). *Opimising the removal by B subtilis and B bacterium of metals found around mining areas: evaluation of the effect of physical and physiological parameters* .
- Golab, Z., Breitenbach, M. (1995). Sites of copper in *Streptomyces pilosus*. *Water Air Soil Pollut* 713-721.
- Jeanne, M. S. (1998). *Encyclopaedia of Occupational Health and Safety*. Geneva: International Labour Office.
- Jian Long, W., Chen, C. (2006). Biosorption of heavy metals by *Saccharomyces cerevisiae*: A review. *Biotechnology Advances* 427–451.
- Kuyucak, N., Volesky, B. (1988). Biosorbents for recovery of metals from industrial solutions. *Biotechnol. Lett.* 137-142.
- Langmuir, Irving. The constitution and fundamental properties of solids and liquids. Part. I: Solids. *Journal of the American Chemical Society*, November 1916, vol. 38, no. 11, p. 2221-2295.
- Liu, Y., Liu, Y.-J. (2008). Separation and Purification Technology 61. *Biosorption isotherms, kinetics and thermodynamics* , 229-242.
- Masud Hossain, S., Anantharaman, N. (2006). Studies on bacterial growth and lead (IV) biosorption using *Bacillus subtilis*. *Indian Journal of Chemical Technology* 13, 591-596.
- Masud Hossain, S., Anantharaman, N. (2006). Studies on bacterial growth and lead(IV) biosorption using *Bacillus subtilis*. *Indian Journal of Chemical Technology* 591-596.
- Muraleedharan, T., Venkobachar, C. (1988). Mechanism of biosorption of copper (II) by *Ganoderma lucidum*. *Biotechnol. Bioeng.* 137-142.
- Nakajimi., A., Sakaguchi., T. (1986). *Appl. Microbiol. Biotechnol.*, 24:.. *Selective accumulation of heavy metal by microorganisms* 59-64.

- Nies, D. (1999). *Appl Microbiol Biotechnol. Microbial Heavy-metal Resistance* , 730-750.
- Pauli, T. K., & James, E. B. (1996). Intracellular Expression of Vitreoscilla Hemoglobin (VHb) Enhances Total Protein Secretion and Improves the Production of  $\alpha$ -Amylase and Neutral Protease in *Bacillus subtilis*. *Biotechnology Progress* , 31-39.
- Rehman, A., Shakoori, F., Shakoori, A. (2006). *Bull Environ Contam Toxicol. Uptake of Heavy Metals by a Ciliate, Tachysoma pellionella, Isolated from Industrial Effluents and Its Potential Use in Bioremediation of Toxic Wastewater.* 77:469-76.
- Rogers, H., Perkins, H. Ward, J. (1980). *Microbial cell walls and membranes*. London: Chapman and Hall.
- Sakaguchi, T., Nakajima, A. (1991). Accumulation of heavy metals such as uranium and thorium by microorganisms. In R. M. Smith, *Mineral Bioprocessing*. Metals and Materials.
- Salton, M. R. (1964). *The bacterial Cell Wall*. Amsterdam, London, New York: Elsevier Publishing Company.
- Salton, M. (1964). *The bacterial cell wall*. Amsterdam: Elsevier.
- Sciwer, S., Volesky, B. (2000). *Biosorption Processes for Heavy Metal Removal*. In: Lovley DE, editor. *Environmental Microbe-Metal Interactions*. Washington D.C: ASM Press.
- Shankar, C., & et al. (2007). Biosorption of chromium and nickel by heavy metal resistant fungal and bacterial isolates. *Journal of Hazardous Materials* , 270-277.
- Sonnenfeld, E., Beveridge, T., Koch, A., Doyle, R. (1996). *J Bacteriol. Asymmetric distribution of charge on the cell wall of Bacillus subtilis.* 163:1167-71.
- US Environmental Protection Agency*. (1997, February). Retrieved August 25, 2010, from *Bacillus subtilis Final Risk Assessments*: [http://epa.gov/biotech\\_rule/pubs/fra/fra009.htm](http://epa.gov/biotech_rule/pubs/fra/fra009.htm)
- Veglio, F., Beolchini, F. (1997). Removal of metals by biosorption: a review. *Hydrometallurgy* (3) 301-316.
- Vijayaraghavan, K., Yun, Y.-S. (2008). Bacterial biosorbents and biosorption. *Biotechnology Advances* 266-291.

- Volesky, B. (1987). *Biosorbents for metal recovery*. 96-101.
- Volesky, B., & Schiewer, S. (1999). Biosorption of metals. In M. Flickinger, S. Drew, editors, *Encyclopedia of Bioprocess Technology* (pp. p 433-53). New York: Wiley.
- Volesky., B., & Holan, Z. (1995). *Biotechnol. Prog.* 11. *Biosorption of heavy metals* 235-250.
- Volesky. (2007). Biosorption and me. *Water Research* 4017-4029.
- Volesky, B., Holan. (1995). Biosorption of heavy metals. *Biotechnology Programming* 235-250.
- Wang, J., Chen, C. (2009). *Biotechnology Advances* 27. *Biosorbents for heavy metals removal and their future* 195-226.
- Wang, X.-j., & et al. (2006). Biosorption of cadmium(II) and lead(II) ions from aqueous solution onto dried activated sludge. *Journal of environmental sciences* , 840-844.
- William, N. (1997). Gram-Positive Bacterium Sequenced. *Science* 478.