FEASIBILITY OF MANGANESE SEQUESTRATION BY GENETICALLY MODIFIED *Escherichia coli* K-12

HUDA BINTI AWANG

UNIVERSITI SAINS MALAYSIA

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FEASIBILITY OF MANGANESE SEQUESTRATION BY GENETICALLY MODIFIED *Escherichia coli* K-12

by

HUDA BINTI AWANG

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LIST OF SYMBOLS

%	Percentage
˚C	Degree Celsius
&	And
=	Equal
+	Plus
-	Minus
/	Divide
±	Plus-minus
<	Less than
→	Yields
x	Times/Multiply
X	Times concentration
Δ^9 desaturase	Delta^9 desaturase
3’OH3’-	3’hydroxyl
5’PO_4 5’-	5’phosphate
Al	Aluminium
As	Arsenic
Ca^+	Calcium ion
$CaCl_2$	Calcium chloride
CH_3COONa	Sodium acetate
Co^{2+}	Cobalt ion
Cr	Chromium
Cu	Copper
$Cyb_5.Fe^{2+}$	ferrocytochromes b_5
$Cyb_5.Fe^{3+}$	ferricytochromes b_5

Fe	Iron
K	Potassium
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
Mn	Manganese
Mn^{2+}	Manganese ion
Mn^{3+}	Manganese ion
$MnCl_2$	Manganese chloride
MnO_2	Manganese oxide
$MnSO_4$	Manganese sulphate
Na^+	Sodium ion
Ni^2	Nikel
P	Phosphorus
Pb	Plumbum
PO_3^-	Phosphite
S	Sulphur
Si	Silicon
SO_4^{2-}	Sulphate
Zn	Zinc

LIST OF ABBREVIATION

µg/g	microgram per gram
µg/L	microgram per litre
µg/mL	microgram per mililitre
µg/kg/month	microgram per kilogram per month
µm	micrometer
µmol/mg	micromole per miligram
µmol.mg dry wt^{-1}	micromol per milligram dry weight
µL	microlitre
µM	micromolar
AAS	Atomic Absorption Spectroscopy
AMP	Adenine Mono Phosphate
AMP-NA	Adenine Monophosphate-Sadium acetate
Amp^r	Ampicillin resistance
Atm	atmospheric pressure
BLAST	Basic Local Alignment Search Tool
Bp	base pair
CCC	Covalently closed circular
cfu/µg	Colony form unit per microgram
C_f	Final concentration
C_i	Initial concentration
cm	centimeter
CR	Chromosomal
CO	Circular open
Cs-mnp1	Ceriporiopsis subvermispora-manganese peroxidase 1

CueO	Cuprous oxidase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FASTA	FAST-All
Fwd	Forward
g/cm^3	gram per centimeter cube
GASP	Growth Advantage in Stationary Phase
gDNA	genomic DNA
GDP	gross domestic product
h	hour
INQWS	Interim National Water Quality Standards
IPTG	Isopropyl β-D-1-thiogalactopyranosidase
IQ	Intelligence quotient
kbp	kilobasepair
KDO	2-keto-3-deoxyoctulosonic
L	liter
LB	Luria bertani
LPS	Lipopolysaccharide
MANOVA	Multivariate analysis of variance
mcs	multiple cloning site
min	minute
mg	miligram
mg/L	milligram per liter
mg/m^3	miligram per meter cubic
mM	milimolar
mmol/g	milimol per gram

MnP	Manganese peroxidase
MRI	Magnetic resonance imaging
NAD(P)H	Nicotanamide adenine dinucleotide phosphate
NAG N	acetyl glucosamine
NAM N	actyl muramic acids
NCBI	National Centre for Biotechnology Information
ng/µL	nanogram per microlitre
nm	nanometer
OD	Optical Density
OM	Outer membrane
OmpA	Outer membrane protein A
OmpC	Outer membrane protein C
ORF	Open reading frame
Ori	Origin of replication
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
PM	Periplasmic
PPi	Pyrophosphate
Ppm	part per million
PVC	Polyvinyl chloride
q	metal uptake
Rv	Reverse
s	seconds
SEM	Scanning electron microscopy
SitABCD	*Salmonella enterica* ATP-binding cassette transporter
sp	species
xg	units of time gravity

Tm	melting temperature
V	Volume ionic solution that contacted to sorbent

KEBOLEHLAKSANAAN PENSEKUESTERAN MANGAN OLEH *Escherichia coli* K-12 TERUBAHSUAI GENETIK

ABSTRAK

Mangan dibebaskan oleh industri melalui air kumbahan menyebabkan kesan jangka panjang seperti menjejaskan fungsi saraf manusia. Penggunaan rawatan bahan kimia akan menghasilkan lebih banyak sisa enap cemar apabila membersihkan bahan cemar logam dalam kepekatan cair. Oleh itu, penerapan *Escherichia coli* K-12 terubahsuai genetik telah cuba menyelesaikan masalah. Objektif kajian ini adalah untuk mengkaji kebolehlaksanaan teknik penyediaan serpihan DNA yang disasarkan, plasmid (pUC19) dan sel-sel tuan rumah (*E. coli* K-12) untuk transformasi yang berjaya., untuk membangunkan manipulasi genetic rawak (terubahsuai genetik) *E. coli* K-12 dan untuk menentukan kesan *E. Coli* K-12 yang terubasuai genetik dalam pensekuesteran mangan. Serpihan DNA telah diasingkan dari *Ceriporiopsis subvermispora* FP105752, diklon ke dalam plasmid pUC19 dan ditransformasi ke dalam *E. Coli* K-12. untuk *E. coli* K-12 *E. coli* K-12 yang ditransformasikan menjadi lebih besar dengan warna oren lembut. DNA yang terubahsuai daripada *E. coli* K-12 telah dijujukkan dan dianalisis menggunakan 'Basic Local Alignment Search Tool' (BLAST) untuk mengenal pasti serpihan DNA yang diklon. Serpihan DNA yang diklon telah dikenalpasti sebagai *Ascomycota sp.* ARIZ RT Ash-1 internal transcribed spacer 1 dengan 82% persamaan yang mennjukkan bahawa manipulasi genetik secara rawak terhadap *E. coli* K-12 telah dicapai. *E. coli* K-12 dterubahsuai genetik telah dibandingkan dengan E. coli K-12 tidak terubahsuai genetik dengan menguji kedua-dua strain dalam media Luria Bertani (LB) yang mengandungi 200, 400, 600, 800 dan 1

000 μM $MnSO_4$. *E. coli* K-12 terubahsuai genetik adalah bahan penjerap yang lebihberkesan berbanding dengan *E. coli* K-12 tidak terubahsuai secara genetik ($p<0.05$). Kepekatan $MnSO_4$ yang optima untuk pensekuesteran mangan adalah 800 μM kerana jumlah tertinggi mangan yang disekuester *E. coli* K-12 terubahsai genetik ialah 0.745 ± 0.027 μmol / mg manakala biojisim sel kering adalah paling rendah pada 8.27 ± 0.105 mg. Kajian menunjukkan bahawa *E. coli* K-12 terubahsai genetik melalui pengubahsuaian genetik secara rawak bekerja dengan baik untuk mensekuester mangan dalam kepekatan cair serendah 800 μM sementara kurang menghasilkan sisa enap cemar biologi.

FEASIBILITY OF MANGANESE SEQUESTRATION BY GENETICALLY MODIFIED *Escherichia coli* K-12

ABSTRACT

Manganese, released by industries through wastewater, caused long term effect on human's neurological functions. Application of chemical treatments will generate more waste sludge when cleaning the metal contaminant in diluted concentration. Thus, application of genetically modified *Escherichia coli* K-12 as a biosorbent was attempted to solve the problems. The objectives of this research were to study the feasibility of the techniques of preparing targeted DNA fragment, plasmid (pUC19) and host cells (*E. coli* K-12) for successful transformation, to develop a random genetic manipulation (genetically modified) for *E. coli* K-12 and to determine the effect of genetically modified *E. coli* K-12 in sequestering manganese. A DNA fragment had been isolated from *Ceriporiopsis subvermispora* FP105752, cloned into plasmid pUC19 and transformed into *E. coli* K-12. The transformed *E. coli* K-12 colonies were bigger in size with light orange colour. The modified DNAof *E. coli* K-12 was sequenced and analysed using Basic Local Alignment Search Tool (BLAST) to identify the cloned DNA fragment. The cloned DNA fragment was identified as *Ascomycota* sp. ARIZ RT Ash-1 internal transcribed spacer 1 with 82% of similarity indicating that random genetic manipulation towards *E. coli* K-12 was achieved. The genetically modified *E. coli* K-12 had been compared to the non-genetically modified *E. coli* K-12 by testing both strains in Luria Bertani media containing 200, 400, 600, 800 and 1 000 μM of $MnSO_4$. The genetically modified *E. coli* K-12 was a more effective biosorbent compared to the non-genetically modified *E. coli* K-12 ($p < 0.05$).

The optimum $MnSO_4$ concentration for manganese sequestration was 800 μM as the highest amount of manganese sequestered by genetically modified *E. coli* K-12 was 0.745 ± 0.027 μmol/mg while the dried cell biomass was the lowest at 8.27 ± 0.11 mg. The study showed that the genetically modified *E. coli* K-12 through random genetic modification worked best to sequester manganese in diluted concentration as low as 800 μM while producing less biological waste sludge.

CHAPTER 1: INTRODUCTION

1.1 Introduction

Manganese is a transition element belongs to VIIB group of periodic table. It is being used for several purposes however the most common use of it is as additive for an alloy to form stainless steel in industry (Clarke & Upson, 2016). Though manganese is an important element for human being and plays a major role in glutamine synthase (a manganese binding protein) which is important to control the brain chemistry, nevertheless excess manganese in environment due to industrial activities led to neurodegenerative disorder in human being (Chellan & Sadler, 2015).

According to World Health Organization (WHO), 0.05 mg/L of manganese is the maximum allowed standard concentration in drinking water (WHO, 2011) which is set by many countries. Malaysia follows Interim National Water Quality Standards which states the maximum permitted manganese concentration as 0.1 mg/L for Class IIA, Class IIB and Class III rivers which become indicators for various uses such as conventional treatment, recreational body contact and requirement for extensive treatment respectively (WEPA, 2016). There are several methods to remove manganese as contaminant, from wastewater such as precipitation, coagulation, and adsorption have been conducted to mitigate the problem (Abbas *et al.,* 2014). Hydroxide, carbonate and oxidative precipitations are chemical methods of treatment but production of excess sludge as a by-product and ineffectiveness towards less concentration make it unfeasible to use it at industrial level, meanwhile, coagulation is an effective method but high cost and excess consumption is not suitable for industrial application (Patil *et al.,* 2016) and hence to minimize the cost and

sludge production, an integrated approach is needed. Microbial biosorption is a process which is observed as eco-friendly and effective even for dilute concentration of contaminant as well (Abbas *et al.,* 2014). However, indigenous bacteria are more suitable for higher concentration of metal ions contaminants because of their robustness in harsh condition (Kumar *et al.*, 2015). Therefore, improving metal recovery by genetically altered bacteria through genetic modification is an important approach to improve the feasibility of manganese sequestration (Joshi *et al.,* 2014; Yang *et al.,* 2015).

The genetic alteration in bacteria improves the adsorption capacity on the surface of bacteria as well as suppress the growth of bacteria which causes less sludge formation and more metal sequestration (Yang *et al.*, 2015).

1.2 Problem statements and objectives

Genetic modification consists of DNA isolation, polymerase chain reactions and cloning. Most of the steps involve enzymatic reactions which are sensitive and require specific conditions (Das & Dash, 2015). Therefore, unfortunately, every pitfall occurrs may lead to failure in the transformation of a host cell (*E. coli* K-12) (Matsumura, 2015).

Ceriporiopsis subvermispora able to sequester manganese due to the ability to uptake the manganese ion up to 320 μM due the presence of manganese - regulated gene known as mnp gene Mancilla *et al.,* 2010). So, feasibility of manganese sequestration by *E. coli* K-12 could be improved by transforming the fungal DNA into the cell. However, genetic information is vast in a living organism, so, recombination of random DNA fragment is an approach to know the DNA fragment that helpful to improve metal sequestration in transformed cell (Yang *et al.*, 2015).

Though genetically modified *E. coli* increases the sequestration of manganese such as overexpressing of CueO gene (encoded for multicopper oxidase) but it is not impressively high and the highest manganese uptake by the strain was reported as up to 1 to 2 mM only (Su *et al.*, 2014). In addition to that, Taudte *et al.*, (2016) reported that genetically modified *E. coli* showed high manganese uptake during the exponential phase in media (Luria Bertanii media) without additional supplements. Since the uptake of manganese occurs at exponential phase consequently, the better sequestration occurs at the price of sludge production (Wei *et al.*, 2003).

Hence, a few considerations in this study have been taken during developing genetically modified *E. coli* K-12 such as random genetic manipulation and testing the strain's effectiveness to sequester manganese ion from diluted concentration while producing less biomass to prevent excess waste sludge production. Therefore, by cloning random DNA fragment isolated from *C. subvermispora* transfered into *E. coli* K-12 to determine the feasibility of manganese sequestration. The study focused on the following objectives:

1. To study feasibility of the techniques of preparing targeted DNA fragment, plasmid (pUC19) and host cells (*E. coli* K-12) for successful transformation.
2. To develop a random genetic manipulation (genetically modified) *E. coli* K-12 for manganese sequestration from aqueous media.
3. To determine the effect of genetically modified *E. coli* K-12 during manganese sequestration.

CHAPTER 2: LITERATURE REVIEW

Since early 1980's, researchers discovered that *E. coli* K-12 was able to sequester metal ions due to strong interaction between metal ions and cell wall of the bacteria (Beveridge & Koval, 1981). The interaction known as biosorption was capable to take up metal contaminants such as manganese from the environment (Fomina & Gadd, 2014).

2.1 Manganese as contaminant

Manganese is applicable for welding in automotive, pharmaceutical, agriculture, batteries, slag and cement manufacturing industries (Hariri *et al.*, 2015; Clarke & Upson, 2016). Contamination of manganese is increasing as the discharge from domestic and industrial effluents are increasing, as well as continuous excavation of the earth's metals (Tiwari *et al.*, 2015; Faradiella *et al.*, 2016). As time passes, the poisoning signs from the metal towards human beings had prevailed for example area that exposed to industries' waste was affecting the quality of drinking water which led to manganese toxicity that caused neurotoxicity (Bjørklund *et al.*, 2017).

2.1.1 Sources of manganese contaminant

Based on Interim National Water Quality Standard for Malaysia (INQWS), safe limit for manganese concentration of INQWS standardization for Class III which applied specifically for extensive treatment required for water supply was 0.1 mg/L (WEPA, 2016). Thus, the concentration of manganese that failed according to INQWS standardization would affect water body significantly (Ashraf *et al.*, 2010). Table 2.1 shows some parameters of Malaysia Interim National Water Quality Standard (INQWS) for Class IIA/IIB and Class III surface water.

Table 2.1: Malaysia Interim National Water Quality Standard for Class II and Class III (WEPA, 2016)

Element	Unit	Class of surface water?	
		IIA/IIB	III[#]
Aluminium	mg/L	-	(0.06)
Arsenic	mg/L	0.05	0.4(0.05)
Barium	mg/L	1	-
Cadmium	mg/L	0.01	0.01*(0.001)
Chromium (IV)	mg/L	0.05	1.4 (0.05)
Chromium (III)	mg/L	-	2.5
Copper	mg/L	0.02	-
Calcium	mg/L	-	-
Magnesium	mg/L	-	-
Sodium	mg/L	-	-
Potassium	mg/L	-	-
Iron	mg/L	1	1
Palladium	mg/L	0.05	0.02*(0.01)
Manganese	mg/L	0.1	0.1
Mercury	mg/L	0.001	0.004(0.0001)
Nickel	mg/L	0.05	0.9*
Selenide	mg/L	0.01	0.25(0.04)
Silver	mg/L	0.05	0.0002
Cyanide	mg/L	-	0.004

Notes

*= At hardness 50 mg/L CaCO3
#= Maximum (Unbracketed) and 24-hour average (bracketed) concentrations

Leachate from landfills was one of the major sources for manganese contamination in the surface water which caused the exceeded permitted limit by INQWS (Taha *et al.,* 2011). Ineffective landfill management such as un-engineered of dump site caused the leachate to run off through hydraulic connection and finally contaminate surface water (Othman *et al.,* 2016). Rapid flow of surface water caused spreading of contaminants from leachate as as it flows, traces of manganese were found in the surface water (Tiwari *et al.,* 2015).

Moreover, the depth of the surface water increased the dilution of manganese concentration. Based on the previous literature, the manganese concentration will decrease as the depth increase (Adiana *et al.,* 2011). For example, Taha *et al.,* (2011) reported that one sample from upstream and three samples from downstream of groundwater in Ampar Tenang landfill (Selangor) contained 0.20 mg/L, 0.01 mg/L, 0.02 mg/L and 0.02 mg/L of manganese, respectively. Based on piezometric level, the groundwater in the landfill site would flow towards southwest known as Sungai Labu which has a surface water contained concentration of manganese that higher than Class II of INQWS standard (Umi Kalsum, 2009). Therefore, cleaning up the manganese in the leachate at upstream region was important to prevent more contamination from spreading to the surface water system (Ashraf *et al.,* 2013).

Furthermore, mining activity in the past and ongoping activity also contributed to the manganese contamination (Farhana & Mohd-Yusoff 2017). Malaysia was a country blessed with mineral resources since long time ago (Louis, 2000). Malaysia produced ore

containing 32-45% manganese, and in 2012, the ore production reached 1,099, 585 metric tons. Mining and quarry industry involving product containing manganese developed progressively in Malaysia as three new mining sites in Sarawak with annual production capacities from 50,000 to 300,000 metric tons were open in third quarter of 2014 (Tse, 2013). Therefore consequently, several rivers surrounding the mining sites were contaminated with manganese and Table 2.1 shows the mining sites with manganese contaminated surface water.

Table 2.2: Surface water of mining sites with manganese contamination.

Mining Sites	Sampling Stations	Concentration of manganese (mg/L)	Reference
Sungai Lembing Mine, Pahang	10 sampling stations of surface water around Sungai Lembing Mine	0.018-41.500	Wan-Zuhairi *et al.* (2009)
Bestari Jaya, Selangor	Ayer Hitam River Udang River Selangor River	0.125 0.325 0.095	Faradiella *et al.* (2016)
Rahman Hydraulic Tin Sdn. Bhd, Klian Intan, Perak	Kepayang River, Perak	4.673±0.122	Farhana & Mohd-Yusoff (2017)

Farhana and Mohd Yusoff (2017) explained that sediment found around the mining site contained high concentration of metal contaminant but the metals were later transported along by the river and accumulated in downstream of the river. Farhana and Mohd Yusoff (2017) reported that even though the mining company had provided limestone treatment at settling pond, unfortunately, the treatment was unable to reduce metal contaminations in the river around the mining site.

2.1.2 A threat to human's neurological functions

Exposure to more than 1 mg/m^3 manganese had increased human's health risk because the magnetic resonance imaging (MRI) analysis showed that accumulation of manganese in the brain caused toxicity (John, 2004).

Furthermore, some researchers discovered that chronic exposure of low level manganese had affected children's intellectual and neurodevelopment (Bouchard *et al.*, 2011). It was shown thatchildren of 6-13 years old with estimated manganese intake from water of approximately 150 to 200 (µg/kg/month) possess intelligence quotient (IQ) below 102 (Bouchard *et al.*, 2011). Another study conducted by Oulhote *et al.*, (2014) found that water contaminated with manganese of more than 100 µg/L caused low memory function and the manganese concentration of more than 180 µg/L led to decrease in attention function among children. These related to the accumulation of manganese in cortical structures, white matters and basal ganglia, as well as disrupt optimal cognitive functioning systems (Oulhote *et al.*, 2014). So, manganese was harmful to human health through contaminated drinking water (Bjørklund *et al.*, 2017).

2.1.3 Chemical reactions of manganese

Manganese is the 12^{th} most abundant element on earth with one isotope 55Mn with valence range from -3 to +7 while occasional valence was +2 and +4 that stable in natural water whereas the rare valence in nature was +3 (Kuleshov & Maynard, 2017). Furthermore, it was discovered that the oxidation state of dissolved manganese in natural water was normally Mn^{2+} and it was more stable compared to Fe^{2+} especially in an aerated condition (Kuleshov & Maynard, 2017). This was due to Irving-Williams stability series

for first-row divalent metal ions: $Mn^{2+}<Fe^{2+}<Co^{2+}<Ni^{2+}<Cu^{2+}>Zn^{2+}$ (Haas & Franz, 2010). The stability of Mn^{2+} activity in equilibrium was within range 0.01 to 100 ppm (Kuleshov & Maynard, 2017). The phase of manganese in the aqueous system summarised in Figure 2.1.

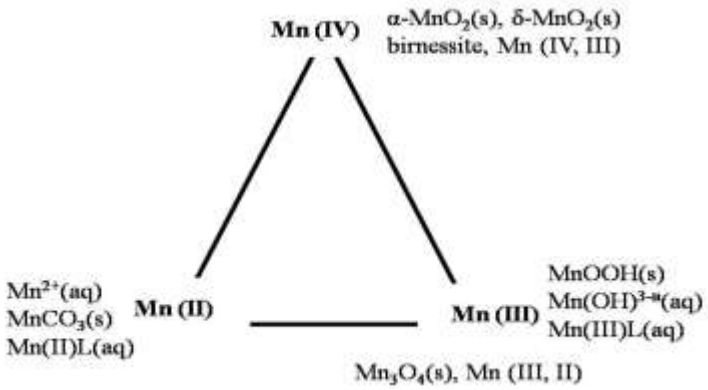

Figure 2.1: Schematic diagram shows 'manganese triangle' in an aqueous system. L denotes for the ligands, such as SO_4^{2-} (Morgan, 2000).

Manganese sulphate ($MnSO_4$) commonly found in natural systems as one of the constituents of contaminants in surface water and its redox reaction affected by natural behaviour at 25°C and 1 atm (Hem, 1985). Thus, $MnSO_4$ appeared as soluble in equilibrium condition because it dissociated into manganese ion (Mn^{2+}) and sulfate oxide (SO_4^{2-}) in water (Hem, 1985).

2.2 Feasibility of genetically modified strains in metal sequestration

Recombinant DNA method was a random genetic mutation approach due to transformation of the host cells later exhibited mutant phenotype (foreign gene) which had been carried and rescued by plasmids (Lodish *et al.*, 2000). Thus, with the aid of biotechnology, exploitation of genetics of bacteria had been carried out for biosorption's

applications (Sauge-Merle *et al.*, 2012).For example, a study conducted by Sauge-Merle *et al.,* (2012) cloned MTII gene originated from sheep encoded for metal binding proteins (metallothioneins) fused with maltose binding proteins (MBP) known as (MBP-MT) into *E. coli* and both genetically modified *E. coli* and non-genetically modified *E. coli* cultivated in media containing salt such as $CdCl_2$ in order to study feasibility of cadmium sequestration. Based on the obtained result, *E. coli* that overexpressed MBP-MT sequestered the metal ions higher than non-genetically modified *E. coli* and genetically modified *E. coli* sequestered 8.31 ± 0.02 $\mu molg^{-1}$ of cadmium rather than the non-genetically modified *E. coli* that sequestered 0.99 ± 0.04 $\mu molg^{-1}$ of copper (Sauge-Merle *et al.*, 2012). Even though metal uptake improved after genetic modification, unfortunately, the study showed that genetically modified strain produced high biomass rather than non-genetically modified (Sauge-Merle *et al.*, 2012) and production of high biomass was unfavourable as it might generate more biological sludge (Gan & Li, 2013). Therefore, this study will embark on recombinant DNA approach to improve metal uptake while reducing production of biomass synergistically.

In addition to that, Singh *et al.*, (2011) highlighted consideration for biosafety aspects for application of genetically modified organism for metal clean up. Based on literature by Verheust *et al.*, (2010), *E. coli* K-12 was a microorganism with biosafety level 1 because it was a non-pathogenic bacterial strain due to its structures. The lack of O-antigen in lipopolysaccharide of *E. coli* K-12 caused the strain absents from glycocalyx; a glycoprotein that surrounded cell's membrane, so, there was no attachment of cells onto the mucosal surface of human's colon (Kimman *et al.*, 2008). Besides that, K antigen was not expressed from *E. coli* K-12, therefore, the strain unable to recognize the mucosal

surface of human's colon for adherence (Kimman et al., 2008). Thus, the characteristics of structures of *E. coli* K-12 caused the strain considered as a safe strain for manipulation (Kimman *et al.,* 2008) for metal clean up (Shankar *et al.,* 2011) while potent to be operated in big scale without compromise biosafety through application of controlled and closed system such as bioreactor (Wright *et al.,* 2013).

2.2.1 Construction of genetically modified strain

Traditional methods in DNA recombinant applied restriction and ligation enzymes suitable for inserting a short DNA into plasmid with high successful rate for bacterial transformation compared to polynary enzymatic method (a method without application of restriction enzyme) (Yu *et al.,* 2017). Even though steps in recombinant DNA to produce genetically modified organism was theoretically straight forward, unfortunately, impurity and degradation of DNA were obstacles when handling genetic modification (Brown, 2006; Rosano & Ceccarelli, 2014). Therefore, this study focused on feasibility of transforming *E. coli* through traditional methods along with the significances and precautions in every sequential step.

2.2.1(a) *Ceriporiopsis subvermispora* as putative source for DNA fragment in random genetic manipulation

Ceriporiopsis subvermispora was a white rot fungus and a strong lignin degrader (Sato *et al.,* 2004) due to manganese peroxidase (MnP) enzyme (a mediator for lipid peroxidation) that capable in degrading compounds consisted of non-phenolic and phenolic compounds (Fernandez-Fueyo *et al.,* 2012). The MnP of the strain showed high oxidoreductase potential compared to another white rot fungi such as *Phanerochaete chrysosporium* (Fernandez-Fueyo *et al.,* 2012). The catalytic reactions of MnP involved

metal ion such as Mn^{2+} (Bao *et al.,* 1994) therefore the fungi contained potent DNA fragment for metal clean (Mancilla *et al.,* 2010). So, this study would assess feasible of random DNA fragment in *C. subvermispora* in sequestering manganese ion.

2.2.1(b) Virtual screening for cloning

Computational approach for virtual screening had been applied to access the existing database so that the DNA fragment could be analysed based on the studies' requirements, and interpreted the information correctly (Donkor *et al.,* 2014). The database of nucleotide sequences retrieved from GenBank of National Center for Biotechnology Information (NCBI) is a comprehensive database due to more than 250 000 formally described species recorded in the Genbank (a gene database). The Genbank is one of the partners of International Nucleotide Sequence Database Collaboration (INSDC) which is an organization that exchange data daily among its members to ensure uniform collection of database worldwide (Benson *et al.,* 2012). The sequences were encoded in FASTA format file which was a library sequence as result of FASTA programme which was an alignment tool based on the heuristic algorithm (Benson *et al.,* 2012). The FASTA programme was a fundamental tool of biology and knowledge on how FASTA operated and its tasks to perform were important in order to interpret accurately the outputs' information retrieved from GenBank (Donkor *et al.,* 2014). So, in this study, the gene from *C. subvermipora* was selected through a unique identifier of GenBank flatfile record known as ‘'gi" 2331294 with accession number of AF013257.

Following that, a plasmid (a double stranded extrachromosomal genetic placement isolated from bacteria and replicated autonomously from chromosome) was important to harbour DNA fragment for cloning so, the plasmid needed to be chosen based on individual

purposes (Rosano *&* *Ceccarelli*, 2014) as for example in this study, a plasmid (plasmid pUC19) was chosen as the host cell for the study was *E. coli.* The size of plasmid pUC19 was 2,686 bp with several considered features such as high copy number of the plasmid (i.e. number of plasmids per bacterial cells in normal growth condition controlled by a replication unit in a prokaryote to initiate DNA synthesis known as origin of replication (*ori*) (Preston, 2003) so the plasmid pUC19 was a high copy number plasmid (approximately 500-700 copies during the growth) able to increase chances for successful cloning as well as potent for industrial scale production (Prather *et al.,* 2003). Another consideration was multiple cloning sites (MCS) that acts as polylinker with a high concentration of many unique restriction enzyme cutting sites (i.e. not available in other parts of the plasmid) contained paired of enzymes that compatible to the restriction cutting sites of the DNA fragment (Preston, 2003). Another important considered feature was selection marker of the plasmid pUC19 known as ampicillin resistance gene (amp^r) encoded β-lactamase (i.e the enzyme catalysed the cleavage of the β-lactam ring of ampicillin) therefore the selection occurred as only transformed cells able to survive on media containing ampicillin (Preston, 2003). Furthermore, inclusion bodies consisted of random protein aggregates found expressed from pUC19 due to environmental stress (Wang *et al.,* 2003) so the stress condition able to ignite expression of protein from pUC19. Hence, based on the mentioned considered features of the plasmid pUC19 so it was a suitable plasmid to harbour foreign DNA fragment in my study.

In addition, Mertz and Davis found cleavage at the specific site produced cohesive termini during 1972. The tool for screening the enzymes like NEB Cutter linked to a comprehensive database regarding restriction enzymes' information known as REBASE

(Vincze, 2003). Thus, selecting suitable restriction endonuclease was important to find compatible restriction endonucleases for both plasmid pUC19 and DNA fragment and linearize DNA which helpful in ligation process (Vincze, 2003). Based on a study conducted by Chen *et al.,* (2000), DNA ligase IV-XRCC4 less active when DNA molecule (substrate) was in nicked structure but modification of DNA ends to linearized DNA either blunt or sticky end showed positive outcome as these ends facilitated alignment between joining DNA molecule during DNA ligase catalytic reaction. In another study conducted by Walker *et al.* (2008) reported that DNA molecule ligated to plasmid pPOP1 through sticky end showed better results rather than blunt end as a pair of different sticky ends able to avoid self-ligation. Therefore, selection of suitable restriction endonuclease in virtual screening was helpful during DNA modification that soon would be carried out in test tube (Brown, 2006).

2.2.1(c) Isolation and preparation of plasmid pUC19

Extraction and isolation of biomolecules like plasmid pUC19 were basic techniques in DNA recombinant (Tan & Yiap, 2009). Generally, extraction and isolation process applied alkaline lysis methods to break the cell, denature chromosomal DNA and plasmid, and denature proteins in the cells (Saunders & Rossi, 2008) to obtain high supercoil plasmid with strong hydrogen bond (Ehrt & Schnappinger, 2003). Besides, isolation of the plasmid required purification steps known as precipitation method through application of chaotropic salt and ethanol to remove denatured DNA and protein which existed as hydrophobic substance during the process (Ehrt & Schnappinger, 2003). Adsorption technique through DNA-adsorbing materials such as silica membrane was introduced as additional step for purifying the DNA to improve the quality of isolated DNA (Becker *et*

al., 2016). Even though advance methods applied for extraction and isolation process, the quantity and quality of isolated DNA affected by phase of growth during harvesting the cells as copy number of plasmid exhibited significant increment during late log phase (Trivedi *et al.,* 2014).

Apart of that, restriction digestion and dephosphorylation were important techniques for preparing plasmid for cloning purposes (Wang *et al.,* 2017). For example, Wang *et al.,* (2017) restriction digest of the CMV promoter of gWiz plasmid by *Not*I (restriction endonuclease) provided a site for insertion of foreign gene and dephosphorylation of 5'-phosphate of the modified end minimized self-ligation of the plasmid (Wang *et al.,* 2017). However, magnesium ion was very important in the restriction endonuclease reaction to trigger *EcoR*1's scission activity at 5'...GAATTC...3' to generate 3'-hydroxyl-G' and 5' –phosphoryl–A termini (Bennet & Halford., 1989) and dephosphorylation activity by Antarctic phosphatase (Rina *et al.,* 2000). However, access of salt might harm for next reaction such as ligation so the plasmid required purification process for example Wang *et al.,* (2017) reported that application of PCR purification kit was efficient for purification of modified gWiz plasmid to achieve successful ligation to harbour the targeted gene. However, conventional affinity-based gel extraction method could damage DNA mechanically (Kalinichenko *et al.,* 2017) so my study would embark the importance of pure and concentration of DNA in order to study feasibility of the techniques in cloning to achieve successful transformation.

2.2.1(d) Isolation of its genomic DNA (gDNA) of *Ceriporiopsis subvermispora*

Isolation of gDNA from fungi was a tedious work that required additional precautions and steps (Shahriar *et al.,* 2011). According to Vasina *et al.,* (2017), fungal should be harvested during the 8th day of cultivation period which indicated as stationary phase because transcription of the whole peroxidases' complex could be observed during the phase. Besides, the fungal strain required additional technique in cryopreservation to maintain good quality of DNA such as snap freezing prior to lyophilization in order to faster the rate of freezing and the snap freezing technique also prevent fragile membrane of the cell from damage (Morgan *et al.,* 2006). Unfortunately, the cell wall of *C. subvermispora* was a constrain to isolate genomic DNA (gDNA) from internal organelle of the cell due to difficulties to disrupt the cell wall by lysis buffer (Shahriar *et al.,* 2011) so the fungi had to be homogenized by hand-operated grinder prior to DNA isolation process (Moslem *et al.,* 2010). For example, as study conducted by Sánchez *et al.,* (2008) showed isolation of DNA of *Penicilium griseofulvum* IBT 14319 by snap freeze and ground to form powder then applied chemical with Proteinase K enzymatic reaction to obtain 81.12 ng/µL of DNA compared to 18.90 ng/µL of DNA which was recovered from lyticase reaction treatment prior to liquid nitrogen treatment. So, combination of physical, chemical and enzymatic reactions was effective to recover desired yield of gDNA with little polysaccharide and protein contamination in the yield with the ratio of wavelength in nanospectrophotometer $A_{260/280}$ within 1.8 to 2.0 which considered as range for pure nucleic acids (Yuan *et al.,* 2015) as contaminants absorbed strongly near A_{260} till A_{280} hence $A_{260/280}$ is ratio to determine protein contaminants (Rio *et al.,* 2011; Psifidi *et al.,* 2015). So, purity was important to indicate quality of DNA and the output also affected by

structure of the fungi treated with alkaline lysis solution as DNA isolated from conidia was purer than mycelia (Rodrigues *et al.,* 2018).

2.2.1(e) PCR and enzymatic digestion to produce targeted DNA fragment

Polymerase Chain Reactions (PCR) was an *in vitro* method with enzymatic synthesis to amplify specific DNA (Erlich, 2015). PCR's principles consisted of template denaturation, primer annealing and annealed primers' extension (Erlich, 2015). The enzyme involved in PCR was *Taq* Polymerase originated from *Thermus aquaticus* was a thermostable DNA polymerase which was robust at high temperature (Erlich, 2015). In addition to that, reagents such as the concentration of primers, deoxynucleotides (dNTPs), magnesium chloride and DNA template were important for successfulness of PCR (Erlich, 2015). Magnesium was cofactor for *Taq* polymerase to facilitate specific binding of dNTP onto DNA template (Ignatov *et al.,* 2003). Unfortunately, accurate replication of DNA occurred naturally so the frequent variations might occur due to technical effect such as small error rate by *Taq* polymerase during PCR (Baldwin *et al.,* 1995). Since *Taq* polymerase was a low fidelity polymerase (Rasila *et al.,* 2009) hence the weakness able to be manipulated to create random mutagenesis of *E. coli* through PCR (Holland *et al.,* 2015). Thus, optimization was required in order to improve the PCR reactions (Erlich, 2015).

Besides, end of DNA fragments would be subjected to modification through restriction digest with two different enzymes to avoid self-ligation of DNA fragments (Rina *et al.,* 2000). The restriction endonuclease applied for catalysing cleavage of phosphodiester bond at end of DNA fragment similar to the enzymes that applied for

plasmid so that the ends for both insert and vector were compatible (Wang *et al.*, 2017). By this way, DNA fragment and vector able to be ligated (Wang *et al.*, 2017).

2.2.1(f) Cloning

Ligation was a thermodynamic based reaction (Dugaiczyk, 1975). According to early work by Ferretti & Sgaramella (1981), optimum temperature of DNA ligase reaction in joining of sticky end was at 4^{o}C. In addition to that, Pascal (2008) reported mechanisms of ligation involved three consecutive steps. The reactions commenced when lysine residue at active site of ligase attacked Adenosine Monophosphate (AMP) to form Ligase-AMP intermediate complex, then, AMP from the complex transferred to 5'PO_4 of adjacent nucleic acids' sticky end to form another intermediate complex (AMP-nucleic acid reaction intermediate (AMP-NA)), later, AMP from AMP-NA reaction intermediate released to achieve successful ligation as 3'OH of nucleic acids' sticky end from opposite strand attacked 5'PO_4 of AMP-NA reaction intermediate to form formed covalent bond between 3'OH and 5'PO_4 of adjacent strands (Pascal, 2008). Thus, low temperature required to compromised catalytic reaction of T4 DNA ligase to complete the reaction as molecules float through solution so rate of collision among molecules was low (Matsumura, 2015). Therefore, ligation was important tool in cloning but needed analytical step to determine successfulness of the reaction (Nordström & Nyrén, 2017).

Following that, ligation product by ethanol precipitation with sodium acetate (CH_3COONa) to remove contaminants such as excess proteins and salts from the nucleic acid developed by Maniatis *et al.*, (1982). According to the protocol by Maniatis *et al.*, (1982), 70% of ethanol used to wash away impurities, however, this stage would become pitfall as ethanol was a threat to cell death during cloning, so a precaution step needed to

ensure that the ethanol would not remain in ligation product prior to transformation (Sarkar *et al.,* 2002).

2.2.1(g) Transformation of *E. coli* K-12

E. coli K-12 was modified to become competent cell for transformation by treating with calcium ion (Mandel & Higa, 1970). The negatively charged of exterior lipopolysaccharide molecule of cell wall repelled DNA molecules that carry the same charge therefore the presence of calcium chloride ($CaCl_2$) as surfactant (i.e. increasing fluidity of lipopolysaccharide at the surface of the cell) especially calcium ion (Ca^+) induced reversible permeabilization at outer membrane (Mandel & Higa, 1970). However, the transferred molecules became less susceptible at inner membrane (Mandel & Higa, 1970). So, additional process to facilitate uptake of DNA such as heat shock required for transformation (Sarkar *et al.,* 2002).

Furthermore, heat shock transformation increased susceptibility of inner membrane towards transferred molecules (Aich *et al*, 2012). Low temperature as low as 0 ^oC needed during transformation to facilitate DNA binding because membrane of the host cell treated with $CaCl_2$ crystallized due to formation cation shield for both phosphate group of cell envelope and phosphate group of plasmid DNA therefore enable transferred of plasmid across the cell envelope (Hanahan & Bloom, 1987). Besides, low temperature during transformation also induced (poly (HB): poly(P)Ca^{2+} complexes) that localized at inner membrane to form transmembrane channel for plasmid DNA uptake (Aich *et al*, 2012). Once the plasmid transformed into the host cell, it formed stable episome (minichromosome) and the stable episome increased when the host cells went through replication during growth (Hanahan & Bloom, 1987). So, combination of calcium ion

treatment and heat shock induced membrane porins to facilitate foreign DNA into the cell to achieve successful transformation (Aich *et al*, 2012).

2.2.1(h) Random genetic manipulation and analysis through Basic Local Alignment Tool (BLAST)

Basic Local Alignment Tool was an important virtual tool in searching for similarities among biological sequences through application of bioinformatic (Donkor *et al.,* 2014). The tool also applied for analysing cloned DNA fragment (Mabizela-Mokoena *et al.,* 2017). For example, Mabizela-Mokoena *et al.,* (2017) cloned modified lipase gene from *Bacillus pumilus* into pGEM-T Easy plasmid then transformed into *E. coli* JM09 (DE3) so based on the BLAST analysis, the cloned was 96 % and 97 % identical to mature protein from *Bacillus licheniformis* and *B. pumilus* respectively. Interestingly, the non-modified lipase gene from *B. pumilus* actually lethal to *E. coli* JM09 (DE3) however the genetically modified *E. coli* JM09 (DE3) containing modified lipase gene survived (Mabizela-Mokoena *et al.,* 2017). So, BLAST analysis provided an explanation on how much divergence occurred in the modified gene (Mabizela-Mokoena *et al.,* 2017).

Furthermore, application of BLAST was significant especially during identification of random gene of an organism that responsible for metal sequestration (Huang *et al.,* 2018). For example, Gutierrez *et al.,* (2008) conducted a study to know Mn-regulated gene from *Ceriporiopsis subvermispora* cultivated in media containing manganese ion, thus, a random cDNA isolated from *C. subvermispora* harboured by PCR-II TOPO TA plasmid had been transformed into *E. coli* and went through BLASTX and BLASTP analysis against NCBI nonredundant database for nucleotide and protein respectively. Unfortunately, analysis through BLASTX showed transcript derived fragment of *C.*

subvermispora had no significant homology (E value $<e^{-7}$) but BLASTP analysis showed putative identical protein from the cloned fragment were CsMn09, CsMn42, CsMn04, CsMn41 and CsMn36 (Gutierrez *et al.,* 2008). Since sequence genome of *C. subvermispora* was wide and not all of the functional regulations gene were defined so an extensive studied should be done in order to identify potent gene for biosorption (Gutierrez *et al.,* 2008).

Apart of that, internal transcriber spacer (ITS) was an 18S Ribosomal DNA for fungal identification (Prakash *et al.,* 2011). The ITS was a non-functional by product during RNA maturation and located between ribosomal RNAs (rRNA) (Prakash *et al.,* 2011). The region of ITS consisted of ITS2, nuclear ribosomal DNA (nrDNA) and ITS1 as in Figure 2.3 (Baldwin *et al.,* 1995). Besides, ITS region frequently used for sequence comparison purpose due to several properties such as ITS was small in size and the nrDNA was a conserved sequence with evolutionarily high (Baldwin *et al.,* 1995). Besides, ITS was used for phylogenetic analysis because nrDNA was produced with high copy numbers at chromosomal locus thus it was useful for detecting small quantities of DNA (Baldwin *et al.,* 1995). Insertion or deletion of nucleotides detected during sequencing caused intra-genomic homogeneity of the repeated units with frequent variations and led to ITS paralogues (i.e. a pair of ITS genes from same ancestor) went through concerted evolution.

Thus, frequent variations impact to evolution up to closely related species level (Baldwin *et al.,* 1995).

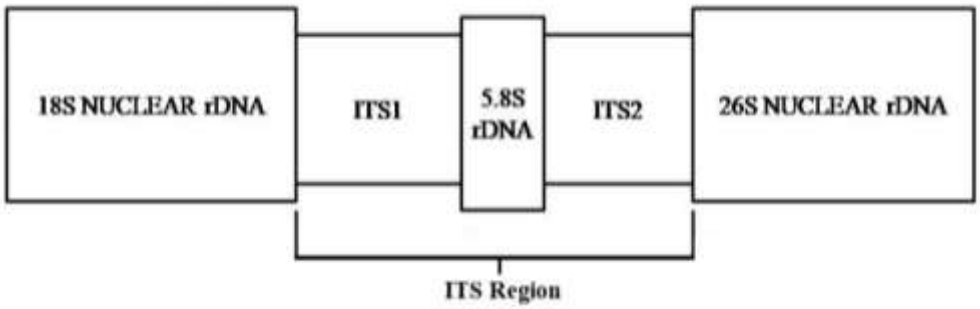

Figure 2.2: Structures of ITS region (Baldwin *et al.,* 1995).

2.3 Metal sequestration

In general, mechanisms of biosorption occurred between interface of cell wall and ion in solution (Hem, 1985). Ions in aqueous solution interacted with the surface outer membrane by a layer surrounding the surface of cell known as hydration shell (Hem, 1985). Following that, divalent cation bound at outer membrane of the cell increased rigidity of water's molecules so the molecules immediately became adjacent to the outer membrane that affected the quantity of bound divalent cation (Hem, 1985). However, capacities of the outer membrane of the cell to provide metallic ions binding in large quantities for long term must be considered (Mullen *et al.,* 1989).

Thus, quality of sorbent material evaluated based on the amount of sorbate that able to be sequestered when the sorbent remains in immobilized form (Volesky, 2007). In order to determine the quality of sorbent, metal uptake (q) was calculated based on amount of sorbate bound by dried sorbent (mmol/g) (Volesky, 2007). The calculation of uptake of metal ion (q) involved sum of metal ion by subtracting final concentration of metal ion (C_f)

from initial concentration of metal ion (C_i) in a sample with specific volume of ionic solution (V) for every dried mass biosorbent (S) as shown in Equation 2.1 (Volesky, 2007)

$$q = V\,(L)\,(Ci - Cf)\,(mmol/L)/\,S\,(g)$$

(Equation 2.1)

However, moisture contents of bacterial were varied due to interstitial spaces between cells which led to inaccuracy of the cells' feasibility in sequestering metal ions (Goyal *et al*, 2003). So, in order to avoid such problem, weight of dried cells was applied in metal uptake calculation (Goyal *et al*, 2003)

2.3.1 Overview the potential of *E. coli* as a biosorbent

Beveridge and Koval, (1981) selected *E. coli* K-12 to study feasibility of metal sequestration because the strain suitable to be modelled for metal binding's study due to its envelope structure. They found that manganese in freshwater systems contacted to the bacterial envelope with amount 0.14 µmol/mg (envelope's dry weight) due to hydrophilic characteristics containing anionic sites of the lipopolysaccharide of *E. coli* K-12 at outer half of the membrane provides metal interaction (Beveridge & Koval, 1981). However, another cell wall component; peptidoglycan also contained active groups for ionic exchange as well (Volesky, 2007).

Later, Beveridge and Hoyle (1984) studied feasibility of peptidoglycan in binding the metal ions and the result showed that 0.052±0.004 µmol.mg dry wt.-1 of manganese ion sequestered by the peptidoglycan of *E. coli* K-12. Based on the obtained result, Beveridge and Hoyle (1984) suggested that manganese ions bound to the carboxyl group of peptidoglycan monolayer. However, uptake of manganese at the outer membrane

reported by Beveridge and Koval (1981) was two times higher than uptake at peptidoglycan as reported by Beveridge and Hoyle (1984). Therefore, the study showed that structures of cell wall affected the uptake of manganese as the ions might entrap at anionic charges of the outer membrane before reaching and binding at peptidoglycan monolayer (Beveridge & Hoyle, 1984).

2.3.1(a) Role of lipopolysaccharides (LPS) of the outer membrane of *E. coli* in an ionic interaction

Lipopolysaccharide (LPS) contributed 30% of outer membrane gross weight (Erridge *et al.,* 2002). The components of LPS consisted of O-antigen, lipid A and oligosaccharide side core (Weinbaum *et al.,* 2016). Structures of LPS in Figure 2.1 shows the inner core of oligosaccharide of LPS contains hydrophilic molecules known 2-keto-3-deoxyoctonic acid (KDO) and heptose (Lüderitz *et al.,* 2016).

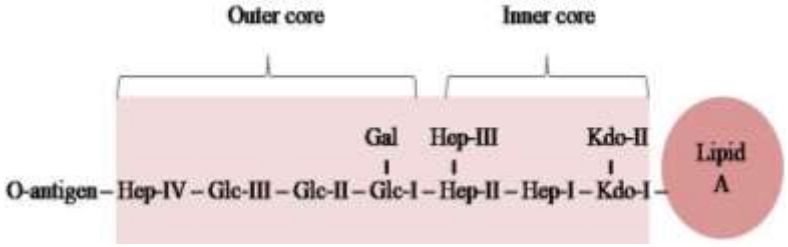

Figure 2.3: The lipopolysaccharide (LPS) structure consisted of outer core and inner core (Jorgenson & Yong, 2016).

The early findings of LPS by Ferris and Beveridge (1986) found one of three carboxyl groups from 2-keto-3-deoxyoctulosonic (KDO) from core polysaccharide of LPS showed higher affinity towards manganese as well as phosphate groups in LPS were also primary sites for manganese ion interaction (Ferris & Beveridge, 1986). Later, Walker *et al.,* (2004) added that heptose substitutions in the structure of LPS also contained carboxyl groups. Then, Meredith *et al.,* (2006) elaborated that carboxylate KDO and Lipid A were

negatively charged, so, they would interact with divalent cations and form ionic bridges that reduced electrostatic repulsion and increased interaction's strength among LPS molecules. Hence, during sequestration of metal ion, the unpaired electrons of the functional groups from cell wall donated electrons to the acceptor (metal ion) until equivalent number electron between acceptor and donor achieved (Schulman *et al.*, 2017).

However, the growth of *E. coli* was dynamic which affected immobilization of ion from solution onto the outer membrane of the cell (Beveridge & Graham, 1991).

2.3.1(b) Metal sequestration according to growth phase

Cell wall was the prime-stress bearing structure divided during cell fission of growth cycle (Beveridge & Graham, 1991). Cell wall cleaved continuously during cell division and 60 % of mature peptidoglycan of *E. coli* broke down and recycled during cell elongation (Mayer, 2012). Cell wall turnover also causes surface-metal sloughed off along with soluble wall polymer such peptidoglycan (Mullen *et al.,* 1989). Thus, cell wall turnover was one reason for low metal sequestration during the most active cell growth phase; exponential phase (Beveridge & Graham, 1991).

Then, a literature reported by Walker *et al.,* (2005) mentioned that ionic strength by *E. coli* K-12 at stationary phase was higher than mid-exponential phase. Firstly, the report discussed that deposition rates of cell grown directly proportional to the ionic strength but the increase of cell concentration during exponential phase insignificantly affected cells' deposition rates (Walker *et al.,* 2005). Secondly, the report by Walker *et al.,* (2005) also mentioned about advantages of heterogeneity surface charges on surface layers due to the existence of functional groups (ligands) because the heterogeneous distribution