COPPER (Cu^{2+}) REMOVAL BY BIO FLOCCULANTS PRODUCED FROM *Bacillus subtillis* BSIA

by

MEGAT AZLAN B MOHAMMED AZMI

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

September 2016

COPPER (Cu^{2+}) REMOVAL BY BIO FLOCCULANTS PRODUCED FROM *Bacillus subtillis* BSIA

MEGAT AZLAN B. MOHAMMED AZMI

UNIVERSITI SAINS MALAYSIA

2016

ACKNOWLEDGEMENT

All praise to almighty Allah S.W.T for the given strength in my way to complete my master. With this opportunity, I would like to express my sincere thanks to my main supervisor, Professor Dr.Norli binti Ismail for her continuous guidance; suggestions and encouragement during the time of my graduate study are highly appreciated. I also would like to thank my co-supervisor, Professor Dr.Norhashimah binti Morad for the advice, guidance and opinion during this research.

I also highly appreciate the lab assistants for their technical assistance and help. My thanks go to my beloved father and mother, Mr. Mohammed Azmi bin Megat Ley and Mdm. Siti Hawa binti Khalid and all my family members for their love, concern, understanding, financial and moral support throughout my research. I appreciate the understanding, support and encouragement of my lovely Wife, Khairiyah bin Abd Rahim for her understanding, loved and courage which driven me to complete this research .

Finally, thanks are dedicated to all my friends and colleagues for all their encouragement during my study. I would like to thank my labmates, Siti Aisyah Ishak, Siti Norfariha Md Nasir, Dr. Adel Alghetti and all 119 lab members. Thanks also goes to my Housemate Suharirul Susah (Asang), Ex Housemates Fadhil Yahya and fellow members in Penang and Perak. Last but not least, I would like to thanks to the person who directly and indirectly involved in my research which not their name stated above, you know who you are.

TABLE OF CONTENTS

Acknowledgement	ii
Table of Content	iii
List of Table	viii
List of Figures	x
List of Plates	xii
Abstrak	xiii
Abstract	xv

CHAPTER ONE: INTRODUCTION

1.1	Introductions	1
1.2	Problems statement	4
1.3	Objective of the study	5
1.4	Significance of the study	6
1.5	Limitations of the study	6
1.6	Organization of the thesis	7

CHAPTER TWO: LITERATURE REVIEW

2.1	Flocculating agent and flocculation	8
2.2	Bio flocculants as an alternative flocculating agent	10
2.3	Mechanism of flocculation process of bio flocculants	11
2.4	Bacteria producing bio flocculants	14
2.5	Polysaccharides based bio flocculants and protein based bio flocculants	21

2.6 Possible use of bio flocculants in environmental technology 22

2.7 Heavy metals and their impact on environmental 23

2.7.1 Copper (Cu^{2+}) ion risk to health and environment 30

2.8 Factor affecting heavy metals removal 31

CHAPTER THREE: MATERIALS AND METHOD

3.1 General 34

3.1.1 Overall methodology and flow chart 34

3.2 Cultivations of Bio flocculants producing Bacteria 37

3.3 Extraction of the exopolysaccharides from *Bacillus subtillis* BSIA (Bio flocculants) 39

3.3.1 Bio flocculants surface morphological 40

3.4 Removal of copper (Cu^{2+}) from synthetic wastewater 40

3.4.1 Preparations of Glassware for experiment 41

3.4.2 Preparation of synthetic wastewater with copper (Cu^{2+}) contaminant 41

3.4.3 Determinations of best culture broth pH for copper (Cu^{2+}) removal 41

3.4.4 Determinations of best culture broth concentrations for copper (Cu^{2+}) removal 42

3.4.5 Determinations of best $CaCl_2$ concentrations for copper (Cu^{2+}) removal (Broth culture) 42

3.4.6 Determinations of best initial copper (Cu^{2+}) concentrations for copper (Cu^{2+}) removal (Broth Culture) 43

3.4.7 Determinations of crude bio flocculants pH for copper (Cu^{2+}) removal 43

3.4.8 Determinations of crude bio flocculants dosage for copper (Cu $^{2+}$) removal 44

3.4.9 Determinations of best $CaCl_2$ concentrations for copper (Cu $^{2+}$) removal (Crude Bio flocculants 44

3.4.10 Determinations of best initial copper (Cu $^{2+}$) concentrations for copper (Cu $^{2+}$) removal (Crude Bio flocculants) 45

3.4.11 Determinations of pure bio flocculants pH for copper (Cu $^{2+}$) removal 45

3.4.12 Determinations of pure bio flocculants dosage for copper (Cu $^{2+}$) removal 45

3.4.13 Determinations of best $CaCl_2$ concentrations for copper (Cu $^{2+}$) removal (Pure Bio flocculants) 46

3.4.14 Determinations of best initial copper (Cu $^{2+}$) concentrations for copper (Cu $^{2+}$) removal (Pure Bio flocculants) 46

3.5 Statistical analysis 47

CHAPTER FOUR: RESULTS AND DISCUSSIONS

4.1 Morphological structure of bio flocculants under Scanning Electron Microscope 48

4.1.1 Morphological structure of bio flocculants under Scanning Electron Microscope 48

4.2 Removal of copper by using *Bacillus subtillis* cell culture 52

4.2.1 Effect of pH toward copper removal efficiency 52

4.2.2 Effect of broth cell concentrations toward copper removal efficiency 55

4.2.3 Effect of Calcium Chloride ($CaCl_2$) toward copper removal efficiency 58

4.2.4 Effect of initial copper concentrations toward copper removal efficiency 61

4.3 Removal of copper by using crude bio flocculants. 64

4.3.1 Effect of pH toward copper removal efficiency 64

4.3.2 Effect of crude bio flocculants concentrations toward copper removal efficiency 67

4.3.3 Effect of inorganic salt ($CaCl_2$) concentrations toward copper removal efficiency 70

4.3.4 Effect of initial copper (Cu^{2+}) concentrations toward copper removal efficiency 73

4.4 Removal of copper by using pure bio flocculants. 76

4.4.1 Effect of pH towards copper removal percentage 76

4.4.2 Effect of pure bio flocculants concentrations toward copper removal efficiency 79

4.4.3 Effect of inorganic salt ($CaCl_2$) concentrations toward copper removal efficiency 82

4.4.4 Effect of initial copper (Cu^{2+}) concentrations toward copper removal efficiency 85

4.5 Types of bio flocculants use to remove copper (Cu^{2+}) 89

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions 92

5.2 Recommendations for Future Study 94

REFERENCES 95

APPENDICES

LIST OF PUBLICATIONS

LIST OF TABLES

		Page
Table 2.1	Several studies carried by other researcher regarding bacteria that produce EPS	18
Table 2.2	Previous study by other researcher on the removal of heavy metals by bacteria	26
Table 4.1	Correlations analysis between pH and copper (Cu^{2+}) removal	54
Table 4.2	Initial, final and Uptake value of copper (Cu^{2+}) concentrations after treated at different pH approaches	54
Table 4.3	Correlations analysis between $CaCl_2$ concentrations and Copper (Cu^{2+}) removal	57
Table 4.4	Initial, final and Uptake value of copper (Cu^{2+}) concentrations after treated at different cell densities	57
Table 4.5	Correlations analysis between pH towards copper (Cu^{2+}) removal	60
Table 4.6	Initial, final and Uptake value of copper (Cu^{2+}) concentrations after treated at different concentrations of $CaCl_2$	60
Table 4.7	Correlations analysis between inorganic salt ($CaCl_2$) concentrations towards copper (Cu^{2+})	63
Table 4.8	Initial, final and Uptake value of copper (Cu^{2+}) concentrations treated by *Bacillus subtillis* cell culture	63
Table 4.9	Correlations analysis between pH towards copper (Cu^{2+}**)** removal	66
Table 4.10	Initial, final and Uptake value of copper (Cu^{2+}) concentrations treated by crude bio flocculants produced by *Bacillussubtillis* at different pH	66
Table 4.11	Correlations analysis between inorganic salt ($CaCl_2$) dosage towards copper (Cu^{2+}) removal	69

Table 4.12 Initial, final and Uptake value of copper (Cu^{2+}) concentrations treated by different crude bio flocculants concentrations produced by *Bacillus subtillis* 69

Table 4.13 Correlations analysis between inorganic salt ($CaCl_2$) concentrations towards copper (Cu^{2+}) removal. 72

Table 4.14 Initial, final and Uptake value of copper (Cu^{2+}) concentrations after treated by crude bio flocculants produced *Bacillus subtillis* at different concentrations of $CaCl_2$ 72

Table 4.15 Correlations analysis between initial copper (Cu^{2+}**)** concentrations towards copper removal. 75

Table 4.16 Initial, final and Uptake value of copper (Cu^{2+}) concentrations treated by pure bio flocculants produced by *Bacillus subtillis* 76

Table 4.17 Correlations analysis between pH towards copper (Cu^{2+}**)** removal. 78

Table 4.18 Initial, final and Uptake value of copper (Cu^{2+}) concentrations treated by pure bio flocculants produced *Bacillus subtillis* at different pH 79

Table 4.19 Correlations analysis between pure bio flocculants concentrations towards copper (Cu^{2+}) removal. 81

Table 4.20 Initial, final and Uptake value of copper (Cu^{2+}) concentrations treated by different pure bio flocculants concentrations produced *Bacillus subtillis* 82

Table 4.21 Correlations analysis between inorganic salt ($CaCl_2$) concentrations towards copper (Cu^{2+}) removal. 84

Table 4.22 Initial, final and Uptake value of copper (Cu^{2+}) concentrations after treated by pure bio flocculants produced *Bacillus subtillis* at different concentrations of $CaCl_2$ 85

Table 4.23 Correlations analysis between initial copper (Cu^{2+}) concentrations towards copper removal. 88

Table 4.24 Initial, final and Uptake value of copper (Cu^{2+}) concentrations treated by pure bio flocculants produced by *Bacillus subtillis* 88

Table 4.25 Duncan and Tukey multiple means comparison analysis for types of bio flocculants used to remove copper (Cu^{2+}) ions 89

LIST OF FIGURES

		Page
Figure 2.1	Proposed flocculation mechanism for bioflocculant DYU500 produced from *Bacillus subtilis* DYU1	13
Figure 2.2	SEM image of the purified bioflocculant by *Bacillus subtilis*	13
Figure 2.3	Cell wall of gram positive and gram negative bacteria	15
Figure 2.4	Cell location of polysaccharides been stored and secreted by gram positive and gram negative bacteria	16
Figure 2.5	Macroscopic appearance of the "ropy" strand formed by cellular mass of a EPS-producing bacteria.	17
Figure 3.1	The flow chart on crude and pure bio flocculants extraction process from *Bacillis subtillis* BSIA.	36
Figure 3.2	Flow chart on the determinations of the best conditions on factor that affecting the ability of bio flocculants to remove copper (Cu^{2+})	37
Figure 4.1	Effect of pH toward the removal of copper (Cu^{2+}) efficiency by *Bacillus subtillis* broth culture	52
Figure 4.2	Effect of cell concentrations toward the removal of copper (Cu^{2+}) efficiency by *Bacillus subtillis* broth culture.	55
Figure 4.3	Effect of $CaCl_2$ concentrations toward the removal of copper (Cu^{2+}) efficiency.	58
Figure 4.4	Effect of initial copper concentrations toward the removal of copper (Cu^{2+}) efficiency.	61
Figure 4.5	Effect of pH toward removal of copper (Cu^{2+}) efficiency by crude bio flocculants produce from *Bacillus subtillis*.	64
Figure 4.6	Effect of crude bio flocculants concentrations toward removal of copper (Cu^{2+}) efficiency produce from *Bacillus subtillis*.	67

Figure 4.7 Effect of inorganic salt ($CaCl_2$) concentrations toward removal of copper (Cu^{2+}) efficiency using crude bio flocculants produce from *Bacillus subtillis*. 70

Figure 4.8 Effect of initial copper (Cu^{2+}) concentrations toward removal of copper (Cu^{2+}) efficiency using crude bio flocculants produce from *Bacillus subtillis*. 73

Figure 4.9 Effect of pH toward copper (Cu^{2+}) removal percentage by pure bio flocculants produce from *Bacillus subtillis*. 76

Figure 4.10 Effect of pure bio flocculants concentrations toward copper (Cu^{2+}) removal percentage produce from *Bacillus subtillis*. 79

Figure 4.11 Effect of inorganic salt ($CaCl_2$) concentrations toward copper (Cu^{2+}) removal percentage 82

Figure 4.12 Effect of initial copper (Cu^{2+}) concentrations toward removal of copper (Cu^{2+}) efficiency using pure bio flocculants produce from *Bacillus subtillis*. 85

Figure 4.13 Comparison on removal percentage for different types of bio flocculants used to remove copper ion. 89

Figure 4.13 Removal percentage of the copper ions using conventional and bio flocculating agent. 91

LIST OF PLATES

		Page
Plates 4.1	SEM micrograph of bio flocculants produce by *Bacillus subtillis* at 500x magnifications	48
Plates 4.2	Bio flocculants precipitation formed at the bottom of the flask after dialyzed with cold anhydrous ethanol	51
Plates 4.3	Crude bio flocculants extracted from *Bacillus subtillis* shown it present in yellowish colour	51

PENYINGKIRAN KUPRUM (Cu^{2+}) OLEH BIO PENGELOMPOK YANG DIHASILKAN OLEH *Bacillus subtillis* BSIA

ABSTRAK

Kuprum (Cu^{2+}) amat mudah didapati pada batu-batuan, air,dan tanah secara semulajadi. Oleh kerana kebolehannya untuk mengalirkan arus dan haba serta mudah untuk dilenturkan, ianya digunakan di dalam pelbagai sektor seperti sektor elektrik dan elektronik,semikonduktor serta industri pelapisan elektro. Olehkerana kuprum (Cu^{2+}) ditafsirkan sebagai sesuatu unsur, ianya tidak dapat dimusnahkan dan keberadaannya di persekitaran amat dititkberatkan. Penggunaan bio pengelompok sebagai kaedah alternatif bagi penyingkiran logam berat telah mendapat perhatian kerana ianya selamat, tidak memberi kesan buruk terhadap persekitaran dan kebolehupayaan nya untuk menyingkirkan kepekatan logam berat yang rendah. Kajian ini memberi penekanan terhadap kebolehan bio pengelompok yang dihasilkan daripada *Bacillus subtillis* BSIA bagi tujuan penyingkiran kuprum (Cu^{2+}) berkepekatan rendah. Faktor seperti pH, kepekatan garam tidak organic, kepekatan bio pengelompok serta kepekatan logam kuprum (Cu^{2+}) juga diberikan perhatian kerana ianya sangat mempengaruhi keberhasilan penyingkiran kuprum. Pada akhir kajian ini, bio pengelompok dan Poiyalumuniumklorida akan digunakan bagi tujuan perbandingan kecekapan penyingkiran kuprum (Cu^{2+}) berkepekatan rendah. pH terbaik bagi penggunaan kultur segar didapati berada pada pH 4 dengan dos 2ml kultur segar serta 4ml dos $CaCl_2$.Walaubagaimanapun penggunaan bio pengelompok mentah boleh bertindak pada pH 4 dengan dos bio pengelompok sebanyak 3 g dan dos $CaCl_2$ sebanyak 4 ml. Bio pengelompok tulen pula bertindak pada pH 6 dengan dos sebanyak 1g bio pengelompok dan 2 ml dos $CaCl_2$. Keputusan hasil kajian ini menyediakan maklumat berkaitan faktor yang mempengaruhi penyingkiran kuprum (Cu^{2+}) dengam menggunakan bio pengelompok yang diperolehi daripada *Bacillus subtillis* BSIA. Faktor seperti seperti pH, kepekatan garam tidak organic, kepekatan bio pengelompok serta kepekatan logam kuprum (Cu^{2+}) perlu diberi perhatian kerana ianya sangat mempengaruhi kecekapan penyingkiran kuprum. Bio pengelompok yang terhasil daripada *Bacillus subtillis* BSIA berkemungkinan mampu menjadi salah satu cara alternatif bagi tujuan penyingkiran kuprum (Cu^{2+}) berkepekatan rendah kerana ianya selamat dan tidak berbahay kepada persekitaran.

COPPER (Cu^{2+}) REMOVAL BY BIO FLOCCULANTS PRODUCED FROM *Bacillus subtillis* BSIA

ABSTRACT

Copper (Cu^{2+}) are commonly metal that been widely spread in the earth due to its availability as an essential element for plants and animal and can be found in rock, water, and soil naturally. Due to their conductivity, and ductile properties copper (Cu^{2+}) was used in various industries such as electrical and electronic, semiconductor and electro plating industries. Since copper (Cu^{2+}) defined as an element which cannot be destroyed, their faith in environment was taken into considerations. The used of bio flocculants as an alternative ways to remove heavy metals was fall into a limelight because of their safeness to health and environment and ability to remove copper (Cu^{2+}) at lower concentrations. This research was focused on the ability of bio flocculants produced from *Bacillus subtillis* BSIA strains to remove lower concentrations of copper (Cu^{2+}). Important factor such as pH, concentrations of inorganic salt, bio flocculants dosage and initial concentrations of copper were also being studied since copper removal was very dependable on stated variables. At the end of the research, the selected bio flocculants was used to treated lower concentrations of copper (Cu^{2+}) and their removal efficiency was compared with Polyaluminium chloride (PAC). Best pH operated for cultural broth to remove lower copper concentration was recorded at pH 4with 2ml dosage of broth culture and 4ml dosage of $CaCl_2$. However, the used of crude bio flocculants to remove lower concentrations of copper (Cu^{2+}) seems to be more interesting since their best operated pH at 4 with 3 g dosage of crude bio flocculants and

4 ml dosage of $CaCl_2$.Pure bio flocculants best operated at pH 6 with 1 g dosage of pure bio flocculants and 2ml dosage of inorganic salt $CaCl_2$. Result obtained from this study provide an information regarding factor that affecting copper removal process using bio flocculants extracted from *Bacillus subtillis* BSIA. Factor such as pH, concentrations of inorganic salt, dosage of bio flocculants and initial concentrations of copper (Cu^{2+}) ion would give a significant effect toward removal efficiency. Bio flocculants extracted from *Bacillus subtillis* BSIA may find possible applications as an alternative ways to remove lower concentrations of copper (Cu^{2+}) since it is safe to human and environment.

CHAPTER ONE

INTRODUCTIONS

1.1 Introductions

Copper (Cu^{2+}) are commonly metal that been widely spread in the earth due to its availability as an essential element for plants and animal. Copper can be found in rock, water, and soil naturally. According to Selvaraj et al, (2004) copper known as transitions metals which can be divided into three transitions states which are Cu^0 (metal), cu+ (cuprous ion) and cu2+ (cupric ions). From these three transitions states, cupric ions know as the most hazardous species for the living organism. Even though copper can be hazardous to living organism, an appropriate concentrations of copper are still needed by organism due to its impotency as functional component to several essential enzymes which also known as copper enzymes. In example, cytochrome C oxidase is one of the copper enzymes which play an important role to organism metabolism by catalyzing the process of reducing oxygen to water. During this process, cytocrome c oxidase will produce an electrical gradient which are needed by mitochondria to create energy for the organism (Angelova et al, 2011).This statement shown us that copper are necessary for organism, but excessive intake of copper would lead to severe problems due to its toxicity.

Copper can be found abundantly through the environment due to its availability from natural sources or anthropogenic sources. Eruptions of volcanic mountain, degradations or decaying of plant and vegetations, forest fire and sea spray phenomenon could be a major factor for copper occurrences into the environment

(Ratnakumar et al, 2009). Mining industries, Agricultural activities, Electrical product productions, electro plating industries, wood industries, power plant stations and incinerators were some example of anthropogenic sources of copper enter to the environment (Klimmek et al, 2001; Hawari and Mulligan, 2006; Vilar et al, 2010; Periasamy and Namasivayam, 1996; Kuppusamy et al, 2004). Due to its abundancy, copper could be deposited and accumulated into water and soil which later entering the food chain (Ratnakumar et al, 2009; Kuppusamy et al, 2004). Besides, Ahsan 2007 and Ola 2007 have reported that, copper could also enter to food chain through the product which have been canned or process by copper containing container. As we know, when copper are introduce into the food chain, it could be very dangerous to organism due to its toxicity.

Several research papers have reported that low concentrations copper poisoning could lead to skin itching and dermatization (Selvaraj, 2004 and Huang et al, 2007). Meanwhile High dosage of copper poisoning could lead to more serious internal organ malfunctioned and damage such as changes in kidney functions and lever damage (Ajmal et al, 1998, Selvaraj et al, 2004, Kalavathy et al, 2005 and Yazichi et al, 2008).Even though there are no clear evidence which can correlate copper poisoning and cancer, several studies from Arsenis and Dimitracopoulos (1986), shown that copper presence has been identified in the central nervous system of human foetuses. This statement supported later by Selvaraj et al, 2004 which stated that heavy metal are non biodegradable and it would tend to accumulate in the living organism. According to World Health Organizations (WHO) and United States Environment Protection Agency (USEPA) the maximum permission level of copper contain in drinking water should not be exceed 1.3 mg/L concentration of copper while according to United States of Food Drug Administration's (USFDA) the maximum permissible limits for drinking water should be not exceed 1.0 mg/L concentrations. Thus, the removal of copper from wastewater should deserve an important attention before it could be discharge into the aquatic system.

Nowdays, more alternative methods were developed in order to remove copper from aqueous solutions such as precipitations technique (Loredo et al. 2003) phytoextractions, membrane filtrations, ultra filtrations, electro dialysis, reverse osmosis, adsorptions method, coagulations/filtrations method, ionic exchange (Inglezakis and Grigoropoulou, 2003), and solvent extractions (Lanagan and Ibana, 2003). Among this method, adsorption technique known as the most adopted method use method to remove copper (Bajpai and Rohit, 2007). Since considering the statement state by Gaszo (2001) inability of current conventional technique such as ion exchange, precipitations process, electrochemical process are not effective treating low concentrations of heavy metal (100mg/L) the use of microorganism to remove heavy metal form waste water seems more promising (Nourbakash et al, 2002).

Several researches reported that the use of microbes could possible remove heavy metals contaminations from aqueous solutions. Hassen et al, (1998) reported that *Psuedomonas aeruginosa* and Bacillus *Thuringiensis* could remove heavy metals from aqueous solutions. *Bacillus sp* also reported has successfully removed lower concentrations heavy metals from aqueous solutions (Green, 2006). Living microorganism such as bacteria has an ability to produce a layered polysaccharide outside their body cell (EPS) to protect their cell from undesired compound. Many researchers have found that this EPS would remove heavy metals through flocculation mechanism, thus end up EPS renown as bio flocculating agent since it derived from biological sources. Flocculating agent define as substance which can flocculate suspended solids, cell colloidal solids etc (Zhang, 2005).

Flocculating agent can be divided into three major group which are inorganic flocculants, organic synthetic flocculants and natural occurring flocculants (Zhang et al, 2007). Flocculating agent widely used for various types of industries such as drinking water industries, food industries, wastewater treatment process and fermentations process (Salehizadeh and Shojaosadati, 2001). The use of bio flocculants has been widely studies to its degradability, cost effectiveness, and does not produce a secondary pollutions during degradations process (Salehizadeh et al, 2000). Thus the aims of these studies are to investigate the potential of *Bacillus subtillis* ability to remove low concentrations of copper and to determine the impotency factor which would affects its removal efficiency.

1.2 Problems statement

Copper know as the first metals that has been used since 10 000 years ago for manufacturing tools, weapons and decorative purposes. Nowadays, copper has been widely used in electric and electronic goods productions, battery industries and agricultural activities According to United Nations Environment Programme report in 2002, electric and electronic goods know as a major export item for Malaysia which contribute RM 197,986 million followed by palm oil with RM 17,193 million. These figures are believed to be increase year by year depends on global economical situations. Industrial wastewater may pollute with copper and it would be discharged directly to the environment. The search for an effective method to remove copper from wastewater is vitals due to its capability to accumulate into the environment. Copper removal from environment has been fall into a limelight due to it toxicity and ability to accumulate. This process becomes more challenging conjunct to several limitations of current method which are not effective when low concentrations of copper involved. Besides, the applications of conventional flocculating agent would resulted in some health and environmental problems such as Alzheimer disease (Arezoo, 2002), cancer, and the derivations of secondary pollutant (Ruden, 2004). In this research pH, initial metal concentrations, bio flocculants concentrations, and inorganic salt concentrations were considered because of it factor essentiality (Converti et al, 2006).Thus, the results from this study could provide a scientific basis as an alternative way to remove copper from aqueous solutions.

1.3 Objective of the study

The objectives of this research are as follow

1) To determine the ability of bio flocculants to entrapped copper ion based on pH, dosage, presence of $CaCl_2$ and initial concentrations of copper.
2) To determine the effectiveness of *Bacillus subtillis* cell culture, crude bio flocculants and pure bio flocculants in treating lower concentration of copper at different factor (pH, dosage, presence of $CaCl_2$ and initial concentrations of copper).
3) To compare the removal efficiency of *Bacillus subtillis* cell culture, crude bio flocculants and pure bio flocculants with conventional flocculants Polyaluminum chloride (PAC) at lower concentrations of copper ion.

1.4 Significance of the study

Removal of copper using conventional method possess various difficulties depend on technique uses. In example, use of chemical precipitations would produce precipitations which are hard to be re dissolute with addition amount of sludge which are not easy to dispose while use of ionic exchange method are believe to generated resin after the desorption process. Mix settler type method known to be very costly due to requirement of large scale of treatment plant and massive inventory of expensive solvent. The use of conventional flocculating agent would raise some environmental problems due to regenerations of secondary pollutions and suspected to be harmful to human being. The use of bio flocculants as a copper removal may benefit to the environment since it is degradable and did not produce any secondary pollutants compared with conventional flocculants. In the other hand, bio flocculant are also able to treating low concentrations of copper whereby a conventional flocculant not able to do so. Therefore these studies are expected to find an alternative way to remove lower concentrations of copper from aqueous solutions with lower impact of environment and health risk.

1.5 Limitations of the study

This study only focused on the lab scale approach which emphasized on the effect on several factor towards copper removal efficiency. The result from this study are only applicable with the condition prevail from this study. Applicability from this to the actual field approach needs further investigations and pilot scale approach. The temperature in this study assumed to be constants and daily fluctuations of the temperature considered negligible.

1.6 Organizations of the thesis

This thesis composed of five extensive chapters including introductions, literature reviews, material and methods, result and discussion and finally conclusion. The first chapter (introductions) presents general overview on copper, bio flocculants, current treatment approach, problems statement, significance of study and objectives. Chapter two (literature reviews) describes literature of this study including previous study carried out by others researchers, classifications of bio flocculants, advantageous of bio flocculants. Chapter three (material and methods) narrate in detail the material used in this study, methods use during conducting an experiments and scientific analysis carried out for data analysis. In additions the statistical analysis such as analysis of variance (ANOVA) , multiple mean comparison test (DUNCAN) and Pearson's correlations analysis were also describe in details. Chapter four (results and discussions) represent research findings from this study, discuss and supported with solid argumentations from the previous research findings by the other researchers. Lastly, chapter five (conclusion and recommendations) provide a cumulative closure from the results obtain from this study whether it fulfil the objective set at the beginning of this study. This followed by the recommendations, suggestions and improvements for future considerations if this study will be conducted in future.

CHAPTER TWO

LITERATURE REVIEW

2.1 Flocculating agent and flocculation

Flocculating agent are usually defines as a macromolecule compound that has an ability to clarify turbid water (Kaplan and Christiaen, 1987) and it has been use widely in various field by human being. Flocculation process define as a process that linking or bridging a small particle to form a larger particle through physical, chemical and biological interactions (Pal et al, 2006; Droppo et al, 2005). The mechanism of flocculation usually carried out by a high molecular weight compound that physically forming a linkage between two or more particle which later united to produce a three dimensional structure which is loose and porous (Pal et al, 2006). This structure later might settle down and suspended resulting a sedimentation particle known as floc (Leppard and Droppo, 2005).Flocculating process has been used in various industries including chemical and mineral industries such as wastewater treatment, water purification process, tap water producing, dredging, fermentation process and food industries (Kaewchai and Prasertsan, 2002a; Salehizadeh and Shojaosadati, 2001; He et al. 2002; Fujita et al. 2000).

Flocculating agent can be classified into three major groups such as inorganic flocculants such as Aluminium sulphate (Alum) and polyaluminium chloride (PAC), organic synthetics flocculants such as polyacarylamide (PAM) and pluethylene imine and organic natural flocculants such as chitosan and bio flocculants (Zhang et al, 2007).The use of polymers as a flocculating agent might be due to their distinctive chemical and physical characteristics. Nowadays, Inorganic and organic synthetic polymers have been the most widely used as a flocculating agent in various industrial fields due to their cost effectiveness and strong flocculating activity properties.

Despite the cost effectiveness and strong flocculating properties of inorganic and organic synthetic flocculating agent, their used has resulted in some health and environmental issues. A study carried out by He et al, 2002 and Shih et al, 2001 revealed that the used of inorganic and organic synthetic flocculants might lead to some health and environmental problems. The aluminium element from polyaluminium chloride (PAC) has been found to induce alzheimer's disease (Arezoo, 2002 and Fujita et al, 2000) while the monomers of acarylamide found to be carcinogenic to human being despite being toxic to health (Lu et al, 2005).

In the other hand, the use of inorganic and organic synthetic flocculating agent may lead to an environmental problems due to it ability to produce a secondary pollutant (Ruden, 2004, and Salehizadeh, 2000). A study carried by Fujita et al, (2000) suggested that, use of inorganic type flocculating agent contributed to the productions of a large volume of chemical sludge and metal residue which later should be taken into considerations. Search for biodegradable, green, safe and environmental friendly flocculating agent are needed as an alternative solutions for existing synthetic flocculating agent such as Polyaluminium Chloride (PAC), Polyacarylamide (PAA) and Potassium aluminium sulphate (Alum).

2.2 Bio flocculants as an alternative flocculating agent

Bio flocculants was grouped into natural flocculating agent which may be produced by microbes during their growth (Lu et al, 2005; Gao et al, 2006). The used of bio flocculants as an alternative flocculating agent have been fall into a limelight due to their biodegradability properties and safe to environmental (He et al, 2004). Besides, bio flocculants can be produced economically in large scale and easy to be recovered from the fermentation broth (Eman, 2012). Bio flocculants may also recognize as a biological polymer which also known as extra cellular polymeric substances (EPS). A study conducted by Kumar et al. (2004) reported that bio flocculants produced by microbes were responsible for flocculation, emulsifications, absorption and the formations of protective layer to prevent threatening compound from the microbial cell structure. Bio flocculation is a dynamic process resulting from the synthesis of extracellular polymer by living cell (Gao et al, 2006; Salehizadeh et al, 2000).

Previous study has found that microbes including yeast, fungi and bacteria, have the capability of generating metabolite product with flocculating activity (Salehizadeh and Shojaosadati, 2001). Bio flocculants is biodegradable macromolecular flocculating agent with strong flocculating activity, harmlessness to humans, environmental friendly and producing no secondary pollution from their degradative intermediates (Qin et al, 2004; He et al, 2002; Fujita et al, 2000). Bio flocculants are nontoxic and biologically degradable (Shih et al. 2001) which their application might not produce a negative effects which may be considered as promising substitute for chemical and inorganic synthetic flocculating agent.

Due to it degradability and safety to environmental, bio flocculants has been used in various application of industrial sector commonly related to textiles industries, detergents productions, adhesive substances and oil recovery purposes (Kumar et al, 2004). Bio flocculants might also be potential to be used in waste water treatment process since it is harmless to human being. Having these special properties, the bio flocculants may be potentially to be applied in drinking water processes, wastewater treatment, downstream processing and fermentation process which are closely related to human and environmental purposes (Salehizadeh and Shojaosadati, 2001).

2.3 Mechanism of flocculation process of bio flocculants

Flocculation is a process where the small particles are connected, bound or bridging together by the formation of bond between them by macromolecular substances (Sahm, 1993). Flocculation can be achieved by the addition of a polymer which acts as a macromolecule substances. During the flocculation process several processes may occurred such as inter-particle collision, reduction of electrical charge, synthetic bridging flocculants and natural bridging polymers. Generally, during flocculation, bio flocculants may aggregate the cells and particles by bridging them and neutralizing their charges (Salehizadeh and Shojaosadati, 2001).

A study carried by Deng *et al*. (2005) and Sonntag (1993) suggested that the bridging mechanism was found to play an important role in flocculating organic particles in wastewater. Most of the flocculants therefore carry either a positive (cationic) or a negative (anionic) charge in for greater aggregation process. These charges would provide an adsorption point for the targeted particle by electrostatic attraction. The charges cause the polymer molecule to extend and uncoil due to charge repulsion along the length of the polymer chain and the molecule is more nearly linear, therefore can accommodate more particle. Several parameters will greatly influence the conformation of the macromolecule polymer on the particle surface including the affinity of the biopolymer for the particle surface, its chemical structure and molar mass and the physiochemical conditions existing at the particles-water interface (Wilkinson and Reinhardt, 2005).

Wastewater which contains positive charges particles such as metal contaminated wastewater will exhibit a good flocculation process when anionic polymers are added. Based on the other researchers, bio flocculants that has been produced from microorganisms such as bacteria was categorized as an anionic polymer which is negatively charges due to presence of carboxyl and carbonyl functional group (OH, COOH, COO) (Gupta et al, 1987,; Deng et al, 2004, ; Aguilera et al. 2008). Figure 2.1 shows the proposed flocculation mechanisms from Wu and Ye (2007) for bio flocculants DYU500 produced from *Bacillus subtilis*. The mechanisms proposed as shown in Figure 2.1 described that the flocculation process may occur when the additions of bio flocculants into the metal contaminated water. The bio flocculants provide an adsorption point for the metal particle which later forming a bond and bridging together due to the presence of the carboxyl groups (COO^-). This process continues until they aggregated and later suspended when the floc formation is heavy enough.

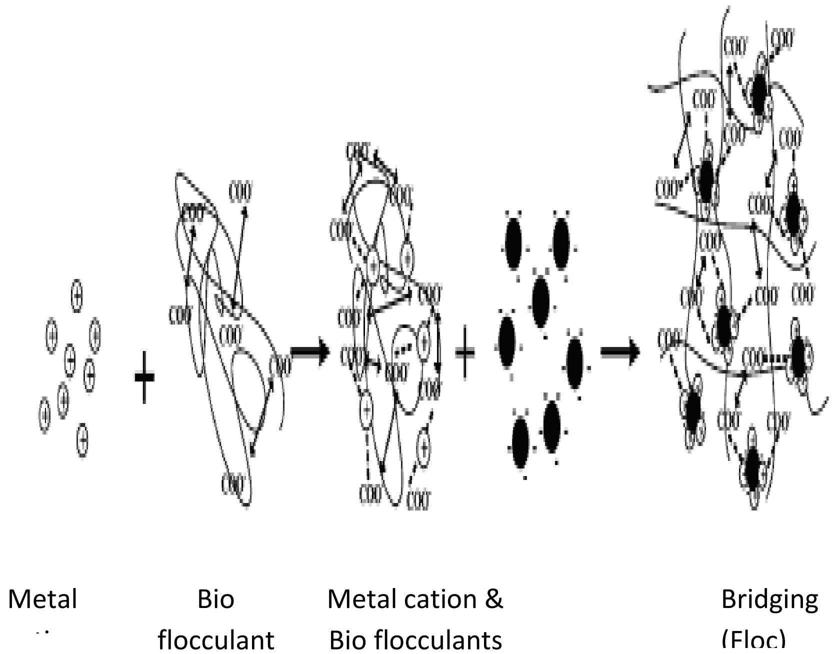

Figure 2.1: proposed flocculation mechanism for bioflocculant DYU500 produced from *Bacillus subtilis* DYU1. Wu and Ye (2007)

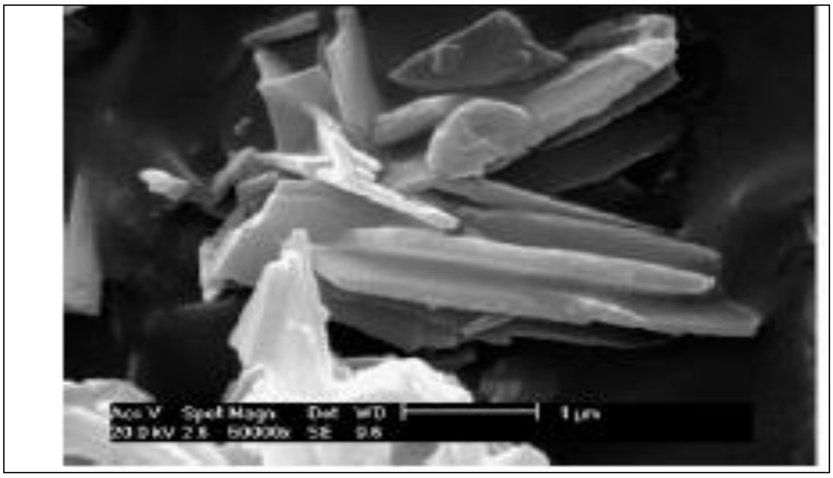

Figure 2.2: SEM micrograph image of the purified bio flocculants by *Proteus mirabilis* TJ-1 having a molecule linear in structure that allowed more particles bind to this bio flocculants (Xia et al, 2007).

The bridging process required an adsorption point which may be provided by hydroxyl, carboxyl, and carbonyl group. The higher the molecular weight of the bio flocculants, the bigger it are, and the more adsorption point they would provide. Therefore, the better flocculation process occurs. Figure 2.2 above show the SEM image analysis of the purified bio flocculants produced from *Bacillus subtilis* which made off linier molecule structure that granted more are for particles binding processes.

2.4 Bacteria producing bio flocculants

Bacteria known as the microorganisms which have been used in various field of studies such as food technology, biotechnology, bio medicals, and environmental technology. Microorganisms such as fungi, alga and actinomyces are already known to have an to produce extracellular polymer outside the cell wall surface. Bacteria, single celled eukaryotes and other microorganism cell can survive and reproduce within a certain range of environmental conditions (Sahm, 1993). Most of the bacteria are very small, with some having length a few micrometers (10^{-6} meters) (Darah and Ibrahim, 2003) and come in wide variety of shapes and sizes which called the morphology of organism. The most common shapes of bacteria are rod-like shape known as bacillus form or spherical shape known as coccus form. The rod forms vary considerable from very short rods that almost look like coccus to very long filaments thousands of microns in length (Rittmann and McCarty, 2001).

Bacteria are divided into two major groups which are either gram positive or gram negative. The differences between these two groups of bacteria might be detected by gram staining procedure. Figure 2.3 shows the cell wall of gram positive bacteria and gram negative bacteria. The gram-positive bacteria have a basic and thick peptidoglycan structure linked to techoic acids whereas the gram-negative bacteria have a membrane outside the peptidoglycan layer consisting of a phospholipic bilayer with lipopolysaccharides and proteins (Volk, 1992). Gram positive bacteria have a thick layer of peptidoglycan cell wall 5 times larger than gram negative bacteria. Gram negative bacteria have a protein pore attachment at the outer layer of the membrane which connected to the inner membrane.

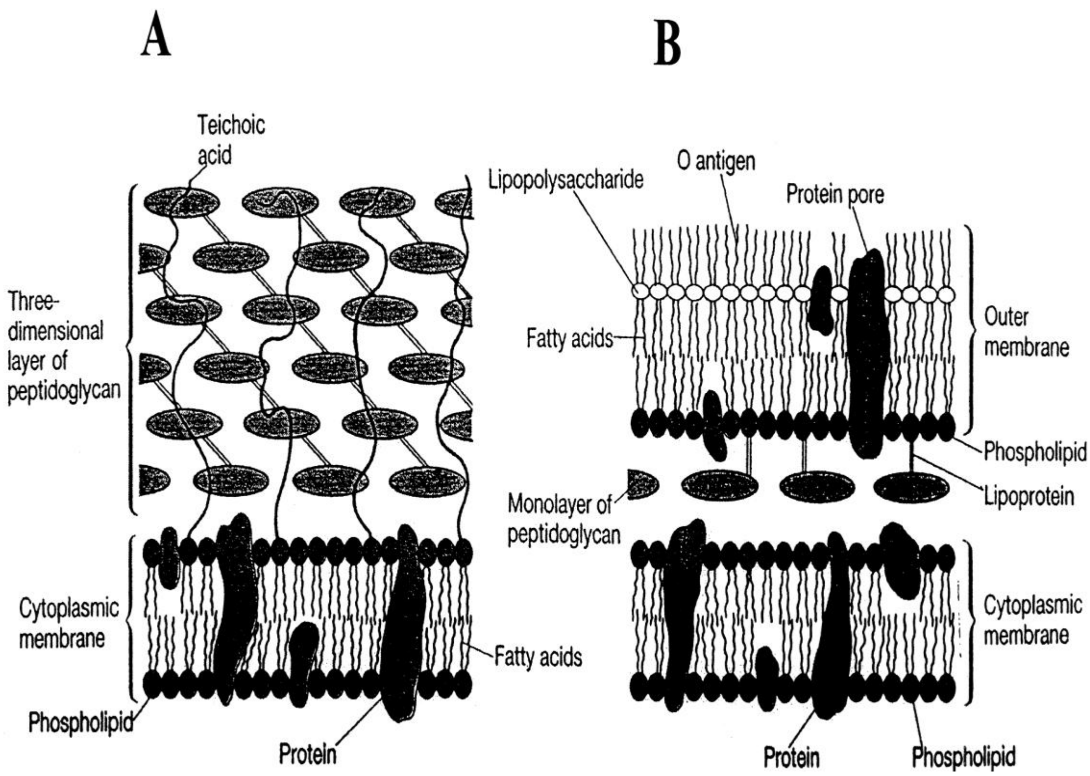

Figure 2.3: Cell wall of gram positive bacteria cell wall (A) and gram negative bacteria cell wall (B) Volk (1992)

The ability of bacteria to produce bio flocculants located at the cytoplasm of the bacteria. The bio flocculants synthesized can be stored into the cytoplasm which referred as glycogen or secreted to the outer layer of the membrane act as a protective layer for defence mechanism. This protective layer referred as glycocalyx' which is consist of glycoprotiens. Figure 2.4 show the location where the glycogen and glycocalyx stored by the bacterial cell.

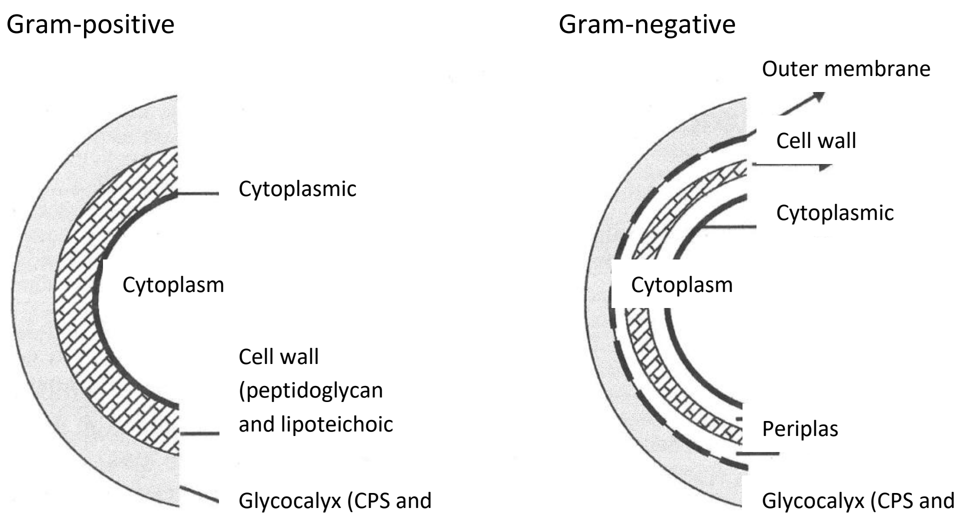

Figure 2.4: Cell location of polysaccharides been stored and secreted by gram positive and gram negative bacteria.
(Ruas and Reyes, 2005)

The secreted bio flocculants or extracellular polysaccharides (EPS) produced by bacteria may used for protections from unnecessary compound that might entered the cell. The ability of bacteria to enclose it cell with this EPS layers may provide it with protections against their natural predator such as protozoa or phage attack (Sutherland, 1996). This EPS also may prevent the accessibility of antimicrobial agent such as antibiotic from entering the cell (Costerton et al, 1987). A study by Dudman (1971) suggested that the exopolysaccharide produced by bacteria are used by them as a shield from the penetrations of the metals ion into the cell.

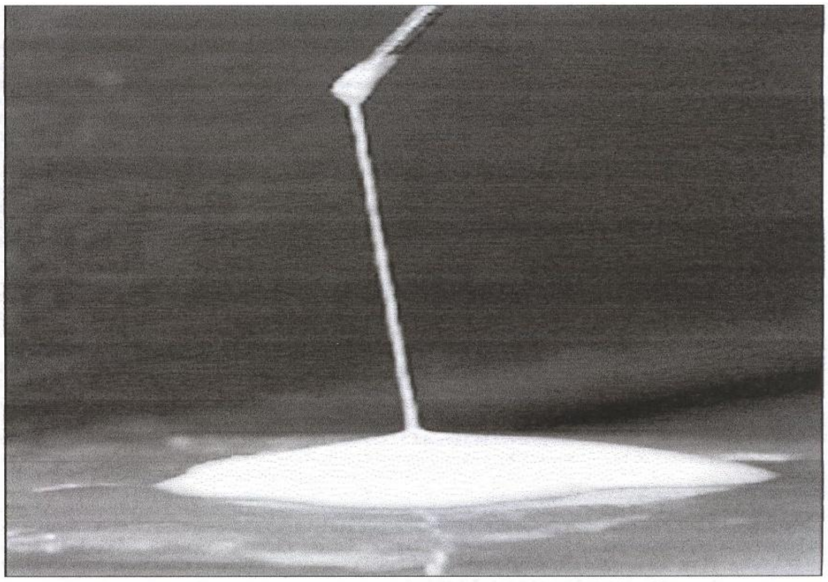

Figure 2.5: Macroscopic appearance of the "ropy" strand formed by cellular mass of a EPS-producing bacteria. (Ruas and Reyes, 2005)

Figure 2.5 shows the macroscopic appearance of the viscous properties of EPS formed by bacteria which is always thought as a type of bacterial with high flocculating activity. The terms such as viscous, sticky and glutinous have been distinctly used in many studies to describe EPS produced by bacteria appearances (Suh et al, 1997; Kumar et al, 2004; Gao et al, 2006).

However, not all EPS produced by bacteria have viscous, sticky and glutinous appearances. Besides, some bacteria might produce a glistening and slimy appearance of EPS on agar plates and unable to produce strands when extended with an inoculation loop (Ruas-Madiedo and De Los Reyes-Gavilan, 2005). Table 2.1 below listed several studies taken by other researcher regarding the bacteria that produce bio flocculants (EPS).

Table 2.1: Several studies carried by other researcher regarding bacteria that produce EPS.

	Microorganisms	References
1	*Acidithiobacillus ferrooxidans*	Studies on Acidobacillus group of bacteria reacted to mineral flocculation process chemical interactions (Natarajan and Das, 2003)
2	*Acidithiobacillus thiooxidans*	Studies on Acidobacillus group of bacteria reacted to mineral flocculation process chemical interactions (Natarajan & Das, 2003)
3	*Bacillus sp.* DP-152	Characterization of pure bio flocculants produced by *Bacillus* sp strains DP-152 with kaolin suspension flocculations (Suh et al, 1997)
4	*Bacillus sp*. I-450	Purification and characterization of bio flocculant produced from haloalkalophillic bacteria*Bacillus* sp isolated from mudflat heavy contaminated soil (Kumar et al, 2004)
5	*Bacillus sp.* PS719	Purification and characterization of bio flocculants produced from alkaliphilic and thermophilic *Bacillus* sp. strain PS-719 isolated from thermal spring soil (Hutadilok-Towatana et al, 1999)
6	*Bacillus sp.* AS-101	Studies on purified bio flocculants produced by *Bacillus* sp strains AS- 101 isolated from activated sludge (Salehizadeh et al, 2000)

7	*Bacillus cereus*	Purification and characterization of extracelllular 1,2-a-L-Fucoside from *Bacillus Creus* in Porcine Gastric Mucin media isolated from soil (Miura et al, 2005; Yang et al, 2007)
8	*Bacillus firmus* MS-102	Application of a novel bioflocculant produced by *Bacillus firmus* strain MS-102 for solid-liquid separation process (Salehizadeh & Shojaosadati, 2002b)
9	*Bacillus subtilis* SM-29	Screening and application of bio flocculants produced by thermotolerant bacteria which is *Bacillus subtillis* WD-90, *Bacillus subtillis* SM-29 and *Enterobacter agglomerans* SM- 38 for palm oil mill effluent treatment (Kaewchai & Prasertsan, 2002a,b)
10	*Bacillus subtilis* WD-90	Screening and application of bio flocculants produced by thermotolerant bacteria which is *Bacillus subtillis* WD-90, *Bacillus subtillis* SM-29 and *Enterobacter agglomerans* SM- 38 for palm oil mill effluent treatment (Kaewchai & Prasertsan, 2002a,b)
11	*Bacillus subtilis* DYU1	Characterization and flocculating properties of an extracellular biopolymer produced from *Bacillus subtilis* strain DYU 1 (Wu and Ye, 2007)
12	*Bacillus polymyxa*	Surface chemical studies on sphalerite and galena using extracellular polysaccharides isolated from *Bacillus polymyxa* (Santhiya et al, 2002)

13	*Bacillus licheniformis* CCRC 12826	Production of a biopolymer flocculants from *Bacillus licheniformis* strain CCRC 12826 and its flocculation properties in kaolin suspension (Shih et al, 2001)
14	*Chryseomonas luteola* TEM05	Utilization in alginate beads for Cu(II) and Ni(II) adsorption of an exopolysaccharide produced by *Chryseomonas luteola* strain TEM05 (Ozdemir et al, 2005)
15	*Citrobacter sp*. TKF04	Characterization of bio flocculants produced by *Citrobacter* sp. strain TKF04 from acetic and propionic acids) isolated from kitchen drain (Fujita et al, 2000)
16	*Corynebacterium glutamicum*	Identification of a novel bioflocculant from a newly isolated *Corynebacterium glutamicum* (He et al, 2002)
17	*Enterobacter aerogenes* WF-1	A novel bio flocculants produced by *Enterobacter aerogenes* and its use in defecating the trona suspension (Lu et al, 2005)

2.5 Polysaccharides based bio flocculants and protein based bio flocculants

Different type of bacteria produced different types of bio flocculants resulting in different types of functional groups which might affect the flocculation process. In examples, *Enterobacter aerogenes* (Lu et al, 2005), *Bacillus sp*. DP-152 (Suh et al, 1997), *Bacillus sp.* 450 (Kumar et al, 2004), *Enterobacter sp.* (Yokoi et al, 1997) and *Vogococcus sp.* W31 (Gao et al, 2006) produced polysaccharide types of bio flocculants while *Bacillus licheniformis* (Shih et al, 2001), *Klebsiella terrigena* (Simphiwe et al, 2010) produced protein types bio flocculants.

The major component that made off bio flocculants would play a major role to determine their molecular weight. Higher molecular weight bio flocculants may consist of major amount of polysaccharide as their structural backboned compared to protein type of bio flocculants normally has a lower molecular weight which have proteins as their structural backboned (Simphiwe et al, 2010).

The backboned of the polysaccharide determine the functional group they would provide during bridging process. Proteinaceous types of bio flocculants provide carboxyl and amine group as their active sites while polysaccharides types of bio flocculants provide carboxyl, carbonyl, and hydroxyl group as their active sites.

Suh et al, (1997) suggest that molecular weight of a flocculants is an important factor in flocculation process because it is related to the chain length of the polymer. Generally, bioflocculant with larger molecular weight usually will have longer polymer chain and sufficient number of free functional groups. These functional groups will act as bridges to bring many suspended particles together

(Michaels, 1954). Bio flocculants with higher molecular weight will give more adsorption points, stronger bridging and higher flocculating activity compared to bio flocculants with lower molecular weight.

2.6 Possible use of bio flocculants in environmental technology

Study of bio flocculants received more attention in environmental technology due to their beneficial properties. Bio flocculants are widely used in various industries including water and wastewater treatment processes. According to Lu et al, (2005) bio flocculants produced by *Enterobacter aerogenes* was able to flocculate several types of material including cell debris. This proves that bio flocculants can be used to treat turbid water resulting increase in clarity. Bio flocculation may play an important role in domestic and wastewater treatment, sludge dewatering and mineral beneficiation (Pal et al, 2006). In wastewater treatment, bio flocculants have been used to treat dyes solution (Zhang et al, 2002; Deng et al, 2005), inorganic solid suspensions such as, solid clay and aluminium oxide (Levy et al, 1992; Shih et al, 2001; Yim et al, 2007), humic acids (Zouboulis et al, 2004) and other synthetic suspensions (Lu et al, 2005). Deng et al, (2005) reported the performance of bio flocculants produced by *Aspergillus paraciticus* for dye removal from synthetic wastewater. Sheng et al, (2006) also reported that bio flocculants produced by *Klebsiella sp.* could flocculate various suspensions such as organic contaminated water.

2.7 Heavy metals and their impact on environmental

Heavy metal pollution has become one of the most serious environmental problems today. The presence of heavy metals in water and wastewater is increasing due to the industrial development. Heavy metal ions are used in various industries due to their technological importance. Heavy metal are usually classified as the following three categories: toxic metal (such as Hg, Cr, Pb, Zn, Cu, Ni, Cd, As, Co, Sn etc.), precious metals (such as Pd, Pt, Ag, Au, Ru, etc.) and radionuclides (such as U, Th, Ra, Am etc.) (Wang and Chen, 2006). Increase use of metal and chemical in industrial process has resulted in generation of large quantities of effluent that contain high level of toxic heavy metal. The presences of heavy metal in environment are not only potential human health but can cause severe damage to other life forms such as aquatic life. Toxic metal ions caused physical discomfort and sometimes life-threatening illness including irreversible damage to vital body systems (Malik, 2004). Lead, mercury, arsenic, chromium, cadmium, copper and zinc are the most frequently found heavy metal contaminants (Ahalya et al, 2005).

Heavy metal can be introduced into the environment through natural occurring process or anthropogenic process. Heavy metals were believed to be an essential element for living organism since it presence would give benefit. In example, the presence of iron (Fe^{3+}) associated with haemoglobin protein in blood are functioned to transport oxygen to muscle and organ in human, presence of chromium (Cr) would facilitate the glucose uptake and presence of zinc (Zn) associated with DNA polymerase enzyme would facilitated the process of synthesizing DNA.

Heavy metals are also identified as major pollutants in water (Iqbal et al, 2005). When heavy metals are present even in very low concentration, due to their recalcitrance and consequent persistence in water bodies their concentration may be elevated through biomagnifications to a level that they start to exhibit toxic characteristic (Atkinson et al, 1998).

The increasing problem of heavy metal contamination of soil, water and other environment has stimulated a search for new mechanisms to remove these pollutants. Many treatment technologies have been developed to remove heavy metal pollution in industrial wastewater (Ahluwalia and Goyal, 2006; Hussein et al, 2004). The existing methods for the removal of heavy metals from the environment can be grouped into biotic and abiotic. Biotic methods are based on the accumulation of the heavy metal by plants or microorganisms while abiotic methods include physicochemical process such as chemical precipitation, chemical oxidation or reduction, electrochemical treatment, evaporative recovery, filtration, ion exchange, membrane technologies and adsorption of the heavy metal by a suitable adsorbent (Kaewchai and Prasertsan, 2002b).

The commonly used physiochemical methods for removing heavy metals ions from wastewater brought many disadvantages such as generation of huge quantity of toxic chemical sludge, not eco-friendly and extremely expensive (Ahluwalia and Goyal, 2006). The conventional method is also not efficient when the heavy metal presence in very low concentration in wastewater. Since considering the statement state by Gaszo (2001) inability of current conventional technique such as ion exchange, precipitations process, and electrochemical process are not effective treating lower concentrations of heavy metal (100mg/L). Yan and Viraraghavan,