ISOLATION AND CHARACTERIZATION OF BACTERIAL ISOLATES FOR BIOREMEDIATION OF DISPOSED X-RAY SHEETS TO PRODUCE SILVER NANOPARTICLES

SITI HAJAR BINTI MOHD RASDI

MASTER OF SCIENCE (BIOTECHNOLOGY)

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

DECLARATION OF THESIS AND COPYRIGHT

Author's Full Name : Siti Hajar Binti Mohd Rasdi

Date of Birth : 05 September 1990

Title : Isolation and Characterization of Bacterial Isolates for Bioremediation of Disposed X-Ray Sheets to Produce Silver Nanoparticles

Academic Session : Semester 2 Session 2017/2018

I declare that this thesis is classified as:

☐	CONFIDENTIAL	(Contains confidential information under the Official Secret Act 1997)*
☐	RESTRICTED	(Contains restricted information as specified by the organization where research was done)*
☑	OPEN ACCESS	I agree that my thesis to be published as online open access (Full Text)

I acknowledge that Universiti Malaysia Pahang reserve the right as follows:

1. The Thesis is the Property of Universiti Malaysia Pahang
2. The Library of Universiti Malaysia Pahang has the right to make copies for the purpose of research only.
3. The Library has the right to make copies of the thesis for academic exchange.

Certified by:

_____	_____
(Student's Signature)	(Supervisor's Signature)
900905-03-5632	
New IC/Passport Number	Name of Supervisor
Date: 29 May 2018	Date: 29 May 2018

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Master of Science in Biotechnology

_____

(Supervisor's Signature)

Full Name : ASSOC. PROF. DR. ESSAM A. MAKKY

Position : ASSOCIATE PROFESSOR

Date : 29 MAY 2018

_____

(Co-supervisor's Signature)

Full Name : ASSOC. PROF. DR. MOHD HASBI BIN AB RAHIM

Position : ASSOCIATE PROFESSOR

Date : 29 MAY 2018

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is based on my original work except for quotations and citation which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Malaysia Pahang or any other institutions.

_____

(Author's Signature)

Full Name : SITI HAJAR BINTI MOHD RASDI

ID Number : MKT14004

Date : 29 MAY 2018

ISOLATION AND CHARACTERIZATION OF BACTERIAL ISOLATES FOR BIOREMEDIATION OF DISPOSED X-RAY SHEETS TO PRODUCE SILVER NANOPARTICLES

SITI HAJAR BINTI MOHD RASDI

Thesis submitted in fulfillment of the requirements
for the award of degree of
Master of Science (Biotechnology)

Faculty of Industrial Sciences & Technology
UNIVERSITI MALAYSIA PAHANG

MAY 2018

Dedicated to my family

ACKNOWLEDGEMENTS

I am grateful and would like to express my sincere gratitude to my great supervisor Assoc. Prof. Dr. Essam Abdellatif Makky Saleh because always helped and give me support to complete my research. He has impressed me with his outstanding professionalism in conduct his duties as supervisor. I was really appreciated with his consistent support for me from the first day I applied to graduate program to these concluding moments. I am also grateful because of his high tolerance when I faced with problems in my research and give suggestions to solve my problems. I also would like to express thanks to my co-supervisor Assoc. Prof. Dr. Mohd Hasbi Bin Ab Rahim for his co-operation throughout the study.

Next, my sincere thanks go to all my labmates who always willing to give help and an encouragement to me. They also give suggestion to me when confronted with the problems. Sincere thanks also to the members of the staff of Faculty of Industrial Science & Technology's laboratory UMP who helped me in many ways to guide me.
I acknowledge my sincere indebtedness and gratitude to my family especially husband and son for their patience, understanding, love, dream and sacrifice throughout my life.

Thanks a lot because of full enthusiasm they give to me in order to perform the entire task as a student in Master of Science (Biotechnology). Thanks also to my siblings who have always been my supporters. Special thanks should be given to my committee members who always give comments and suggestion, so that I can correct and improve my task.

ABSTRAK

Penghasilan lembaran x-ray menggunakan sehingga 1000 tan perak yang dihasilkan secara kimia di seluruh dunia. Pencemaran berlaku apabila lembaran x-ray yang digunakan tidak dikitar semula. Teknologi mikrob boleh digunakan untuk menguruskan masalah ini untuk menghilang dan meneutralkan pencemaran. Kajian ini bertujuan untuk mengkaji penggunaan teknologi mikrob dengan menggunakan lupusan lembaran x-ray dalam pengeluaran nanopartikel perak (SNP) dan peranan enzim nitrat (NR) yang terlibat dalam proses ini. Dalam penyelidikan ini, lupusan lembaran x-ray digunakan sebagai sumber tunggal karbon untuk sintesis SNP dan menjadi pemangkin pengurangan nitrat perak kepada nitrit perak. Pengasingan bakteria berbeza yang telah dipilih daripada yang paling berpotensi telah digunakan dan dioptimumkan dengan menggunakan beberapa parameter seperti keadaan pengeraman, kepekatan substrat, tempoh pengeraman, nilai pH dan sumber nitrogen. Kemudian, kuantifikasi protin dan aktiviti enzim dianalisis dengan menggunakan Pembaca Microplate. Untuk pencirian SNP, analisis menggunakan Pembelauan Sinar X (XRD) dan Spektrometri Massa Plasma yang Digabungkan secara Induktif (ICP-MS) telah digunakan. Hasil kajian menunjukkan bahawa pengasingan CL4C dan GL7 yang paling kuat masing-masing menunjukkan saiz purata SNP dihasilkan sehingga 19.53 nm dan 52.35 nm. Di samping itu, aktiviti NR didapati optimum dalam keadaan pengeraman statik, tempoh pengeraman 15 hari dalam kegelapan dan menggunakan natrium nitrat sebagai sumber nitrogen. Kemudian, kepekatan substrat untuk mikrob CL4C dan GL7 masing-masing didapati optimum apabila 0.5 dan 0.8g lupusan lembaran x-ray ditambah. Nilai pH optimum CL4C dan GL7 masing-masing adalah pada pH 7.0 dan 8.0 dengan aktiviti enzim dan kandungan protin sehingga 69.952±0.090 IU/ml dan 1.139±0.029 mg/ml untuk CL4C. Sementara itu, aktiviti NR bagi mikrob GL7 adalah 108.882±0.354 IU/ml dengan kandungan protin sehingga 1.454±0.024 mg/ml. Oleh itu, mikrob CL4C telah diramal sebagai *Morganella morganii* dan GL7 diramal sebagai *Enterobacter aerogenes* dengan menggunakan analisis Biolog. Ini dapat disimpulkan bahawa dengan menggunakan kaedah biologi, ia kurang menggunakan bahan kimia yang beracun dan alternatif yang baik untuk bioremediasi. Dalam kajian ini, impak menggunakan CL4C dan GL7 sebagai mikrob paling berpotensi yang dapat menggunakan lembaran x-ray yang dilupuskan sebagai substrat adalah penemuan baru dalam bidang penyelidikan ini kerana sebelum ini tidak ada kajian mengenai penggunaan substrat ini.

ABSTRACT

The production of x-ray sheets can utilize up to 1000 tons of silver chemically produced worldwide. The pollution occurs when the used x-ray sheets are not recycled. Microbial management could be used to manage this problem in order to remove and neutralize the pollutants from contamination. This study aims to examine the microbial management of disposed x-ray sheets in silver nanoparticles (SNPs) production and the role of the nitrate reductase (NR) enzyme involved in the processes. In this research, disposed x-ray sheets were used as the sole carbon source to synthesis SNPs and catalyse the reduction of silver nitrate to silver nitrite. Different bacterial isolates which had been chosen from the most potent isolates were used and being optimized by using several parameters including incubation condition, substrate concentration, incubation period, pH values and nitrogen source. Then, the protein quantification and enzyme activity were analyzed by using microplate reader. For characterization of the SNPs, the X-Ray Diffraction (XRD) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis were used. The results revealed that the most potent isolates CL4C and GL7 showed the average size of SNPs produced was up to 19.53 nm and 52.35 nm respectively. In addition, NR activity was found to be optimum at static incubation condition, 15 days incubation period in darkness and using sodium nitrate as the nitrogen source. Then, substrate concentration of CL4C and GL7 isolates found to be optimum when 0.5 and 0.8g of disposed x-ray sheets were added respectively. The optimum pH values of CL4C and GL7 were exhibited to be 7.0 and 8.0, respectively, with the enzyme activity and protein content up to 69.952±0.090 IU/ml and 1.139±0.029 mg/ml for CL4C. Meanwhile, the NR activity of the GL7 isolate was 108.882±0.354 IU/ml with the protein content up to 1.454±0.024 mg/ml. Consequently, the CL4C isolate was predicted to be *Morganella morganii* while GL7 was predicted to be *Enterobacter aerogenes* by using a Biolog analysis. It can be concluded that, by using the biological method, it requires less toxic chemicals and as a good alternative for bioremediation. In this study, the impact of having isolated CL4C and GL7 as the most potent microbe which able to use disposed x-ray sheets as substrate was a new finding in this research area because there was no previous study yet regarding the uses of this substrate.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

°C	degree Celsius
µg	microgram
mg	milligram
ml	milliliter
M_r	relative molecular mass
nm	nanometer
pH	potential of Hydrogen
λ_{max}	maximum of light wavelength absorbed
kDa	kilodalton

LIST OF ABBREVIATIONS

A	Apple
Abs	Absorbance
Ag	Argentum/Silver
AgBr	Silver Bromide
ATP	Adenosine Triphosphate
BPB	Bromphenol Blue
BSA	Bovine Serum Albumin
Cb	Cytochrome b
CbR	Cytochrome b reductase
CcR	Cytochrome c reductase
CL	Chicken Liver
DNA	Deoxyribonucleic acid
EC	Enzyme Code
FAD	Flavin Adenine Dinucleotide
GL	Gambang Lake
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
JF	Jackfruit
KK2	Kolej Kediaman 2
LSPR	Linear Surface Plasmon Resonance
Mo-MPT	Molybdenum-molybdopterin
mRNA	messenger Ribonucleic Acid
NA	Nutrient Agar
$NaBH_4$	Sodium Borohydride
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
Nap	periplasmic dissimilatory
Nar	membrane-bound respiratory
Nas	cytoplasmic assimilatory
NB	Nutrient Broth
NEED	N-(1-Naphthyl)ethylenediamine dihydrochloride
NR	Nitrate Reductase
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PMF	Proton Motive Force
ppb	parts-per-billion, 10^{-9}
ppm	parts-per-million, 10^{-6}
ppq	parts-per-quadrillion, 10^{-15}
PU	Polyurethane
RIU	Refractive Index Units
rpm	revolutions per minute
RSS	Sungai Belat Soil
SAED	Selected Area Electron Diffraction
SD	Standard Deviation
SNPs	Silver Nanoparticles
SPR	Surface Plasmon Resonance
STEM	Scanning Transmission Electron Microscopy
SXE	*Solanum xanthocarpum* berry

TEM	Transmission Electron Microscopy
UV-Vis	Ultraviolet–visible spectroscopy
WHO	World Health Organization
XRD	X-Ray Diffraction

CHAPTER 1

INTRODUCTION

1.1 Introduction

The purpose of this chapter was to provide an overview on the research that has been conducted. This chapter provides an explanation on what constitutes of pollution, bioremediation, disposed x-ray sheets and the enzyme responsible for microbial degradation. This chapter also presents the background of study, problem statement, research objectives and the overview of this research.

1.2 Background of Study

In recent years, rapid industrialization has caused a lot of pollution around the world. This occurs due to the lack of awareness on the harmful effects of pollutants to nature, especially to the aquatic environment. Water pollution occurs due to the presence of chemical, physical, radioactive or pathogenic substances which cause illness and deaths. Therefore, this study was done in order to reduce the pollution by using biological methods called bioremediation. In this regard, introducing fast and trustworthy processes for the synthesis of nanosized materials is very important in the field of nanotechnology; nanobiotechnology refers to the process which includes biological entities in the method such as viruses, fungi, bacteria and plants.

Bioremediation should be done to remove and neutralize pollutants from contamination. It is a natural treatment that uses biological agents, such as fungi and bacteria to break down or remove hazardous substances into less or non-toxic substances. It is the safest way to remove hazardous substance as it does not require any

toxic, harsh and costly chemical. In this study, the bioremediation of disposed x-ray sheets was conducted for silver nanoparticles production. Disposed x-ray sheets contain silver nanoparticles which are white, soft, thermal conductive and lustrous metal with high electrical properties. The silver nanoparticles were found to have medical and therapeutic benefits before it was realized that microbes was actually agents for infections (Firdhouse & Lalitha, 2015).

In recent years, studies have demonstrated the abilities of biological entities to absorb and accumulate inorganic metallic ions from their surrounding environment. These desirable properties make the biological entities capable to significantly reduce environmental pollution and reclaim metals from industrial waste. Nowadays, their abilities to accumulate, interact and extract metallic materials from surroundings have been capitalized through bioremediation and bioleaching (Shah, Fawcett, Sharma, & Tripathy, 2015) where microorganisms are able to detoxify the hazardous components such as silver nanoparticles in the environment through the natural process. This process can be improved by some factors such as the use of nutrients and the addition of electron acceptors (Ayangbenro & Babalola, 2017).

In this project, reductase enzyme specifically the nitrate reductase (NR; EC 1.7.99.4) enzyme was produced and used to reduce silver nitrate into nitrite (Gupta, Mukherjee, & Singh, 2014) inside the samples of x-ray sheets which act as substrates during the synthesis period through the use of microbial power. Nitrate reduction involves assimilatory and dissimilatory reduction. During assimilatory reductions, energy is used and assimilatory nitrate reductions are performed by bacteria, fungi or algae, while dissimilatory reduction yields energy. Both processes form byproducts by reducing the electron acceptor. Furthermore, SNPs were produced as a byproduct, and the enzyme activity and protein quantification were measured. Lastly, the silver nanoparticle was characterized by using X-Ray Diffraction (XRD) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

1.3 Problem Statements

X-ray was discovered over 100 years ago and it still used as the fundamental part of diagnostic examination and treatment. The x-ray is responsible in the field of radiotheraphy where it is commonly used as dental imaging, medical imaging and non-destructive testing in the manufacturing industry. Ionizing reduction is commonly used in x-ray examination. As the x-ray beam can penetrate into most of the object, x-ray sheets were used to record the image. The production of x-ray sheets are controlled by several global companies such as Fuji Medical System, Kodak and Sterling Diagnostic Imaging/Agfa. According to the Silver Institute, the total world consumption of x-ray sheets is about 2.16 billion images each year. Used sheets are often not recycled, and this creates wastage and produces as hazardous waste due to the presence of silver in the sheets. As a result, disposed silver sheets increases solid waste, especially in Malaysia and this brings negative effects the environment. The production of these wasted sheets can utilize up to 1000 tons of silver annually and if the silver used was in high concentration, it will affect the living organisms. In order to manage this problem, this study conducts bioremediation by using disposed x-ray sheets as substrates for biological entities required reducing the silver pollutants in the sheets and changing them into less hazardous forms. In the meantime, the enzyme produced in this process was also studied. The biological method was used in this study as it is fast, environmentally safe, and requires a minimal cost. Microbial management was used to study the silver nanoparticles production and the related enzymes involved in this study. Through the bioremediation conducted in this study, wastage of X-ray sheets can be reduced as they can classified as waste that can be turned to wealth. This is because the silver was found in the x-ray sheets can be used in various applications that give benefits to the industry.

1.4 The Objectives of The Research

1. To isolate, purify and identify the microorganisms from different sources - soil, water and spoiled food samples for most potent bacteria.

2. To screen, determine and optimize microbial silver nanoparticles and nitrite production.

3. To characterize the microbial silver nanoparticles (SNPs) and nitrate reductase (NR) produced using X-ray Diffraction (XRD) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

1.5 Overview of The Report

This report was about the overall research that has been conducted including the literature review, research methodology, results achieved, discussion, conclusion and recommendation for future research. The literature review focused on hazardous waste and x-ray sheets, SNPs, the application of SNPs and their mechanisms, microorganism producing SNPs, enzyme mechanism as well as microorganism producing NR enzyme and their applications based on previous paper research. Meanwhile, the research methodology chapter details the methodology used in this research. For this study, the bacteria were isolated from various sources (soil, water and spoiled food) to obtain specific bacterial strains which were used for the biosynthesis of SNPs. After that, the enzyme and protein assay was done to measure the enzyme activity and protein content in the samples and consequently, the most potent microbial isolates were chosen. Then, the size of silver nanoparticles and concentration of silver elements produced were characterized by XRD and ICP-MS analysis, respectively.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The purpose of this chapter is to review past research related to the biosynthesis of SNPs production and its enzyme production.

2.2 Hazardous Waste and X-Ray Sheets

A hazardous waste is a waste potentially dangerous or harmful to our health and the environment. There is 400 million tons of various hazardous waste produced yearly which could be in solids, liquids or gaseous forms. They are also produced as by-products of manufacturing processes, discarded used materials, or discarded unused commercial products, such as cleaning fluids (solvents) or pesticides. Wastes can be classified as hazardous if they are corrosive or exhibit ignitability, reactivity or toxicity (The World Count, 2017).

Ignitability means that the waste can spontaneously combust or have a flash point less than 60 °C (140 °F) under certain conditions, for example using solvents and oil waste. A corrosive waste is the waste that produces an acidic or alkaline solution and other materials including acids or bases solids. Such wastes are in aqueous forms with a pH less than or equal to 2.0 or greater than or equal to 12 which are classified as corrosive. These liquid wastes may also be corrosive and can corrode metal containers. Reactivity means that the waste that can cause explosions or release toxic gases or vapors when heated, compressed or mixed with water. These wastes are unstable under normal conditions, for instance, a lithium-sulfur battery. Meanwhile, toxic wastes are

harmful or fatal when they are absorbed or ingested. Improper disposal of toxic wastes could cause toxic wastes such as mercury or lead to enter and pollute the water ways. (California Code of Regulations, 2013).

Generally, the term hazardous is deemed as synonymous with dangerous. In this regard, hazardous wastes are labelled according to their specific class. These wastes will be dangerous to living things and the environment when handled, transported or disposed of in an unsafe manner; hazardous substances can potentially come from three major waste streams. The first stream comprises of municipal waste, such as components of electronic, household chemicals and products with nano-sized ingredients such as titanium oxide in cosmetics and nano-silver in skin gel. The second stream comprises of commercial and industrial wastes such as heavy metals while the third stream of waste comprises of construction and demolition wastes such as asbestos (White & Heckenberg, 2011).

In the photographic industry, wastes such as from x-ray film processing are regulated by Minnesota Pollution Control Agency (MPCA) under the Hazardous Waste Rules. This is because they are corrosive and have toxic reactions. X-ray films could either have low or high pH and could release silver or chromium. In this light, x-ray sheets usually contain silver concentrations that are not high enough to be toxic depending on the concentration available (Minnesota Pollution Control Agency, 2011).

The basic composition of an x-ray film is similar to a photographic film, however, an x-ray film has a photographic receptor that consists of photographically active or radiation sensitive emulsion. The emulsion coats both sides of a thin, sheet-like materials as shown in Figure 2.1. It is responsible to record the images of the object exposed to the x-ray. The x-ray sheets composed of a film base, a film emulsion, an adhesive layer and a protective layer. The function of this film base is to support the fragile photographic emulsion on both of its surfaces. The base is flexible and quite strong so that the films can be snapped frequently into x-ray illuminators. It must also be thick enough to withstand any geometric distortion due to the heat generated during the film development process and the base must provide a uniform, highly transparent, optical background (Houston, 2007).

The film emulsion is the heart of a radiographic film. The information is transferred to the sheets during the interaction between x-ray and emulsion. It is composed of a very homogenous mixture of gelatin and small silver halide crystals (grains). The gelatin keeps the silver grains well dispersed and prevents them from clumping. The use of the developing and fixing solutions cause the gelatin to penetrate rapidly without changing the strength or permanence of the gelatin. The gelatin is used as a binder because it can act as a medium that the silver nitrate and sodium bromide can react with and release fine AgBr before being dispersed. Meanwhile, the small silver halide crystal (1.0 to 1.5 microns in diameter) comprises of the light-sensitive substance in the emulsion where bigger grain size will increase the speed of the film. The crystal may form tabular, globular, polyhedral or irregular shape. These grains are known as silver-iodo- bromide and are typically comprised of 90 to 99% silver bromide and 1 to 10% silver iodide (Houston, 2007)

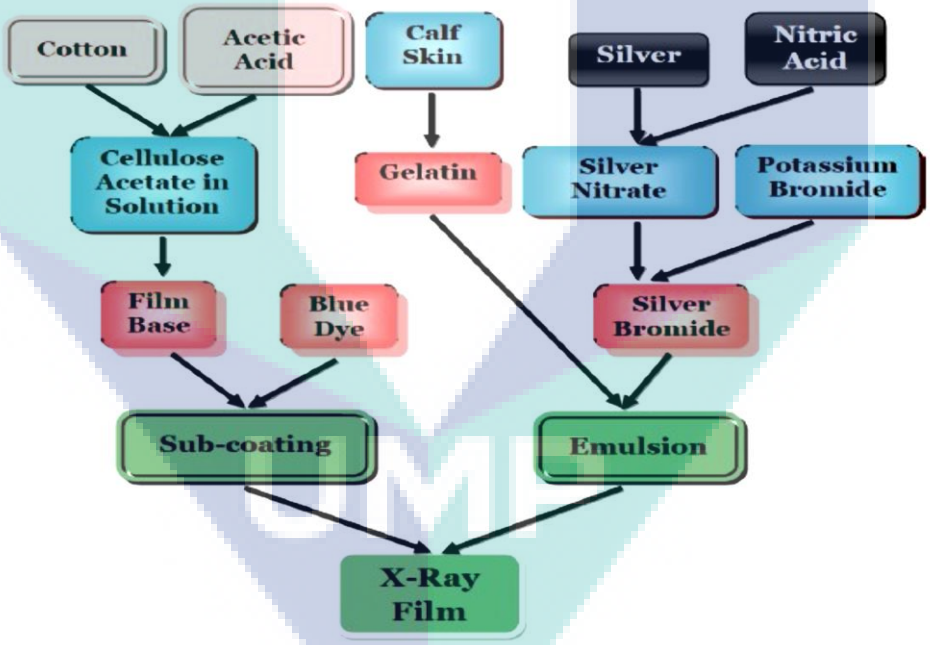

Figure 2.1 The composition in an x-ray film

Source: Houston (2007)

The function of the adhesive layer is to attach the emulsion to the film base because in general, both are not able to adhere to each other. Thus, a clear, thin layer of gelatin is suitable to be used as an adhesive. Then, the protective layer functions to protect the emulsion from being scratched and damaged by normal handling, hence a

very thin outer protective layer usually made of gelatin could also be applied (Feldstein & Sloan, 2015).

2.3 Silver Nanoparticles (SNPs)

The study on nanoparticles is particularly interesting as working with and synthesizing extremely small structures are much more complicated than their macroscopic counterparts. This is because nanostructures possess unique properties and characteristics. One of these characteristics is that nanoparticles have a very high surface to volume ratio. Consequently, nanoparticles can be used in the catalytic industry and some nanoparticles actually have been recognized as good catalysts. They also show bactericidal effects, but their most significant effect is its high surface to volume ratio. The size of nanoparticles often ranged between 1 – 100 nm, which is similar to the size of human proteins as shown in Figure 2.2 (Zhang, Liu, Shen, & Gurunathan, 2016).

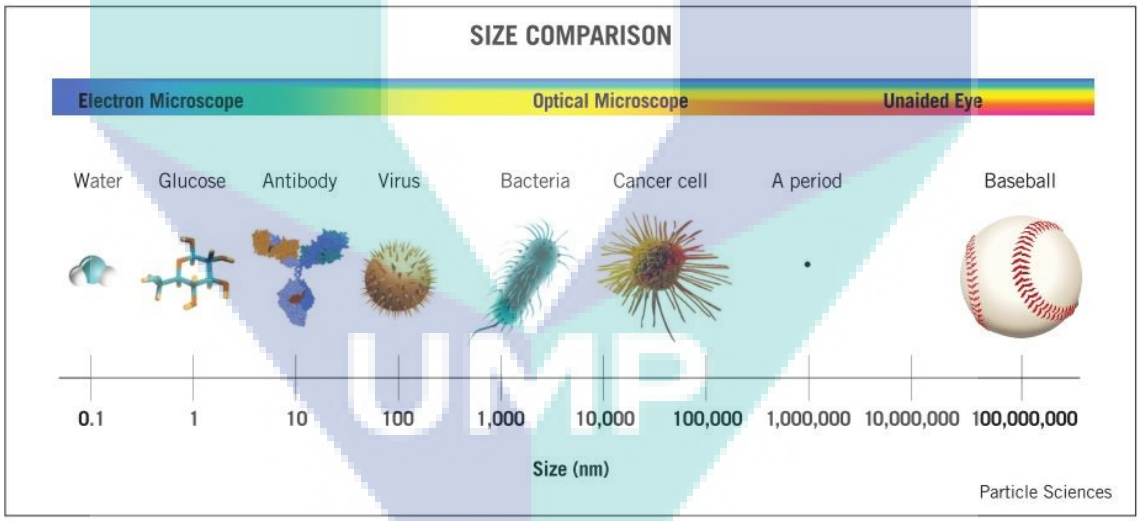

Figure 2.2 Size comparisons in nanometer scale

Source: Particle Sciences (2012)

In the field of pharmaceutical nanotechnology (nanopharmaceuticals), there are various kind of nanoparticles including magnetic nanoparticles, polymeric nanoparticles, carbon nanotubes, quantum dots, metallic nanoparticle and dendrimers. Their presence has brought huge changes in the general medical service system, particularly the delivery of drugs (Bhatia, 2016). In the meantime, scientists from the

Chinese Academy of Science recommended the use of gold nanoparticles to improve Polymerase Chain Reaction (PCR). Nanoparticles have also used in the production of valuable anti-reflective optical coatings. In this regard, nanoparticles do possess many exciting qualities and over time, more of these qualities will be exploited (Diantoro, Fitrianingsih, Mufti, & Fuad, 2014).

Three preparation techniques, physical, chemical and biological methods can be conducted to synthesize the SNPs. However, most researchers used biological synthesis because this technique provides particles with good control over the size distribution. The procedures structured by nature for the synthesis of inorganic components on nano and microscales have contributed to the growth of a relatively new and mostly unexplored area of research which focuses on the use of bacteria in the biosynthesis of nanomaterials (Mandal, Bolander, Mukhopadhyay, Sarkar, & Mukherjee, 2006).

Biosynthesis of nanoparticles can be categorized into two - intracellular and extracellular synthesis, based on the location where the nanoparticles are formed. An intracellular synthesis is a method consists of transporting ions into the microbial cell to form nanoparticles in the presence of enzymes. Whereas, extracellular methods involves trapping metal ion on the surface of cells and reducing ions with the presence of enzymes (Zhang, Yan, Tyagi, & Surampalli, 2011).

Elahmohammad & Adeebauniesshareef (2014) used biological method to synthesize the nanoparticles as an alternative method to complex chemical synthesis. By conducting this method, the SNPs could be effectively produced by pathogenic *Pseudomonas aeruginosa*, which show the highest microbial activity compared to *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Enterococcus* sp.

According to Zhang, Liu, Shen, & Gurunathan (2016), biological methods are simple, rapid, non-toxic, dependable, and green approaches to produce well-defined size and morphology under optimized conditions for translational research and chemistry approach for the synthesis of SNPs . On the other hand, particle characterization is necessary as part of the synthesis. This is because the physicochemical properties of a particle have a significant impact on its biological properties. It is necessary to characterize the prepared nanoparticles before their application to address safety issue in

the use of any nanomaterials for the purpose of human welfare in the healthcare industry, particularly in nanomedicine.

Meanwhile, the evaporation-condensation process involves the use of a tube furnace at atmospheric pressure and laser ablation to physically prepare the nanoparticles through the physical method. The advantages of the physical methods include they are often fast and do not involve the use of hazardous chemicals as radiation is used as a reducing agent. However, these methods have low yield and consume a large amount of energy, they are also prone to solvent contamination and there is a lack of uniform distribution. A typical tube furnace requires more kilowatts of power consumption and more preheating time to reach a stable operating temperature. Through the use laser ablation, the silver nanoparticle was synthesized using metallic bulk materials in solution. The advantage of this technique in the production of metal colloids is the absence of chemical reagents in the solution. However, the efficiency of the ablation and the characteristics of the nano-silver produced depend on various parameters including the wavelength of the laser which influences the metallic target, the duration of laser pulses, laser fluence, time duration and the effectiveness of the liquid medium (Iravani, Korbekandi, Mirmohammadi, & Zolfaghari, 2014).

In the chemical methods, water or organic solvents are used to prepare the silver nanoparticles. Three main components are involved, which are metal precursors, reducing agents, and stabilizing or capping agents. Basically, the reduction of silver salts involves two stages -nucleation and subsequent growth. However, both methods are extremely expensive. According to Gudikandula & Maringanti (2017), there are various methods suggested for the chemical synthesis of silver nanoparticle formation such as chemical reduction method, radiolytic process and the polyol method. In this light, the chemical reduction method is deemed as the easiest, low cost and high yield method for yielding nanoparticles without aggregation.

2.4 Applications of SNPs

Due to their unique physical and chemical properties, SNPs are increasingly used in various fields, including for medical, food, healthcare, consumer, and industrial

purposes. Some of the important applications of SNPs are shown in Figure 2.3. The silver nanoparticle is one of the most commercialized inorganic nanoparticles among the other nanoparticles. One of the possible applications of SNPs is as a catalyst.

It also can be immobilized on silica spheres that have been tested for their ability to catalyze the reduction of dyes by sodium borohydride ($NaBH_4$). The samples showed very little or no reduction of the dyes in the absence of SNPs, and a reduction was only observed when SNPs were added into the solution. This shows that the reaction time strongly dependent on the SNP concentration as when the concentration was doubled, the reaction time was reduced to less than 30 percent. Furthermore, the images of the nanoparticles obtained using the scanning electron microscopy (SEM) revealed that they were unbroken after the reaction and still immobilized on the silica spheres. This shows that the particles act as catalysts because they are not absorbed in the reaction (Diantoro, Fitrianingsih, Mufti, & Fuad, 2014).

Important applications of Silver Nanoparticles
Treatment of ulcerative colitis & acne
Treatment of dermatitis
Inhibition of HIV-1 replication
Enhanced raman scattering spectroscopy (SERS)
Detection of viral structures (SERS & silver nanorods)
Antimicrobial effects against infectious organisms
Remote laser light-induced opening of microcapsules

Figure 2.3 Important applications of silver nanoparticles
Source: Iravani et al. (2014)

The SNPs are useful in controlling and suppressing bacterial growth due to their good antimicrobial potency against viruses, bacteria and eukaryotic microorganism (Elahmohammad & Adeebauniesshareef, 2014). Silver is deemed as a biocide because it is highly toxic to most microbial cells and its presence can cause the destruction of life.

However, several bacterial strains are resistant to silver and could be used to derive silver from the cell wall up to 25% of their dry weight biomass. This indicates their potential use for industrial recovery of silver from ore materials. The *Pseudomonas stutzeri* AG259 can derive the silver bacterial strain within the size range of 35–46 nm, as well as the silver sulphide in the SNP cells (Silver, 2003; Vaidyanathan et al., 2010).

Consequently, several applications had been using the bactericidal effect of SNPs. Jain & Pradeep (2005) described the procedures to produce and test bactericidal foam. The aim of their research was to demonstrate the bactericidal effect of silver nanoparticle-coated foam. In this light, SNPs were present in the form of foam which was caused by the coating process. After being washed with water, the SNPs also remained in the foam form. These results showed that it was possible to coat Polyurethane (PU) foams with SNPs by a simple and cheap process. According to the assessment outcome performed on the bacteria, the coated foam had a bactericidal effect on the *Escherichia coli*. In order to validate the result, they had conducted three different tests. But all the results revealed that no bacteria were recognized afterwards. The assessments and results are in line with World Health Organization (WHO, 2017) specifications for drinking water. The overall outcome from the article obviously reveals that the PU foams can be coated with SNPs and the bactericidal effect of these was verified. It proved that SNPs-coated foams can be used as water filtration. As the chemicals used are low-cost and normally available and the coating procedure is simple, it should be possible to make this available to undeveloped countries.

There are two groups of bactericidal effect of silver which are the reactive component being either silver ions or SNPs. Silver ions (Ag^{+}) are charged atoms whereas SNPs comprise of single crystals with nanosize dimensions. On the other hand, even though the bactericidal effect of silver ions is well known, their uses are still not widely recognized. In this light, tests have proven that silver ions are able to change the structure of the cell membrane. There are many sulfates with enzymes in the bacteria's membrane and studies suggest that silver ions' interactions with these sulfate groups cause changes to the membrane morphology by mollifying the enzymes. This inactivation makes the membrane susceptible and easier to penetrate by the silver ions. Silver ions keep destructing different parts of the cell by interacting with sulfate-groups, which are frequently located in the active site of enzymes. This interaction with the

active site causes the inactivation of the enzymes (Diantoro, Fitrianingsih, Mufti, & Fuad, 2014).

Other than the sulfate-groups, silver ions are also able to interact with phosphorus-groups of molecules which have shown severe impacts such as the interaction between silver ions and the DNA backbone which stops the microbe's ability to replicate itself or to transcribe the mRNA for new proteins. All these changes stun the growth of the bacteria and consequently kill it (Diantoro, Fitrianingsih, Mufti, & Fuad, 2014).

A series of experiments were carried out to test the bactericidal effect of SNPs by using four different Gram-negative bacteria treated with SNPs in different sizes and shapes. The bacteria used are *Pseudomonas aeruginosa, Vibrio cholera, Salmonella typhus* and *E. coli*. These experiments showed that SNPs interact with the membrane and interior of the bacteria. These interactions between SNPs and the bacteria look quite similar with the interactions between silver ions and bacteria. In this light, both interactions could cause the same types of loss to the bacteria (Morones et al., 2005).

Meanwhile, the antibacterial activities increase in the presence of biologically synthesized silver nanoparticles. This increase was observed for Bacitracin against *E. coli* and *Salmonella paratyphi B.*, Ampicillin against *Corynebacterium diphtheriae*, Kanamycin against *Klebsiella pneumonia,* gentamycin against *P. aeruginosa* and for Bacitracin, Gentamycin, Erythromycin, Ciprofloxacin against *Staphylococcus aureus* (Gandhi & Khan, 2016).

Another possible application of SNPs is as an optical sensor of zeptomole (10^{-21}) sensitivity. The surface plasmon resonance caused SNPs to be highly sensitive which allows real-time measurements to be performed. According to McFarland & Duyne (2003), this is due to the effect of localized surface plasmon resonance (LSPR) and as a result, SNPs display a peak in the extinction caused by a collective excitation of the conduction band electrons of the nanoparticle. The λ_{max}-value of this plasmon resonance peak is dependent upon the size, shape and the solvent and adsorbates used. However, when UV-vis spectroscopy was used, the signal-to-noise ratio makes it difficult to identify the signal of a single nanoparticle even under the most optimal conditions.

The experiments conducted in McFarland & Duyne (2003) showed a slight difference between the sensitivity of a single SNPs and the array (sensitivities of 191 and 197 RIU^{-1} respectively). This indicates a linear relationship between the LSPR λ_{max} with respect to the refractive index. This sensitivity depends on the shape and size of the nanoparticles. Moreover, the sensor's real-time response in yielding a real-time response comparable with that of an array was examined. The study found that an array of unique nanoparticles can concurrently work as individual detectors by controlling their size, shape and chemical modification. One of the possible applications of high sensitivity LSPR is in vivo detection. It can monitor the quantity of chemical species inside a cell and monitor the dynamical processes that occur.

2.5 Microorganisms Producing SNPs

The booming demand for "green" nanoparticle synthesis processes has inspired the development of a new research routes for nanoparticles in recent years. This includes the utilization of living bacteria, fungi or plant extract including *P. stutzeri*, *Verticilium* sp., *Fusarium. oxysporum*, *Thermomonospora* sp., alfalfa and geranium plants. One of the examples of such studies is Sastry, Ahmad, Islam Khan, & Kumar (2003) which produced extremely stable silver hydrosols, using the fungus (*F. oxysporum*). However, even though the green methods of producing particles are stable and are not mono-dispersible, they have a different rate of synthesis compared to the chemical synthesis methods. This creates a need to find new microorganisms that can be used for such synthesis and to improve the synthesis methods (Mohammadian, Shojaosadati, & Rezaee, 2007).

The biological synthesis of SNPs using *Bacillus thuringiensis* was also reported by Dash (2013). The bacterial strain was cultured on a shaker at room temperature. The SNPs was found in detailed characterization by using UV-visible spectroscopy, Scanning Electron Microscopy (SEM), XRD and Fourier Transform Infrared Spectroscopy (FTIR). UV-visible spectroscopy recorded the absorption peak at 420nm, while SEM image confirmed the spherical shape of SNPs. Next, the XRD analysis found SNPs crystalline in nature as shown by the FTIR peak at 518 cm^{-1}.

Natarajan, Selvaraj, & Murty (2010) study used the *E. coli* bacterial strain. This bacterial strain was cultured and incubated on an orbital shaker at 27 °C in order to investigate the microbial production of silver nanoparticles. This research focused on the effect of different basal medium compositions, which are nutrient broth and Lysogeny broth (LB) broth, on the nanoparticles produced. They characterized the nanoparticles by using UV-visible spectroscopy and a laser diffraction particle size analyzer.

Fungi could also be potentially used to produce SNPs because they can secrete a large amount of enzymes. Ganesh et al. (2008) used *Aspergillus flavus* and found that the SNPs have accumulated on the surface of its cell wall when the fungi were incubated with silver nitrate solution. In this light, *Aspergillus fumigatus* can rapidly synthesize the SNPs and monodisperse particles were formed which can be used in bacterial applications.

Meanwhile, the cell walls of the Gram-positive bacteria were found to bind with large quantities of metals compared to the Gram-negative bacteria. In this regard, the role of bacterial cells in the destiny of metals in the environment has not been methodically analyzed even though they signify an important component of metal characteristics. The first evidence of SNPs synthesis was established in 1984 using the *P. stutzeri* AG259, a bacterial strain that was originally isolated from silver mine (Nair & Pradeep, 2002).

Bacillus licheniformis is one of the organisms used to synthesize SNPs at 1 mM concentration. When the concentration of the silver ion in the environment is 1 mM, the organism can synthesize SNPs without undergoing an apoptosis. However when the concentration of the silver ions is higher, for example, 10 mM, the organism would undergoes cell death within minutes, especially when the concentration crosses the threshold level (Vaidyanathan et al., 2010).

Pugazhenthiran et al. (2009) proved silver precipitation by observing *Bacillus* sp. by using TEM after exposure to a 3.5 mM aqueous $AgNO_3$ solution. Compared to many other reviews of metal-resistant bacteria, where efflux of harmful ions is the main detoxification mechanism (Vaidyanathan et al., 2010), the majority of the accumulated

silver here was deposited as seed-like particles between the outer membrane and the plasma membrane.

Meanwhile, the Abd, Al-Kawaz, & Al-Dahmoshi (2013) presented images of silver nanoparticle (36.9 nm) in the *Morganella morganii* samples after 20 h reaction obtained from their TEM analysis. Moreover, Saifuddin and his co-researchers posited the presence of varied nanoparticle morphology of in the micrographs, such as spherical and triangular nanoparticles. As the nanoparticles were not in direct contact with the aggregates, there is a presence of capping agents such as protein, which is secreted by the bacteria to stable itself (Saifuddin et al., 2009).

Instead of using biological entities, a green synthesis route for the production of silver nanoparticles using methanol extract from *Solanum xanthocarpum* berry (SXE) was reported by Amin, Anwar, Janjua, Iqbal, & Rashid (2012). In the study, SXE (as capping as well as reducing agent) reacted with $AgNO_3$ resulting in SNPs with a surface plasmon resonance (SPR) band centered at 406 nm, during a 25 min process at 45 °C. The results showed that the time of reaction, temperature and volume ratio of SXE to $AgNO_3$ could accelerate the reduction rate of Ag^+ and affect the SNPs size and shape. The nanoparticles were found to be about 10 nm in size, mono-dispersed in nature, and spherical in shape.

The images of the nanoparticles are uniformly sized across the different medium (Vaidyanathan et al., 2010). By comparing the results with Pugazhenthiran et al. (2009), the substrate that they used is 3.5 mM $AgNO_3$ (silver nitrate) and the biogenic nanoparticles sized 10 – 15 nm were observed in the periplasmic space of the bacterial cells between the outer and inner cell membranes using the higher magnification TEM.

Meanwhile, an experiment using fresh leaf extract found the average size of particles to be 50 nm within the size range of 30 nm to 80 nm. Some irregular shaped of SNPs were observed. However, as this research is more focused on smaller sized particles, the solution was centrifuged at 1200 rpm for 15 min until the particles present in the supernatant were homogenous and reached the average size of 7 nm. Since there are numerous methods for synthesizing SNPs, the shapes obtained were also varied as shown in Figure 2.4. These include octahedral, spherical, cubic, disc, shell shapes,

hexagonal, tetrahedral, wire, triangular prism, triangular mark, coaxial cable and belt (Jawaad, Sultan, & Al-Hamadani, 2014).

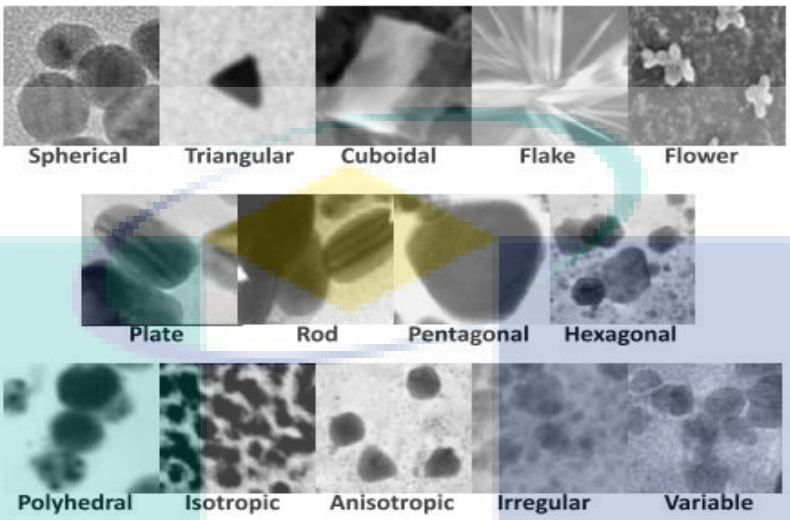

Figure 2.4 Various shape of SNPs

Source: Srikar, Giri, Pal, Mishra, & Upadhyay (2016)

According to a previous study by Natsuki, Natsuki, & Hashimoto (2015), there are several factors that affected the size of SNPs including dispersants, reducing agents, amine and temperature. In their study, the sizes of SNPs obtained were from 7 nm to 40 nm by TEM analysis and the particle's diameter distribution was measured with Zetasizer Nano Series. During their study, when the reaction was performed at 50 °C, the large silver particles formed and the shape appeared to be linked, not spherical. From Al-Marhaby & Seoudi (2016) studied, during TEM analysis, they were found that the silver nanoparticles found to be roughly spherical in shape and irregular distribution as shown in Figure 2.5 when the samples were prepared under different concentrations of trisodium citrate.

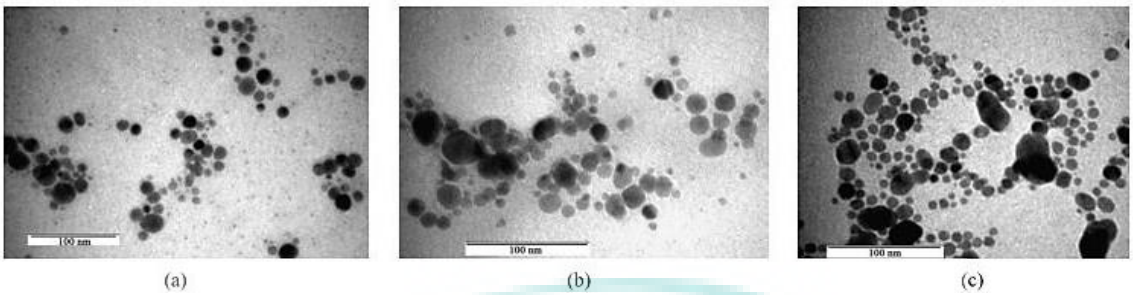

Figure 2.5 TEM image for SNPs in roughly spherical shape and irregular distribution

Source: Al-Marhaby & Seoudi (2016)

2.6 Nitrate Reductase Enzyme

In production of silver nanoparticles, the nitrate reductase was the important enzyme that responsible in the reduction process of silver ions to silver nanoparticles (bioreduction of silver ions). The presence of nitrate reductase enzyme and the involvement nitrogen gas were observed in the process. Nitrogen plays an important role in proteins and nucleic acids. It was also important biological macromolecules which form the basic elements of life. There are two processes for nitrogen removal - nitrification and denitrification (The Water Planet Company, 2009).

Nitrate reductase (NR; EC 1.7.99.4) comprised of molybdoenzymes that reduced nitrate to nitrite. It is also known as a respiratory nitrate reductase which is an important reaction in protein production. Nitrate reduction can be carried out for three different purposes, which are nitrate assimilation, nitrate respiration and nitrate dissimilation. The assimilation is a process of utilizing the nitrate as a source of nitrogen for growth. The assimilatory nitrate reduction is performed by bacteria, fungi, algae and higher plants while nitrate respiration means that the metabolic energy is generated by using nitrate as a terminal electron acceptor. Lastly, dissimilation is a process of excreted the excess reducing power for redox balancing. During the nitrification, the nitrogen is removed from the environment by oxidative conversion of ammonia to nitrate. In the meantime, in denitrification process, the nitrate is successfully reduced to nitrite, N oxides (NO and N_2O), and dinitrogen (N_2). The reduction of nitrate plays an important role in the nitrogen cycle.

The nitrate reductase catalyzes the two electron reduction of nitrate to nitrite by four types which are eukaryotic assimilatory nitrate reductase and three different bacterial enzymes made up of cytoplasmic assimilatory (Nas), membrane-bound respiratory (Nar) and a periplamic dissimilatory (Nap) nitrate reductase. The eukaryotic and prokaryotic reductase contain a molybdenum cofactor at their actives sites while in both assimilatory nitrate reductases, even though there are no similar sequence, they share a common physiological function.

In regard to the bacterial enzymes, there are two types of dissimilatory nitrate reductase present which are membrane-bound respiratory (Nar) and periplasmic dissimilatory (Nap) nitrate reductase. In Nar, a transmembrane proton motive force (PMF) is generated which allows ATP synthesis. The periplasmic seems to be dissimilatory enzyme to some Gram-negative bacteria, such as *Shewanella* genus (Chen & Wang, 2015). In their study, the *Shewanella* genus contains special traits allowing the bacteria to possess two periplasmic nitrate reductase systems (Nap-α and Nap-β).

Nitrate induces the enzyme in darkness only when suitable sources of reducing equivalent is added which permits the formation of nitrite. The nitrite itself facilitates about induction of the enzyme in darkness without the supplementation of exogenous reducing cofactors. The inhibition of constitutive nitrate reductase by tungstate prevents the induction of the enzyme by nitrate but not by nitrite. Nitrate induces the enzymes only under conditions which allow the production of nitrite while the induction capacity of nitrite is free of this constraint, hence, nitrate reductase can be considered a product-inducible rather than a substrate-inducible enzyme. In this regard, the NR monomer is composed of a 100 kDa polypeptide and each contains flavin adenine dinucleotide (FAD), heme-iron (Fe), and molybdenum-molybdopterin (Mo-MPT).

NR was identified as a flavoprotein which contains a heme-Fe and molybdenum complexed with a unique pterin or molybdopterin (refer Figure 2.6). NR was shown to catalyze partial reactions including a dehydrogenase functionality characterized by the NADH-dependent reduction of ferricyanide and mammalian cytochrome c, which could be inhibited at a sensitive thiol protected by NADH. The nitrate reduction could also be handled with reduced flavins, methyl viologen, and bromphenol blue, which inhibited

from the dehydrogenase function. All the catalyzed reactions, including natural NADH/NADPH, were deduced subsequently.

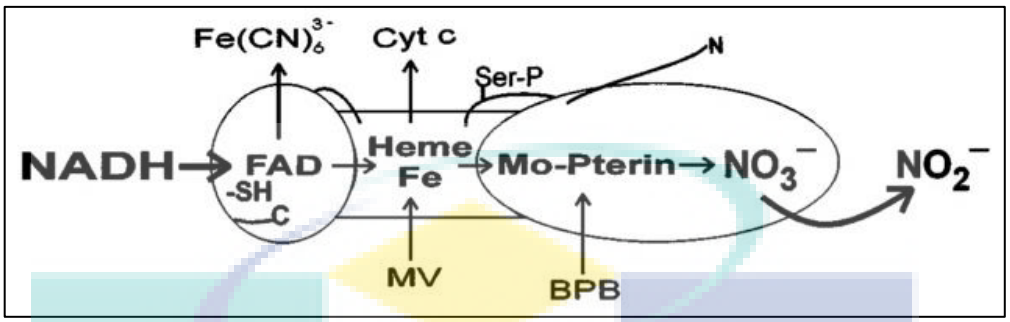

Figure 2.6 Functional model of enzyme which contains a heme-Fe and molybdenum complexed with a unique pterin or molybdopterin

Source: Campbell (1999)

Figure 2.7 shows the unique set of proteins similar to the conservation region of NR sequences with known proteins and enzymes. NR has eight sequence segments which are 5 structurally distinct domains and 3 sequence regions that differ from another protein. The distinct domains are Mo-MPT domains with a nitrate-reducing active site, interface domain, cytochrome b domain, FAD domain and NAD(P)H domain. The cytochrome b reductase fragment (CbR) is formed when FAD and NADH domain is combined. Meanwhile, the cytochrome c reductase (CcR) was formed when cytochrome b (Cb) domain is joined to CbR. The cytochrome b reductase fragment contains the active site where NAD(P)H transfers electrons to FAD. A complete three-dimensional dimeric NR structure modelwas built from structures of sulfite oxidase and cytochrome b reductase.

The 3 sequence region that differs to another protein and varying in sequence among NR forms are N-terminal "acidic" region, Hinge 1 and Hinge 2 N-terminal region is rich in acidic residues but limited in NR forms. Hinge 1 contains serine phosphorylated in reversible activity regulation with inhibition by 14-3-3 binding protein. Both Hinge 1 and 2 contain a proteinase site (Campbell, 1999).

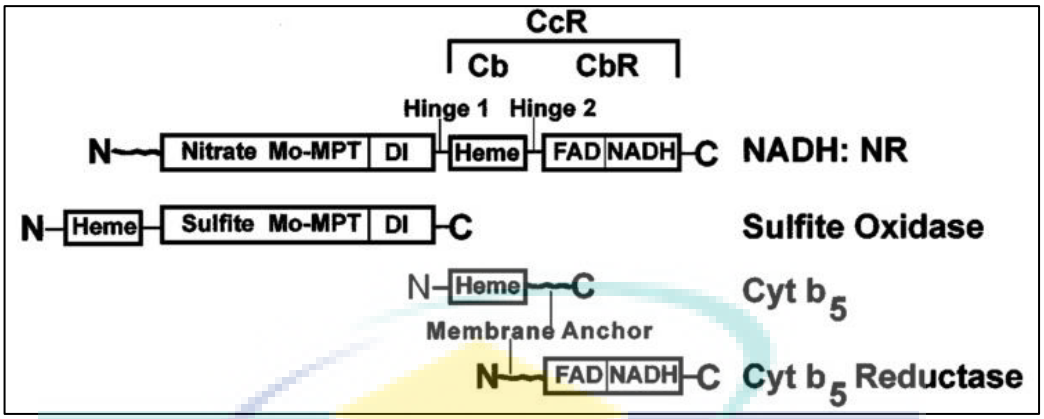

Figure 2.7 Unique set of proteins similar to the conservation region of NR sequences with known proteins and enzymes.

Source: Campbell (1999)

2.7 Microorganisms Producing NR Enzyme

Studies found that *Pseudomonas aeruginosa* has the ability to produce nitrate reductase enzymes; *P. aeruginosa* is a Gram-negative bacterium which is rod-shaped, asporogenous and monoflagellated. This microorganism can be found in water, soil, plants, animals, humans, sewage and hospital. It is an obligate respire that using aerobic respiration as it metabolism and also able to respire anaerobically on nitrate or other electron acceptors.

The cell-free supernatant of *P. aeruginosa* culture was used to measure the nitrate reductase and nitrite reductase activities. The result revealed that nitrite reductase activity was greater than the nitrate reductase activity along with the presence of molybdenum-enhanced extracellular enzyme activities, as shown in Figure 2.8. Nitrate reductase plays an important role in the biosynthesis of metal nanoparticle and the important cofactor that enhances nitrate reductase activity is molybdenum even though the activity of nitrite reductase does not depend on the presence of molybdenum. The previous experiment showed when the activity of nitrate reductase increased by 96% molybdenum was added and there was a significant increase in the activity of nitrite reducatase (Tiwari, Narayanan, Thakar, Jagani, & Rao, 2013).

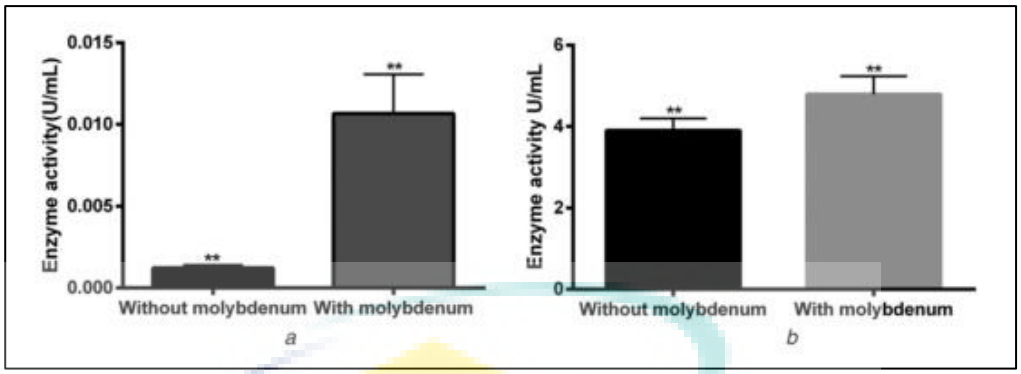

Figure 2.8 Enzyme activity (U/ml) in cell-free supernatant of *P. aeruginosa* culture with and without molybdenum for (a) Nitrate reductase; (b) Nitrite reductase

Source: Tiwari et al. (2013)

Bacillus subtilis was also found to produce nitrate reductase enzymes. It is a gram-positive bacteria which can found in water, soil, decomposed plant matter and in air. This bacterium is widely used in genetic research as opposed to health research. It is because this bacterium is highly responsive to genetic mutations. Other than nitrate reductase, these bacteria also able to produce amylase and protease enzyme. In the previous study reported by Saifuddin, Wong, & Yasumira (2009), the nitrate reductase activity about 152 nmol/ h/ ml was detected in the culture supernatant of *B. subtilis*. It showed that the reductase together with electron shuttling compounds and other peptides/proteins may be responsible for the reduction of Ag^+ ions and the subsequent formation of silver nanoparticles. Shuttling compounds and other peptides/proteins may be responsible for the reduction of Ag^+ ions and the subsequent formation of silver nanoparticles.

The *Rhizobium* sp. and *Azotobacter* sp. isolates also able to aerobically reduced nitrate by adding 0.2 ppm of silver nanoparticles in the culture medium. In previous research, they showed that both isolates were the best strains in aerobically reducing nitrate. Both microbes were isolated from the meliloti nodules and soil of Isfahan (Shahrokh, Hosseinkhani, & Emtiazi, 2014). They are Gram-negative bacteria and can fix nitrogen. The *Rhizobium* sp. and *Azotobacter* sp. can create symbiosis relationship with certain plants such as legumes, then the nitrogen was fixed from the air into ammonia and it was used as a natural fertilizer for the plants.

A previous study by Sohaskey & Modesti (2009) revealed that *Mycobacterium tuberculosis* and *Mycobacterium bovis* contain low nitrate reductase activity during aerobic growth but they showed strong hypoxic induction. *M. tuberculosis* and *M. bovis* are the members of *M. tuberculosis* complex. Both microorganism able to cause tuberculosis in humans. To prevent tuberculosis, a live attenuated vaccine strain, *M. bovis* Bacilli Calmette-Guerin (BCG) was used. Historically by the fact, *M. tuberculosis* was distinguished from *M. bovis* in which only *M. tuberculosis* can reduce a significant amount of nitrite. However, under longer incubation period and anaerobic conditions, the production of nitrite has been reported for some *M. bovis* strains.

Instead of microorganisms, a previous study by Redinbaugh & Campbell (1985) isolated Blue Sepharose and zinc-chelate, the nitrate reductase (EC 1.6.6.1) squash cotyledons (*Cucurbita maxima L.*) by using chromatography followed by gel filtration. By UV/visible spectrum, the homogeneous nitrate reductase contains a typical b-type cytochrome. The enzyme was found to contain one each of flavin (as FAD), heme iron, molybdenum, and Mo- pterin/M_r = 115,000 sub-unit. The two features observed on two-dimensional gels which are native gel electrophoresis and denaturing during the enzyme purification by using Blue Sepharose and zinc column chromatographies. The result revealed that there were multiple and variable protein staining bands corresponding to the native nitrate reductase, indicative of limited proteolytic cleavage of the native enzyme. *Cucurbita maxima L.* is a type of herbaceous vines genus in gourd family. This species is grown worldwide variously known as squash, gourd or pumpkin depends on their species.

Chow, Capociama, Faria, & Oliveira (2007) studied the nitrate reductase activity in marine red alga *Gracilaria caudata* by conducting an *in vitro* assay. The presence of nitrogen is the most important abiotic factor in seawater that limits the growth of algal. The study revealed that the nitrate reductase activity showed maximal peaks at 20 °C and pH 8.0. The nitrate reductase was managed by several environmental and intracellular factors which are molybdenum, the concentration of nitrogen compound, carbon dioxide, iron, phytohormones, carbon metabolites, plastidic factors and light.

Other than *Cucurbita maxima L.* and *G. caudata*, there also have some plants that able to produce nitrate reductases, such as *Dactylis glomerata L.* and *Festuca ovina*

L. The previous study by Scheurwater, Koren, Lambers, & Atkin (2002) showed that slow-growing grass species perform a greater proportion of total plant nitrate reduction in their roots that fast-growing grasses. The result indicates that the slow-growing grass species, *Festuca ovina L.* perform the same proportion of total plant nitrate reduction in their root as exhibited by fast-growing grasses, *Dactylis glomerata L.* The shoots act as predominant site of whole plant nitrate reduction in both fast and slow growing grass when they are grown with free access to nutrients.

2.8 Properties of NR Enzyme

2.8.1 As Main Role in Nitrate Assimilation

The nitrate reductase plays a role in the regulation of nitrate assimilation in lower and higher plants. In higher plants, assimilatory NR is a most interesting enzyme, both from its central function in plant primary metabolism and control of the catabolic activity. The major external of light and plant hormone availability is triggered for the rapid and reversible modulation of NR activity. As it is well known that products of light reaction reflect the need to coordinate carbon (C) and nitrogen (N) assimilation. In the NO_3 assimilation pathway, NR is the first enzyme and represents the rate-limiting step in this process. It is able to generate NO_2 in the cytoplasm of a plant cell, which is translocated into the plastids for further reduction and metabolization. The indigenous enzyme is a dimer in higher plants and Chlamydomonas, and a tetramer in chlorella.

NR is a molybdenum (Mo) protein enzyme. From a number of plants, Mo has bound to the enzyme purified. The biosynthesis of molybdenum cofactor (MoCo) involves the complex interaction of six proteins and is a process of four steps, which requires Fe, ATP and Cu. MoCo is distributed to the apoproteins of Mo-enzymes by MoCo-carrier/binding proteins after the synthesis. A deficiency in the biosynthesis of MoCo has lethal consequences for the respective organisms. The redox potential of the heme-Fe in the enzymes depends greatly on the form of NR used for the movement. The reduction of NR activity is more declared at the reproductive stage of the plant (Mazid, Khan, & Mohammad, 2012).

2.8.2 As Heavy Metal Detection Tool

By the 1970s, in United States, Canada, and Europe, serious nitrate pollution had occurred in some surface and groundwater which reached the Maximum Contaminant Limit (MCL) for potable water (Burow, Nolan, Rupert, & Dubrovsky, 2010). The methemoglobinemia or blue baby syndrome posed by a high concentration of nitrate in drinking water. This was caused by strong binding of nitrite to hemoglobin and oxidation of the iron center, which is more serious in infants since fetal hemoglobin binds nitrite more strongly and can result in death. The health risks such as cancer and long-term exposure to high nitrate concentrations in drinking water are not well enough documented to justify the further restriction of the nitrate MCL. The excess N in the environment as a result of agricultural practices to maximize crop yield and animal wastes causes the pollution when they were released into the environment without undergoing a tertiary process to remove nutrients. This has happened since the end of the Second World War. Other than that, the industrial processes and air pollution also contribute to nitrate pollution. The N contamination has potential health effects in drinking water, nutrient enrichment of aquatic and terrestrial ecosystem and can cause global warming (Puckett, Tesoriero, & Dubrovsky, 2011).

Nitrate reductase is an efficient enzyme and done the important job in nitrogen metabolism which essential in every plant on earth. It has been used as nitrate testing method and this is very important in environmental biotechnology. In the previous study by Sandu et al. (2014) reported that, instead of cadmium or enzymes, the nitrate testing in the environment was done with zinc powder which is heavy metals. This testing has high potential to harm the environment. So that, the nitrate reductase based nitrate testing was replaced the older method because of this method was environmentally friendly, more accurate and safe to conduct. On a real-time, nitrate must be monitored in surface and ground waters.

A nitrate electrode was developed recently based on corn leaf NR, where electrons are directly supplied to the enzyme for nitrate reduction by an electrode coated with methyl viologen. It has the capability to quantify nitrate in fertilizer solutions. On the other hand, NR enzyme also can be used in the biosensor for nitrate determination (Campbell, 2015; Sohail & Adeloju, 2016). A Biosensor is an analytical device

composed of bioreceptor, detector and transducer. Because of a commercial nitrate biosensor based on NR, the other biosensors such as those based on glucose oxidase for monitoring blood glucose also produced. Removing nitrate from potable water methods would be a solution for many communities and individuals where nitrate pollution has contaminated their drinking water source.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter details the research design of this project. The research began with the isolation of microbial from three different sources soil, water and spoiled food. Subsequently, all of the unknown bacteria obtained underwent a purification process and microbial inoculation was added to the growth medium. The protein assay was first studied to obtain the most potent isolate among all the bacteria and after the most potent isolate was identified, microbial inoculation was performed once again and added to the growth medium. In this step, the several parameters were studied so that the most potent isolate able to produce and improved the enzyme and nanoparticle production. The microbial cells were harvested after 15 days and were centrifuged to obtain a supernatant. With the supernatant, protein and enzyme assays were performed to derive the value of proteins and enzymes activity presented in each sample. Next, the supernatant was sent for ICP-MS analysis. Freeze drying was conducted to obtain the sample in powder form to be sent for XRD analysis. Finally, the colonies of the most potent bacteria were sent to the central lab for bacterial identification. The details descriptions of the research methodology were provided in the next section. The summary of the research methodology is presented in a flowchart in Figure 3.1.

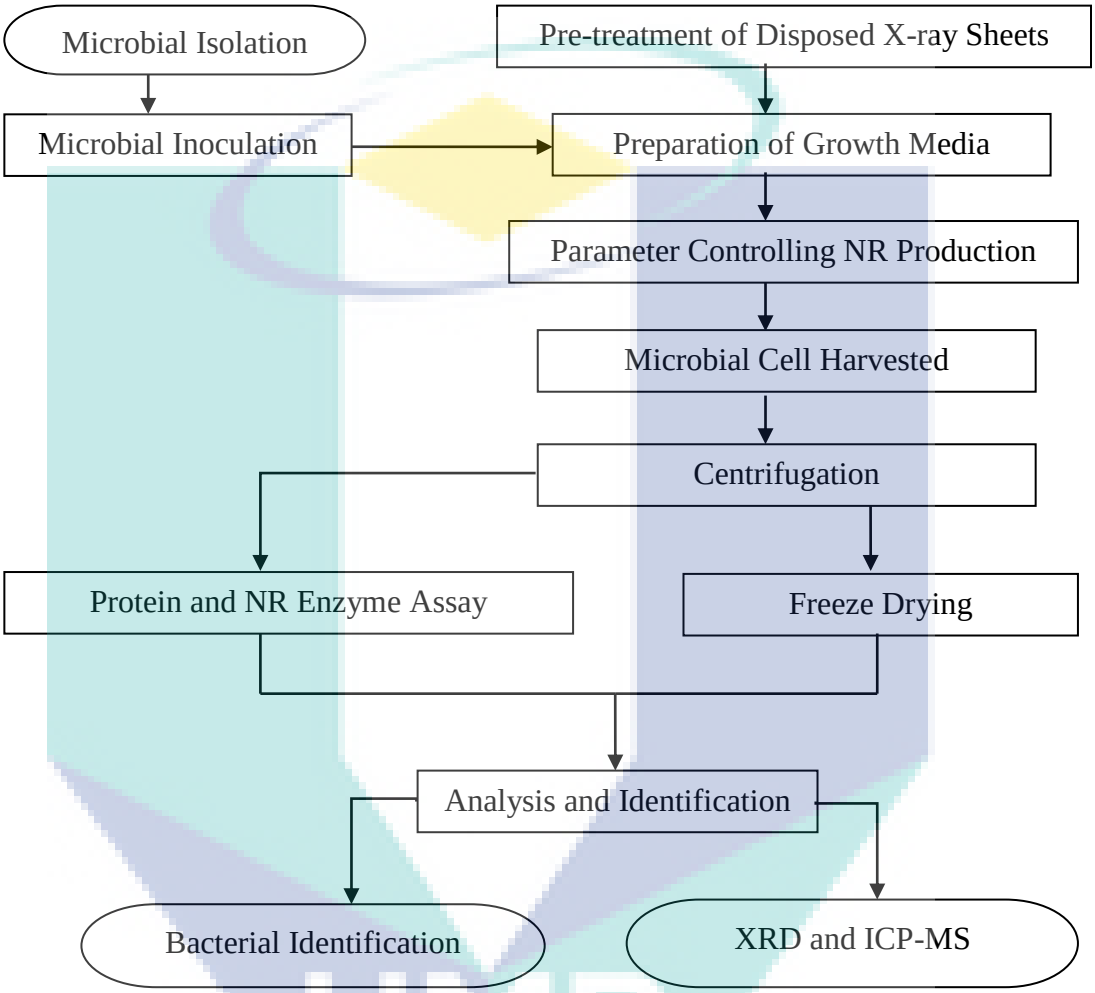

Figure 3.1 The flow chart of the research methodology

3.2 Microbial Isolation from Different Sources

3.2.1 Samples Collection

Soil, water and spoiled foods were collected from different sites. Soil samples were collected from Kolej Kediaman 2 (KK2) in University Malaysia Pahang and Panching area, Kuantan, Pahang while the water samples were collected from Sungai Belat (500 ml) located in front of Gambang Sports School and from Gambang Lake (600 ml). The spoiled food samples were collected from rotten fried chicken livers, jackfruits and apple fruits.

The soil samples from Kolej Kediaman 2 and Sungai Panching were labeled as 'KK2S' and 'PS', respectively. The water samples from Sungai Belat and Gambang Lake were labeled as 'RSS' and 'GL', respectively while the spoiled food samples in forms of rotten fried chicken liver, jackfruit and apple fruit were labeled as 'CL', 'JF' and 'A', respectively. After that, the soil samples were stored at room temperature and protected from humidity and sunlight while the water and spoiled food samples were stored in the chiller at 4°C.

3.2.2 Serial Dilution Method for Isolation

One gram of each soil sample was weighed in a small beaker and 30 ml distilled water was added. The mixture was mixed well for 3 to 5 minutes continuously and left to sediment. The 9 ml of distilled water were pipetted into each 5 blue cap tubes and 1 ml of soil extract from Kolej Kediaman 2 (KK2S) was added into the first tube and shakes well and labeled as 10^{-1} dilution. Then, 1 ml of the mixture from the first tube was pipetted into the second tube and labeled as 10^{-2} dilution. This step was repeated until get the 10^{-5} dilution as shown in Figure 3.2. After that, these steps were repeated for other samples collected from Panching soil (PS), Sungai Belat (RSS) and Gambang Lake (GL). For the spoiled food samples, a direct inoculation loop was used to retrieve the bacteria from the spoiled area and mixing them with 9 ml distilled water. Then, similar to the previous steps, a serial dilution was done until the 10^{-5} dilution.

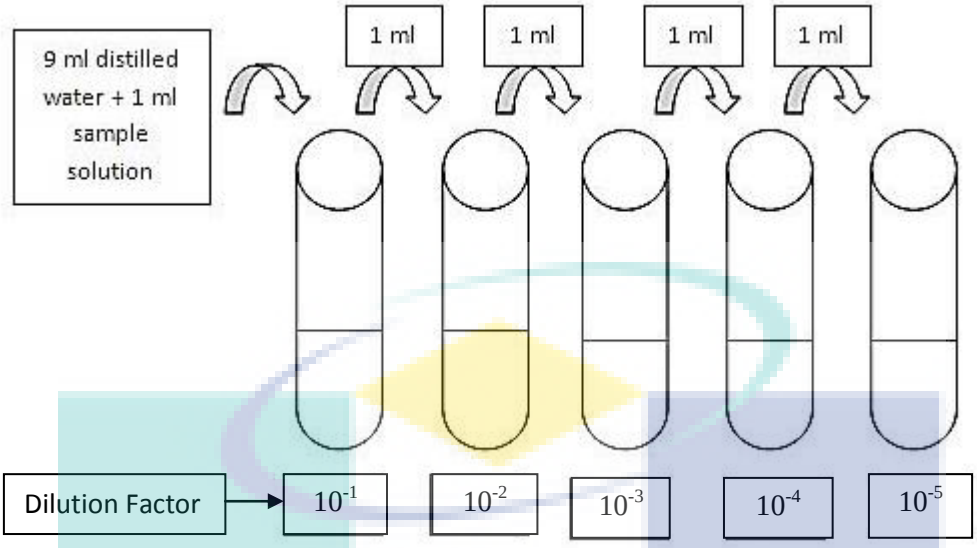

Figure 3.2 Serial dilution of collected samples for microbial isolation

3.2.3 Preparation of Nutrient Agar Medium

About 28 g of nutrient agar (NA) powder (Oxoid) was weighed before being suspended in 1 liter of distilled water. The mixture was stirred continuously on the hot plate stirrer until it was completely dissolved. After that, the mixture was autoclaved for 121 ℃ for 15 minutes by using autoclave machine (Hirayama) to make sure the unwanted microorganisms were killed and to avoid contamination. After that, the nutrient agar was poured into the sterile petri dishes and left to solidify in the laminar air flow.

3.2.4 Preparation of Potato Dextrose Agar (PDA) Medium

The 9.76 g of PDA powder (Merck) was weighed on the weighing balance, then; it was suspended with 250 ml of distilled water and stirred on the hot plate until the PDA was totally dissolved. After that, the PDA was autoclaved for about 15 minutes at 121 ℃ using autoclave machine (Hirayama). The autoclaved PDA was poured quickly into sterile Petri dishes and left to completely solidified. The process was conducted under a laminar air flow to avoid any contamination.

3.2.5 Spreading and Streaking Method

The bottom of each agar medium plate was labeled with the name of the samples, date and the temperature. Then, 0.1 ml of the bacterial suspension from the serial dilution was pipetted onto the center of an NA plate. The L-shaped glass rod was sterilized by dipped it into a beaker of ethanol and tapped on the side of the beaker to remove any excess ethanol. After that, the ethanol-soaked spreader was briefly passed through the flame to burn off the alcohol. It was allowed to cool before the bacterial suspension was spreaded evenly over the agar surface. Then, the spreader was immersed again in ethanol and re-flamed. These steps were repeated for the other samples. On the other hand, for the spoiled food samples, the direct streak was applied, as shown in Figure 3.3. Finally, all of the petri dishes were inverted and incubated for 24 to 48 hours at 37°C and 50°C.

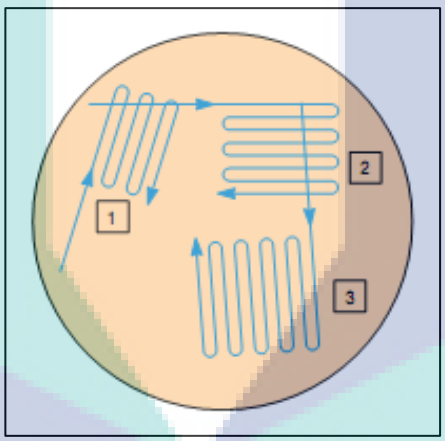

Figure 3.3 Direct streaking method for microbial isolation

3.3 Subculture of Pure Bacterial Isolates

After isolation process was completed, the bacteria were purified to another NA to specify their characteristic. The process was conducted under a laminar air flow for ensure sterility. In this light, some petri dishes might contain 2 or 3 species of bacteria from different genera. Therefore, the bacteria were carefully taken and streaked to another NA. This step was repeated with other petri dishes that contained different microbes. The purified microorganisms were incubated at their respective temperatures which were 37°C and 50°C for 48 hours.

3.3.1 Preparation of Agar Slants Culture

About 2 liter of NA was prepared and autoclaved at 121 °C for 15 minutes. Then, 5 ml of the autoclaved NA was poured into each slant tube to fill less than half of the tube. About 144 slants were prepared from 1.5 liter of NA and autoclaved for 15 minutes, then, 500 ml of nutrient agar was used to fill in the petri dishes. After that, the slants were left on the bench at a slant position for 24 hours to solidify (refer Figure 3.4 in Appendix B).

After being fully solidified, the bacterial isolates from petri dish were spreaded evenly over the agar surface with the sterilized spreader (refer Figure 3.5 in Appendix B). All the processed were done under a sterile condition.

3.4 Preparation of Bacterial Inoculation

About 8g of NB medium was weighed and put into the beaker. Subsequently, 1 liter of distilled water was added to each beaker. The mixture was stirred continuously on the hot plate stirrer until it was completely dissolved. Then, 50 ml of the medium was measured and put into a 100 ml conical flask (refer Figure 3.6 in Appendix C). Next, the medium was autoclaved using autoclave machine (Hirayama) at 121 °C for 15 minutes to make sure the unwanted microorganisms were killed and to avoid the contamination. Then, it was cooled under laminar air flow. After that, the bacterial isolates from slants were inoculated in the prepared flasks. The control, which does not contain any bacterial inoculum, was also prepared. Then, the flasks were incubated in 37°C and 50°C incubators for 24 hours. Finally, all the changed of the samples in flasks were observed.

3.5 Production of Silver Nanoparticles

3.5.1 Pre-treatment of Substrate (X-Ray Sheet) Using 4M NaOH

The substrate was cut into small pieces (0.2 x 0.2 cm), as shown in Figure 3.7. Then, 35g of the substrate was put into a 250 ml beaker. Then, 4M NaOH (refer Appendix D for their preparation) was added to cover the substrate surface as shown in

Figure 3.8(a). After that, the mixture was mixed well using the Lab Companion S1-600R Benchtop Shaker for overnight at 150 rpm, then the suspension was washed using tap water to remove the colored of the substrate as shown in Figure 3.8(b) while the pH was adjusted until it achieved the pH of 7. Then, it was dried in an incubator at 50°C overnight for the next step.

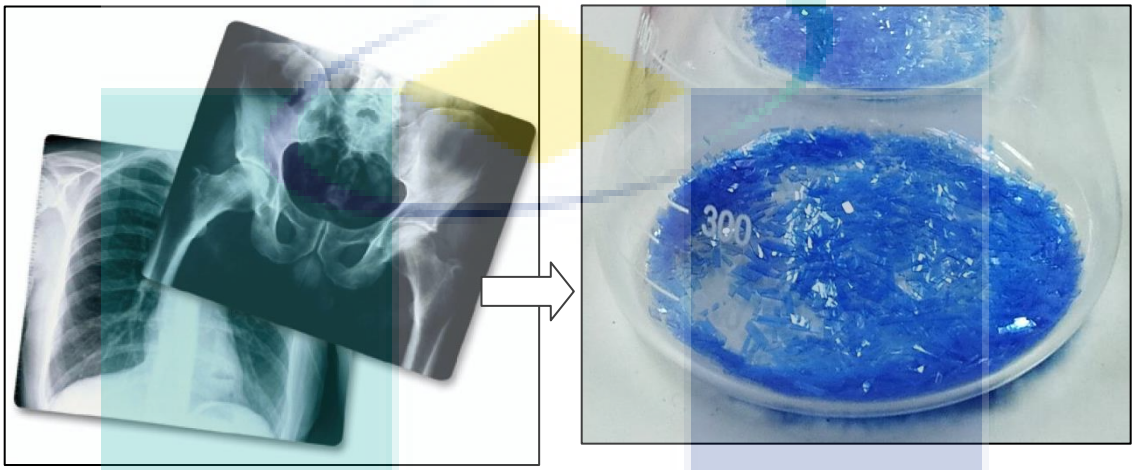

Figure 3.7 The x-ray sheets before and after cutting into small pieces

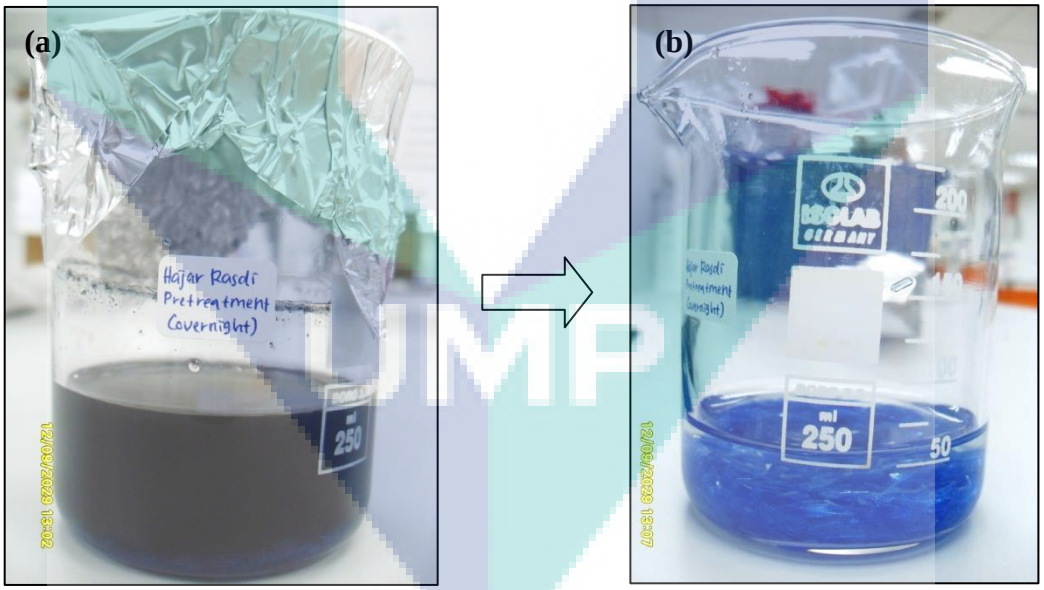

Figure 3.8 The substrate pre-treatment of x-ray sheets using 4M NaOH.(a) The substrate covered with 4M NaOH. (b) The substrate after washing with tap water.

3.5.2 Preparation of Growth Medium (Modified Minimal Medium, MM9)

According to the manual for preparation of Minimal medium (refer Appendix A, three stock solutions were prepared and labeled as solution A, B and C. Solution A, was prepared by removing NH_4Cl and adding 20% D-Glucose. Subsequently, 25.6 g/l of Na_2HPO_4. 7(H_2O) (Merck), 6 g/l of KH_2PO_4 (Merck) and 1 g/l of NaCl (Merck) were prepared. All of chemicals were weighed and suspended in 500 ml distilled water inside 1 liter beaker and distilled water was further added until the volume reached 1 liter. This solution was prepared twice (2x) concentrations as it is stock solutions. It was labeled as solution A. For solution B, 1x concentration of MM9 was prepared by transferring 500 ml of the stock solution A into another 1 liter beaker and 2 ml of solution B (1.0 M $MgSO_4$ solution) and 1 ml of solution C (1.0 M $CaCl_2$ solution) were added. Distilled water was further added until the volume reached 1 liter. The calculation to determine the amount of of $MgSO_4$ and $CaCl_2$ needed to prepare the solutions B and C is presented in Eq. (3.2) in Appendix D.

3.5.3 Preparation of Modified Minimal Medium (MM9) Containing X-Ray Sheet

For this preparation, 50 ml of the complete 1x concentration production medium (MM9) was added into 100 ml conical flasks. After that, 0.5 g of the substrate (pre-treated x-ray sheets) were weighed and added into each conical flask (refer Figure 3.9 in Appendix C) and autoclaved at 121 °C for 15 minutes using autoclave machine (Hirayama) to make sure the unwanted microorganisms were killed and to avoid contamination. After cooled, 1 ml of medium was removed and 1 ml of 20% D-glucose was added. The mixture was mixed thoroughly and 5 ml was discarded from the flask. Then, it was replaced by 5 ml of bacterial inoculum which was pipetted into each flask. This process was conducted under a laminar air flow. The flask for control only contains MM9 and bacterial inoculum but no x-ray sheets, was also prepared. All of the flasks were incubated at 37°C and 50°C incubators for ten days in darkness.

After the incubation period, each mixture was transferred into a 50 ml centrifuge tube and was centrifuged for 5 min, 5000 rpm and 4°C by using refrigerated centrifuge

machine (Heraeus Biofuge Primor) to separate the microbial cell with the supernatant. Then, all of the tubes contained supernatant was kept in a chiller for the next step.

3.6 Lowry Protein Standard Curve and Assay

The Bovine Serum Albumin (BSA) with different concentrations was prepared which were 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg/ml as the protein standard curve. The experiment was carried out in darkness because BSA is sensitive towards the presence of light. Lowry, Rosebrough, Farr, & Randall (1951) protocol was used to determine the protein content.

Generally, there were 3 solutions in Lowry assay which were solution A, B and C. Then, the mixture of solution A, B and C which known as Lowry solution was prepared freshly before used using appropriate ratio. In solution A, 2.86 g of NaOH with 14.30 g of Na_2CO_3 was dissolved in 500 ml distilled water. For solution B, 1.42 g of $CuSO_4$. $5(H_2O)$ was dissolved in 100 ml distilled water and for solution C, 2.85 g of Na_2. Tartarate. $2(H_2O)$ was dissolved in 100 ml distilled water. The Lowry solution was prepared in the ratio 48: 1: 1.

The Folin-Ciocalteu's phenol reagent was prepared with the ratio of 1:1. For example, if 4 ml of complete reagent is needed, 2 ml of the Folin-Ciocalteu's phenol reagent (Merck) need to be diluted in 2 ml distilled water. This solution was kept in an amber container because it is light-sensitive. It was prepared fresh i.e 5 minutes prior to the incubation of the first sample.

Lowry assay began by pipetted 5 ml of Lowry solution into each test tube and 0.5 ml of the chilled sample (refer section 3.5.3) was added while 0.5 ml of distilled water was added to the control sample. Each mixture was mixed and kept for 15 minutes at room temperature. Then, 0.5 ml of diluted Folin-Ciocalteu's phenol reagent was added into each mixture. It was mixed and kept for 30 minutes at room temperature to observe color changed. Finally, each solution was transferred into a cuvette. Each mixture's absorbance was measured at a wavelength of 700 nm by using a microplate reader (Infinite M200 Pro Tecan). The mixtures with the highest microbial isolates absorbance's reading were considered as most potent isolates.

3.7 Nitrate Reductase Biochemical Test

A nitrate reductase biochemical test was performed to confirm the most potent isolates had been successfully produced nitrate reductase enzyme before proceeded for enzyme assay. The reagent for Nitrate A and Nitrate B was prepared for this test. The reagent for Nitrate A consists of 0.5 ml dimethyl-naphthylamine or alpha-naphthylamine dissolved in 5N acetic acid. The reagent for Nitrate B consists of 0.2 g sulfanilic acid dissolved in 5N acetic acid. Both reagents were used to detect the presence of nitrite produced by the reduction of nitrate in nitrate broth. First, the samples were inoculated in 5 ml nitrate broth and incubated overnight. After that, about 1 ml of each reagent was added into the culture. It was observed that the culture turned red which indicates the nitrate was successfully reduced to nitrite.

3.8 Nitrate Reductase Standard Curve and Assay

Different concentration of sodium nitrite ($NaNO_2$) were prepared viz; 0, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml as standard concentrations. The concentrations were prepared through dilution with distilled water.

The enzyme activity was measured using the assay method described by Saifuddin et al. (2009). In this method, two assay medium which were 30 mM KNO_3 and 5% propanol in 0.1 M phosphate buffer pH 7.5 was prepared. Then, the nitrite assay reagents consist of Sulfanilamide solution: 1% (w/v) in 25% (v/v) HCl and N-(1-napthy) ethelenediamine dihydrochloride solution: 0.02% (w/v) in distilled water was also prepared.

By using 10 ml of bacterial supernatant of most potent isolates (refer section 3.5.3), the enzyme assay was determined. The enzyme assay was done by prepared negative and positive control. The negative control were prepared by placed the samples contained 10 ml bacterial supernatant and 10 ml assay medium in a boiled water bath to kill the enzyme. After being boiled for 5 minutes, the tubes were kept at room temperature before the absorbance was measured. This was done in triplicates. After that, for positive control, 10 ml of assay medium were added to 10 ml bacterial

supernatant and were incubated in darkness for 60 minutes before being placed in a boiled water bath for 5 minutes. This was done in triplicates also.

After the samples were cooled, 5 ml of sulfanilamide solution 1% (w/v) in 25% (v/v) HCl was added to each tube. Next, 5 ml of 0.02% (w/v) N-(1-napthy) ethelenediamine dihydrochloride solution was added and quickly mixed by inverted the tubes. A color change was observed after 20 minutes and 250µl of each sample was loaded into appropriate microplate wells. Finally, the absorbance at 540 nm was measured by using a microplate reader (Infinite M200 Pro Tecan).

The amounts of nitrite found in negative controls were subtracted from the amount in positive controls to calculate the amount produced by the enzyme activity in 60 min. One unit of nitrate reductase (IU) is define as the amount of enzyme liberates one micromole of nitrite per minute under standard condition.

3.9 Parameter Controlling NR Enzymatic Activity on Disposed X-Ray Sheet

The parameters studied of NR enzyme and SNPs production was carried out only for the most potent microbial isolates identified in the screening process by using protein assay (refer section 3.6). These isolates were CL4C and GL7. In the meantime, the parameters were important to determine the suitable condition and sources that can boost and improve the nitrate reductase enzyme activity and silver nanoparticles production.

3.9.1 Effect of Different Incubation Condition

In this study, 50 ml of complete production medium (MM9) contained bacterial inoculum (refer section 3.4) were prepared for each isolate (CL4C and GL7) including control as elaborated in section 3.5.3. In the completed MM9, 5ml of bacterial inoculum (10 % v/v) with $5x10^8$ cells/ml was added into 45 ml of medium. The control did not contain x-ray sheets. Each of them was prepared in 2 batches which was for static (standing incubation) and not static (shaker incubation condition). Then, 0.5g of the substrate was added to each flask. After that, the first batch of flasks was incubated in a standing culture and second batch in non-static condition by using 140 rpm Lab

Companion S1-600R Benchtop shaker. Both were incubated at 37°C for 10 days in darkness. After finished incubation, each sample was transferred into 50 ml centrifuge tube and centrifuged for 5 min, 5000 rpm and 4°C using refrigerated centrifuge machine (Heraeus Biofuge Primor) to separate the microbial cell with the supernatant and the supernatant underwent a Lowry protein assay (refer section 3.6) and NR enzyme assay (refer section 3.8).

3.9.2 Effect of Different Substrate Concentration

About 50ml of complete production medium (MM9) contained 5ml bacterial inoculum (refer section 3.4) were prepared for each isolate (CL4C and GL7) including control as elaborated in section 3.5.3. The control for this studied was the medium without any x-ray sheets. After that, the flasks contained different substrate concentrations were prepared which are 0.2g, 0.5g, 0.8g, 1.0g and 1.5g for each most potent isolate. Finally, both were incubated at 37°C for 10 days in darkness. After the incubation period, each sample was transferred to a 50 ml centrifuge tube and centrifuged for 5 min at 5000 rpm and 4°C using refrigerated centrifuge machine (Heraeus Biofuge Primor) to separate the microbial cells from the supernatant. Consequently, the supernatant underwent a Lowry protein assay (refer section 3.6) and NR enzyme assay (refer section 3.8).

3.9.3 Effect of Different Incubation Period

Fifty ml of complete production medium, MM9 (refer section 3.5.3) contained 5 ml of bacterial inoculum (refer section 3.4) were prepared for each isolate (CL4C and GL7). In this study, the different substrate concentrations as previous parameter (refer section 3.9.2) was prepared for each isolate (CL4C and GL7) but they were prepared in three batches. The first batch was incubated for 10 days, second for 15 days and third for 20 days. All of them were incubated at 37°C in darkness. The control for this studied is the medium that do not contained any x-ray sheets. After the incubation period, each sample were transferred to a 50 ml centrifuge tube and centrifuged for 5 min at 5000 rpm and 4°C using refrigerated centrifuge machine (Heraeus Biofuge Primor) to separate the microbial cells from the supernatant prior to undergoing a Lowry protein assay (refer section 3.6) and NR enzyme assay (refer section 3.8).

3.9.4 Effect of Different Initial pH Values

In regard to the pH values, the similar procedure was applied by preparing 50 ml of the complete production medium (MM9) containing 5ml bacterial inoculum for each isolate which are CL4C and GL7 (refer section 3.5.3). The pH of MM9 medium for each isolate was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 by using hydrochloric acid (HCl) and sodium hydroxide (NaOH). Then, 1.0 g of substrate was added to each flask. The control for this study is medium that do not contain any x-ray sheets. All the samples were incubated at 37°C for 15 days in darkness. After the incubation period, each of the sample was transferred to a 50ml centrifuge tube and centrifuged for 5 min at 5000 rpm and 4°C using refrigerated centrifuge machine (Heraeus Biofuge Primor) to separate the microbial cells from the supernatant before the supernatant underwent a Lowry protein assay (refer section 3.6) and NR enzyme assay (refer section 3.8).

3.9.5 Effect of Different Nitrogen sources

One gram each of different nitrogen sources which are yeast extract, malt extract, ammonium chloride, potassium nitrate and sodium nitrate, was added into the 50 ml of complete production medium (MM9) containing 5 ml bacterial inoculum for each isolate (refer section 3.5.3). Then, 1.0 g substrate was added to each flask. The control for this study did not contain any x-ray sheets. All the samples were incubated at 37°C for 15 days in darkness. After the incubation period, all the samples were transferred to a 50 ml centrifuge tube and centrifuged for 5 min at 5000 rpm and 4°C using refrigerated centrifuge machine (Heraeus Biofuge Primor) to separate the microbial cells from the supernatant and the supernatant underwent a Lowry protein assay (refer section 3.6) and NR enzyme assay (refer section 3.8).

3.9.6 Effect of Different Concentrations of Nitrogen Source

After the optimum nitrogen source was chosen through the protein and enzyme assay, it was observed that the sodium nitrate provides the optimum conditions for NR and SNPs production, hence, the effects of different sodium nitrate concentrations was analyzed. The main purposed of this studied was to investigate whether in minimum usage of sodium nitrate, the most potent isolate was able to produce optimum enzyme

activity or not. Nitrogen sources with different concentrations which is 0.01, 0.02, 0.03g, 0.04g and 0.05g was studied by added into 50 ml of complete production medium (MM9) containing 5ml bacterial inoculum for each isolate (CL4C and GL7). Different concentrations of each substrate also was added and presented in ratio as shown in Table 3.1 to monitor the nitrate reductase activity. In this light, the control medium for this study contained 1g of x-ray sheets but no nitrogen source. All the samples were incubated at 37°C for 15 days in darkness. After the incubation period, each sample was transferred to a 50 ml centrifuge tube and centrifuged for 5 min, at 5000 rpm and 4°C using refrigerated centrifuge machine (Heraeus Biofuge Primor) to separate the microbial cells from the supernatant. Then, the supernatant underwent a Lowry protein assay (refer section 3.6) and NR enzyme assay (refer section 3.8).

Table 3.1 NR monitoring using different sodium nitrate and x-ray concentrations

$NaNO_3$: X-Ray Sheets (g/50ml)
0.050 : 1.00
0.040 : 1.50
0.015 : 1.00
0.010 : 1.25
0.003 : 1.00

3.10 Production of Nitrate Reductase by Compiling All the Optimum Parameters by the Most Potent Bacterial Isolate

Flasks that contained all the optimum parameters were prepared based on the result obtained from each parameter studied (refer section 3.9.1 until 3.9.6). The similar procedure was applied by preparing 50 ml of the complete production medium (MM9) containing 5ml bacterial inoculum for each isolate which are CL4C and GL7 (refer section 3.5.3). At the end of incubation, the supernatant underwent a Lowry protein assay (refer section 3.6) and NR enzyme assay (refer section 3.8). The pellet was kept in chiller for analysis to check whether SNPs deposited in or outer the bacterial cells (intracellular or extracellular).

3.11 Analysis and identification

After the Lowry protein assay and NR enzyme assays were completed, the remaining samples were kept in a chiller (supernatant obtained during centrifugation) before being transferred into freeze-dry flasks to undergo a freeze-drying process to obtain the powder for XRD analysis. The samples were frozen in the freezer at the temperature of -20 °C for 5 days. Once the freeze-drying process, the powder obtained was sent for XRD (Rigaku, Miniflex II) analysis at FIST Laboratory, Universiti Malaysia Pahang, Gambang. The pellet was then sent for Transmission Electron Microscopy (TEM) at Imaging Centre (iMACE), Faculty of Pharmacy, Universiti Technology Mara (UiTM) Puncak Alam Campus, Selangor to see the image of SNPs produced and the supernatant was sent to ICP-MS at Central Laboratory, Universiti Malaysia Pahang, Gambang to analyze the concentration of several elements present in the samples. Then, the most potent isolates were identified by using a Biolog Microbial Identification System conducted in the Central Laboratory, Universiti Malaysia Pahang, Gambang.

3.12 Gram staining

Gram staining was conducted to test whether the most potent isolate is either Gram positive or Gram negative. In the beginning, the heat-fixed smears of two different types of bacteria labeled as CL4C and GL7 were prepared. The slides were placed on the staining rack and the primary staining (crystal violet) was flooded for 30 seconds. After that, it was rinsed with water for 5 seconds. Next, both slides were covered with Gram's iodine mordant for 1 minute to fix the stain to the cell before being rinsed with water for 5 seconds. Then, the decolorizer containing a mixture of acetone was added drop by drop for 30 – 60 seconds or until the crystal violet failed to wash from the slide. It was rinsed again with water for 5 seconds. Later, the counter-stain (safranin) was added for 60 – 80 seconds and rinsed again with water for 5 seconds. Finally, both slides were dried by flame and were examined under oil immersion bright field microscope (100 x magnification). The Gram-positive isolate stained from blue to purple stain and Gram-negative isolates stained pink to red, as shown in Figure 3.10 (refer Appendix E).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of Bacterial Isolates

All the samples were collected from different sources which were soil, water and spoiled food. These samples have undergone isolation processed to purify them. The bacterial colonies were obtained from the spreading and streaking method on nutrient agar and potato dextrose agar according to their respective circumstances. Since bacteria can grow in various environments, all the unknown bacterial colonies were incubated at two different temperatures which are 37 °C and 50 °C due to uncertainty on which temperature was suitable for them to survive.

The results indicated that the entire unknown bacterial colony was able to survive in 37 °C and can be classified as mesophilic, whereas, some of them were able to survive in high temperature (50 °C) and can be classified as facultative thermophiles. In this light, a mesophile bacterium can grow best in medium temperature which is neither too cold nor too hot while a thermophile bacterium can thrive at a relatively high temperature between 45 °C to 122 °C.

The significant thermophilic bacteria analysis was inspired by a previous research by Deljou and Goudarzi which showed that the thermophilic *Bacillus* sp isolated from hot spring could be used for the extracellular synthesis of silver nanoparticles. Based on their result, the biosynthesis of silver nanoparticles were confirmed by dark brown color formation in the mixture and silver surface plasmon resonance band which appeared during the UV-Vis spectroscopy (Deljou & Goudarzi, 2016). Besides that, Syed, Saraswati, Kundu, & Ahmad (2013) reported the successful

use of thermophilic fungus *Humicola sp* in the biosynthesis of silver nanoparticles. The result was confirmed by TEM analysis which showed the morphology of spherical shape nanoparticles.

The data recorded in Table 4.1 showed the different isolation sources of unknown bacterial isolates chosen after 5 x 10^8 cells/ml subcultures in the purification process. About 36 isolates were successfully isolated at the incubation temperature of 37 °C for the incubation period of 24 – 48 hours. Only 3 isolates were obtained from soil samples, 11isolates were derived from the water samples and 22 isolates obtained from the food samples.

Table 4.1 Isolation of bacterial isolates at 37 °C. (a) Soil (b) Water (c) Spoiled Food

No	Isolate code (Soil source)
1	KK2S4
2	KK2S5
3	PS1

No	Isolate code (Water source)
1	GL1
2	GL4
3	GL 5C
4	GL 6
5	GL 7
6	RSS 2B
7	RSS 3
8	RSS 4
9	RSS 5
10	RSS 6
11	RSS 7

Table 4.1 Continued

No	Isolate code (Spoiled food source)
1	A1
2	A5
3	A6
4	A9
5	CL 2 (Branch)
6	CL 2C
7	CL 3
8	CL 4B
9	CL 4C
10	CL 5
11	CL 7
12	CL 8A
13	CL 9
14	CL 11
15	CL 13A
16	CL 13B
17	CL 14
18	CL 16
19	JF INNER (NA)
20	JF OUTER 2
21	JF OUTER 2A (PDA)
22	JF OUTER 2B (PDA)

Meanwhile, the data recorded in Table 4.2 showed the different isolation sources of unknown bacterial isolates chosen after being purified and incubated at 50 °C. 19 isolates were successfully isolated with the NA medium at the temperature of 50 °C for 24 – 48 hours incubation period. Only two isolates were obtained from soil samples, 6 out of 19 were obtained from water samples and 11 out of the 19 isolates were obtained from food samples.

Table 4.2 Isolation of bacterial isolates from soil samples at 50 °C. (a) Soil (b) Water (c) Spoiled food

No	Isolate code (Soil source)
1	KK2S 4
2	KK2S 5

No	Isolate code (Water source)
1	GL 1
2	GL 4
3	RSS 2B
4	RSS 5
5	RSS 6
6	RSS 7

No	Isolate code (Spoiled food source)
1	A1
2	A5
3	A6
4	A9
5	CL 4B
6	CL 4C
7	CL 7
8	CL 13A
9	CL 13B
10	CL 16
11	JF OUTER 1 (NA)

As much as possible unknown bacterial isolates were derived from different sources to find the best or strongest microbe that can be used to produce SNPs, and has the highest ability to reduce the nitrate to nitrite. The bacteria had undergone several subcultures during the purification process in order to obtain pure culture and the best cultures. After all the bacterial isolates had been successfully grown, the screening process specifically the protein assay was conducted to find the most potent isolates.

4.2 Screening for Most Potent Microbial Isolates

About 36 and 19 unknown bacterial isolates obtained previously were incubated at 37 °C and 50 °C respectively. All of these isolates had undergone the primary screening processed to choose the most potent bacteria before the next step. In this regard, protein acts as an indicator of all biological activity. Hence, in any biological estimation or bio-process, the protein assay was done instead of biomass studied because to determine the quantification of protein produced by the bacterial isolate in certain environment. In this studied, the total protein content in a bacterial culture was determined to estimate the potency growth of the microbes when the disposed x-ray sheet was used as substrate because there was no previous studied yet regarding the uses of this substrate . The most potent bacteria were chosen depended on the highest protein quantification and it is important to know first when the enzyme assay was expected.

To measure the protein quantification, the Lowry assay proposed by Oliver H. Lowry in 1951 was chosen in this study instead of UV absorption (280nm), Bicinchoninic acid, and Bradford assay. Even though each assay has its own advantages and disadvantages, the Lowry assay is highly sensitive and more precise compared to other methods. On the other hand, the Lowry assay might be incompatible with reducing agents and detergents and the assay requires a long procedure.

The Lowry protein assay was done to determine the total level of protein content in the bacterial supernatant in order to screen the most potent isolates. This is exhibited through the color changes shown in Figure 4.1 (see Appendix F). The color of the solution changed from colorless to blue indicated the presence of protein in the samples. This color changed was due to the reduction of Folin-Ciocalteu reagent by the copper-peptide bond complex.

As the color goes darker, the total protein concentration will be higher. Here, the value recorded using the colorimetric technique was compared in all bacterial isolates samples. In this step, the isolates with the highest protein contents were chosen for the next process. The data recorded in Table 4.3 show the values of protein content (mg/ml) and the standard deviation (SD) obtained from the triplicates measurement of

absorbance reading at 700 nm by using a microplate reader (Infinite M200 Pro Tecan). The bacterial isolates samples include control were incubated at 37 °C.

Table 4.3 Primary screening of microbial isolates for quantification of the protein obtained from disposed x-ray sheets as substrate at 37 °C

NO.	Isolate Code	Protein Content (mg/ml) Mean ± SD
1	Control	0.000
2	A6	0.548 ± 0.012
3	RSS6	0.391 ± 0.045
4	RSS4	0.480 ± 0.026
5	CL3	0.618 ± 0.005
6	A5	0.456 ± 0.030
7	GL6	0.602 ± 0.013
8	JF INNER (NA)	0.551 ± 0.006
9	RSS 2B	0.529 ± 0.002
10	CL2 (BRANCH)	0.564 ± 0.018
11	CL4C	0.670 ± 0.014
12	A1	0.491 ± 0.020
13	CL 2C	0.456 ± 0.026
14	JF OUTER 2	0.542 ± 0.003
15	CL 4B	0.464 ± 0.016
16	RSS7	0.575 ± 0.009
17	CL 13B	0.575 ± 0.010
18	CL 16	0.491 ± 0.005
19	CL7	0.580 ± 0.002
20	RSS5	0.437 ± 0.002
21	CL 13A	0.578 ± 0.013
22	CL 14	0.591 ± 0.002
23	A9	0.377 ± 0.007
24	KK2S 4	0.624 ± 0.003
25	GL 7	0.692 ± 0.001
26	RSS 3	0.567 ± 0.001
27	CL 11	0.475 ± 0.008
28	GL 4	0.496 ± 0.019
29	PS 1	0.692 ± 0.013
30	GL 5C	0.654 ± 0.002
31	JF OUTER 2A (PDA)	1.055 ± 0.009
32	KK2S 5	0.540 ± 0.006
33	CL 9	0.491 ± 0.001
34	GL 1	0.529 ± 0.008
35	CL 8A	0.553 ± 0.001
36	CL 5	0.683 ± 0.012
37	JF OUTER 2B (PDA)	0.778 ± 0.004

Meanwhile, the data recorded in Table 4.4 show the protein content (mg/ml) of protein assay obtained from the measurement of absorbance reading at 700 nm by using microplate reader (Infinite M200 Pro Team). The microbial isolates samples included control was incubated at 50 °C.

Table 4.4 Primary screening of microbial isolates for quantification of the protein obtained from disposed x-ray sheets as substrate at 50 °C

NO.	Isolate Code	Protein Content (mg/ml) Mean ± SD
1	Control	0.000
2	KK2S 5	0.621 ± 0.026
3	RSS 2B	0.594 ± 0.004
4	CL 13B	0.632 ± 0.008
5	A9	0.635 ± 0.008
6	JF OUTER 1 (NA)	0.464 ± 0.001
7	A6	0.483 ± 0.045
8	CL 4C	0.458 ± 0.006
9	GL 1	0.627 ± 0.003
10	CL 4B	0.534 ± 0.004
11	GL 4	0.689 ± 0.011
12	KK2S 4	0.575 ± 0.006
13	RSS6	0.542 ± 0.040
14	RSS7	0.697 ± 0.001
15	A1	0.548 ± 0.011
16	CL 7	0.727 ± 0.094
17	A5	0.632 ± 0.040
18	RSS 5	0.559 ± 0.028
19	CL 16	0.515 ± 0.004
20	CL 13A	0.477 ± 0.009

The most potent isolates were chosen according to their highest value of protein quantification. This is done by compared total protein content obtained from the bacterial isolates incubated at different incubation temperatures of 37 and 50°C. By chosen the highest protein quantification, the enzyme activity can be measured more accurately. In this light, the protein content needs to be measured accurately since to get the accurate data for the enzyme activity if the protein purification was studied (Olson and Markwell, 2007).

At first, the isolates code CL4C, GL7, JF Outer 2A (PDA) and JF Outer 2B (PDA) for 37 °C and 2 bacterial isolates at 50 °C (KK2S5 and RSS7) were reserved for secondary screening to select the best and most potent bacteria to proceed the studied. However, due to the no growth of some isolates which are JF Outer 2A (PDA), JF Outer 2B (PDA) at 37 °C and all bacterial isolates at 50 °C after several subculture was repeated, only the best two most potent isolates were decided to further the next studied. They were showed no growth due to physical and nutritional factors that affected the microbial growth. The physical factors that influence microbial growth are pH, temperature, oxygen concentration, hydrostatic pressure, osmotic pressure, moisture and radiation. Whereas, the nutritional factors are carbon, nitrogen, sulfur, trace elements, phosphorus and vitamins (Karki, 2017). In this study, the moisture factor might cause the bacteria to show no growth. It is probable that the bacteria did not have accessed to constant moisture as they were stored too long in a chiller before the subculture processed and the environment becomes unsuitable for them, hindering their growth.

Table 4.5 Secondary screening of the most potent isolates for quantification of the protein content obtained from disposed x-ray sheets at 37 °C.

NO.	Isolate Code	Protein Content (mg/ml) Mean ± SD
1	Control	0.000
2	CL4C	0.651 ± 0.002
3	GL7	0.673 ± 0.009

In the meantime, according to the Table 4.5, the protein quantification for selected samples was successfully determined during the secondary screening. The CL4C and GL7 showed the highest protein content compared to other samples. This obtained result was used in further steps which are parameters controlling, SNPs and NR enzyme productivity.

A protein standard curve was commonly plotted after the protein content in all the selected samples was determined in the secondary screening. The data recorded in Table 4.6 (see Appendix F) showed the results of protein content for BSA. The final concentration of the stock solution was 10 mg BSA/ml.

The Lowry protocol was followed because it has been used widely to determine the amount of particular activity present in biological samples in a quantitative fashion. First, the protein is pre-treated with copper ion in alkali solution and then the aromatic amino acids in the treated sample reduced the phosphomolybdic-phosphotungstic acid present in the Folin Reagent. The color of the end product of this reaction changed from blue to transparent.

After measuring the absorbance of the standards at 700 nm by a using microplate reader (Infinite M200 Pro Tecan), the calibration curve as shown in Figure 4.2 in Appendix G, was plotted. The purpose of the protein standard curve is to compare and measure the unknown concentration of protein content in the samples with the known concentration. Both protein assay and protein standards used the same method by combining the unknown or known samples with assay reagent and used the colorimetric techniques to measure the absorbance.

After the graph was plotted, the linear regression or straight line was obtained by assuming the overall relationship between concentration and absorbance. The equation for this line is:

$$y = mx + c \tag{3.3}$$

$$Absorbance = 0.3687x + (y\ intercept)$$

$$X\ (mg/ml) = \frac{Absorbance}{0.3687}$$

The protein content (mg/ml) for all the most potent isolate samples in primary and secondary screening (refer Table 4.3, Table 4.4 and Table 4.5) were calculated based on this equation. The absorbance in the equation was the absorbance reading got from the measurement of selected samples at 700 nm by using UV-Vis spectrophotometer. The protein standard curve was plotted after the primary and secondary screening to make sure the standard curve is in the expected range.

4.3 Nitrate Reductase Biochemical Test

Next, the nitrate reductase biochemical test was performed to confirm whether the selected most potent isolate was able to reduce the nitrate to nitrite during this studied or not. This test was commonly done before the enzyme assay was carried out. Figure 4.3 showed the results of this test for two isolates (CL4C and GL7), as well as the control. After incubating the inoculated nitrate broth with a heavy growth of isolates, the samples showed a positive result where their color changed from yellow to dark red compared to the control. This indicates more nitrates had been reduced to nitrite.

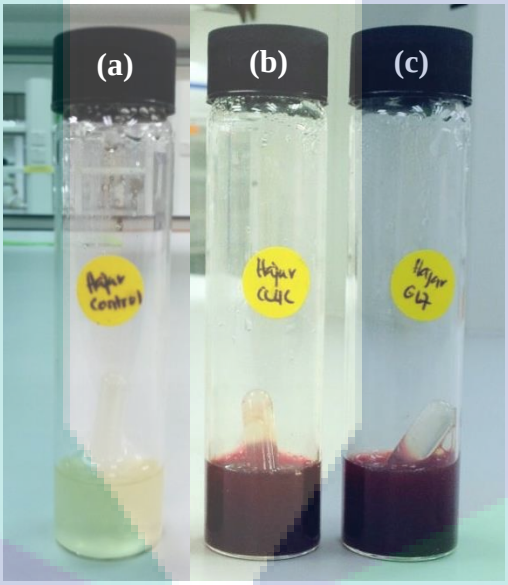

Figure 4.3 The color changes for nitrate reductase test. (a) The result for control which the color remain unchanged. (b) The result for CL4C isolates which the color turns to dark red. (c) The result for GL7 isolates which the color turns to dark red.

This experiment was conducted to prove that the unkown bacterial isolates could successfully reduce the nitrate to nitrites. This is shown by the red color which indicates the presence of nitrite in the tube. The color was formed because the formation of nitrous acid resulted from the reaction between sulfanilic acid and naphthylamine. In this light, if the organism is able to reduce nitrate to nitrite, the nitrous acid was formed in the medium. Consequently, the nitrous acid will react with sulfanilic acid and diazotized sulfanilic acid was produced. At the end, it will react with naphthylamine and a red colored compound was formed.

$$NO_3^- + 2H^+ + 2e^- \xrightarrow{\text{Nitrate reductase}} NO_2^- + H_2O$$

Sulfanilic acid + α-naphthylamine + Nitrite ions → Sulfobenze azo – α-naphthylamine

(colourless) (red coloured)

Nitrate reduction commonly occurs in anaerobic respiration where organisms uses nitrate as an electron acceptor. However, there are also many other ways that a microbe can utilize the nitrate as their final acceptor. The product of reduction could be observed through the present of nitrogen gas (N_2) before any reagents were added. The gas is trapped in the Durhm tube and appears as a bubble at the top of tube if the nitrogen gas present. In this studied, there was no gas bubble observed in Durhm tube, so, it can be concluded that there was no nitrogen gas detected but it was still possible to identify that nitrate was reduced to nitrite. According to the Nitrate Test Handbook, to support this evidence, a red color will be produced when nitrite is present in the medium and if the nitrate was converted to ammonia, the color remains unchanged compared to the control (Reynolds, 2011). However, if the organism was unable to reduce the nitrate or able to denitrify the nitrate or nitrite to produce ammonia or molecular nitrogen, the medium will not turn to red after reagents were added. In this case, the small amount of powdered zinc need to be added and if the mixture in the tube turns red, it could be confirmed that the unreduced nitrate was present, confirming the negative result (Acharya, 2015).

4.4 Parameters Controlling NR Productivity

After the most potent isolates and biochemical test for nitrate reduction were chosen and done, they were proceeded to undergo parameters controlling to study their optimum condition in order to produce nitrate reductase enzyme. Most of the microorganism especially bacterial species induced responses to environmental stress (Keerthirathne, Ross, Fallowfield, & Whiley, 2016). For example, for microbial in soil, the temperature and water content influence their activity and growth (Demoling, Daniela, & Baath, 2015). There are physical and nutritional requirements in order to study the optimum environment for the bacteria. Instead of temperature, the other physical requirements are oxygen, pH, and osmosis factor. For nutritional requirements,

other than water, the energy source, carbon source, nitrogen source, minerals and growth factor also can be studied as reported by Kaiser (2017).

In this study, five parameters were chosen which were incubation condition, substrate concentration, incubation period, pH values and nitrogen source. The isolates were stable in these conditions as they were promoted their growth. The amount of net increases (IU/ml) in all samples was calculated by subtraction the amount of sample enzyme activity (positive control) with their negative control produced after 60 min incubation in darkness (Saifuddin et al., 2009). The nitrite standard curve was plotted (refer Appendix I) to measure the NR activity. In the enzyme assay, when the color reagents which are sulfanilamide solution and N-(1-naphthy) ethelenediamine dihydrochloride solution (NEED) was added into the samples, the color was changed from colorless to pink (Adikaram, Perera, & Wijesundera, 2012) as shown in Figure 4.4 (see Appendix F) after stand for 20 min at room temperature. The darker the color, the more NR enzymes was contained in samples. Then, all the samples were loaded in microplate wells as shown in Figure 4.5 (see Appendix H) before absorbance was measured by using microplate reader (Infinite M200 Pro Tecan).

4.4.1 Effect of Different Incubation Condition

According to data represented in Figure 4.6, the result revealed that CL4C isolate in static incubation reached the optimum condition with the NR enzyme activity of 0.617±0.001 IU/ml compared to shaker incubation which produced only 0.363±0.000 IU/ml. Similar for GL7 isolate, in static incubation conditions, the NR enzyme activity was up to 0.943±0.000 IU/ml compared to the shaker which was 0.544±0.001 IU/ml under the same initial pH, substrate concentration and incubation periods. In fact, while incubated in shaking condition, although it provided the aeration and evenly distributed nutrient to culture environment, the microorganism may consume oxygen during the growth but when the maximum population was reached, the oxygen and nutrient was limited thus cause the nitrate reductase was induced compared to incubate in static condition.

The previous studied by Talon, Walter, Chartier, Barriere, & Montel (1999) revealed that by using an incubator shaker, the growth rate of microorganisms was only

higher in strains *Staphylococcus xylosus* 873, 16 and *Staphylococcus saprophyticus* 852. They found that the synthesis of NR was maximum during exponential growth phase in static condition but in incubator shaker, the synthesis was maximal at the beginning of the stationary phase. On the other hand, the presence of nitrate in shaking condition did not influence the growth rate of the strains. This is supported by Rogosa (2017) who studied the nitrate reduction by certain strains of the genus *Lactobacillus* and found that in static culture, agar and anaerobiosis were not essential for nitrate reduction. Unfortunately, there was limitation in this studied since the effect of growth rate in most potent bacterial isolate was not done in this project.

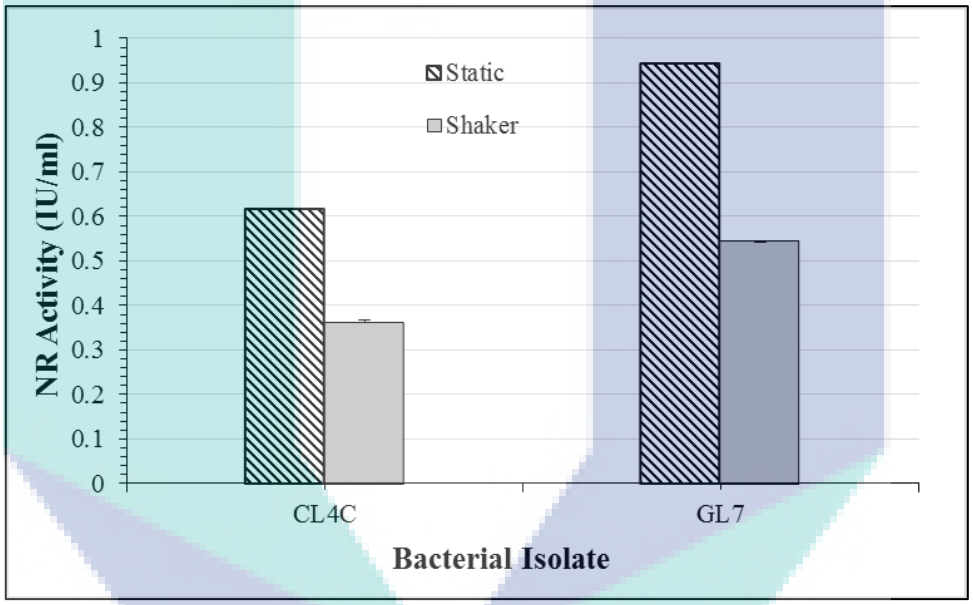

Figure 4.6 Values for NR activity in different incubation condition for CL4C and GL7 isolate

4.4.2 Effect of Different Substrate Concentration and Incubation period

In terms of substrate concentration and incubation period, the experiment was carried out at once. According to the Figure 4.7, the result for the CL4C isolates revealed that it was optimized at 0.5g substrate concentration with protein content up to 0.765±0.010 mg/ml and the NR activity was up to 1.197±0.020 IU/ml. Meanwhile, for the GL7 (refer Figure 4.8), 0.8g substrates reached the optimum NR activity. The protein content was 1.047±0.021 mg/ml and the NR activity was 0.653±0.003 IU/ml.

The substrate concentration is one of the main factor that influences the intensity of velocity in catalyzed the biochemical reaction of the enzyme. This is because they will be bounded to the active sites in the enzyme-protein. Normally, when the substrate concentration was increase, the rate of enzyme reaction may also increase until the enzyme becomes saturated when the substrate concentration was too high. By referred to the CL4C isolate graph, the NR activity was optimum during 0.5g substrate concentration instead of 0.2g, 0.8g, 1.0g and 1.5g.

The rate of reaction of NR activity graph supposed to show the gradually decreasing trends after the optimum substrate concentration was achieved. But during 0.8g substrate was added, the NR activity was lower than 1.0g although it was repeated and this become the limitation in this studied.

By referring to the graph for GL7 (refer Figure 4.8), the NR activity was increased until the optimum substrate concentration was achieved and it was decreased rapidly after 1.0g and 1.5g substrate was added. This can be concluded that, when the small amount of substrate is present in the mixture, the active sites (a place inside the enzyme where substrate go and binds for conversion into product) are left free and the enzyme works less than its maximum ability (Bobade & Khyade, 2012). However, after the optimum concentration was achieved any increase will no longer show the effect on the rate of the reaction since the substrate concentration no longer becomes the limiting factor.

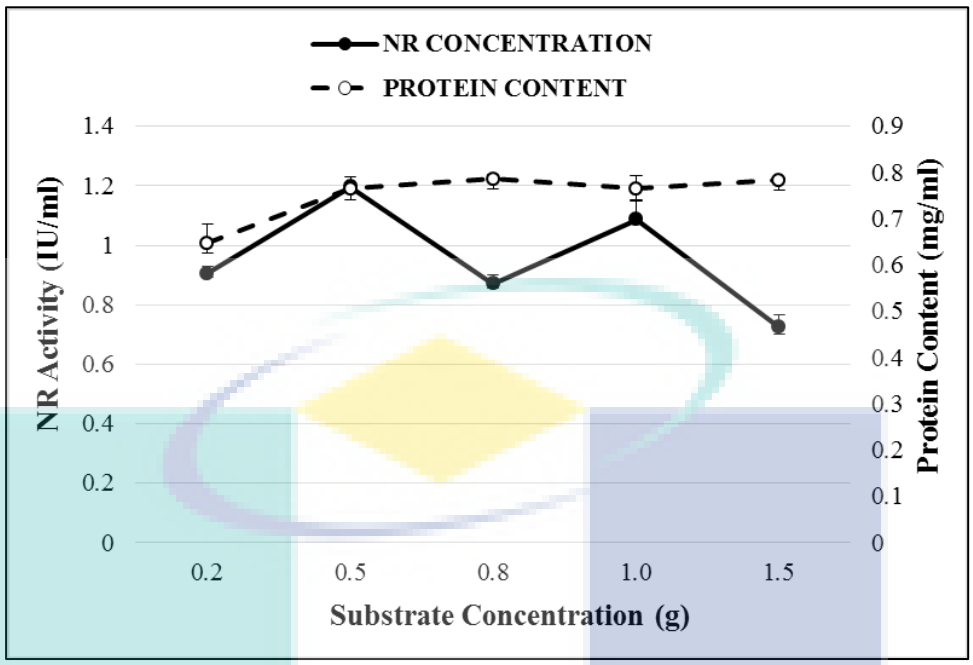

Figure 4.7 Values for NR activity and protein content in different substrate concentration for CL4C

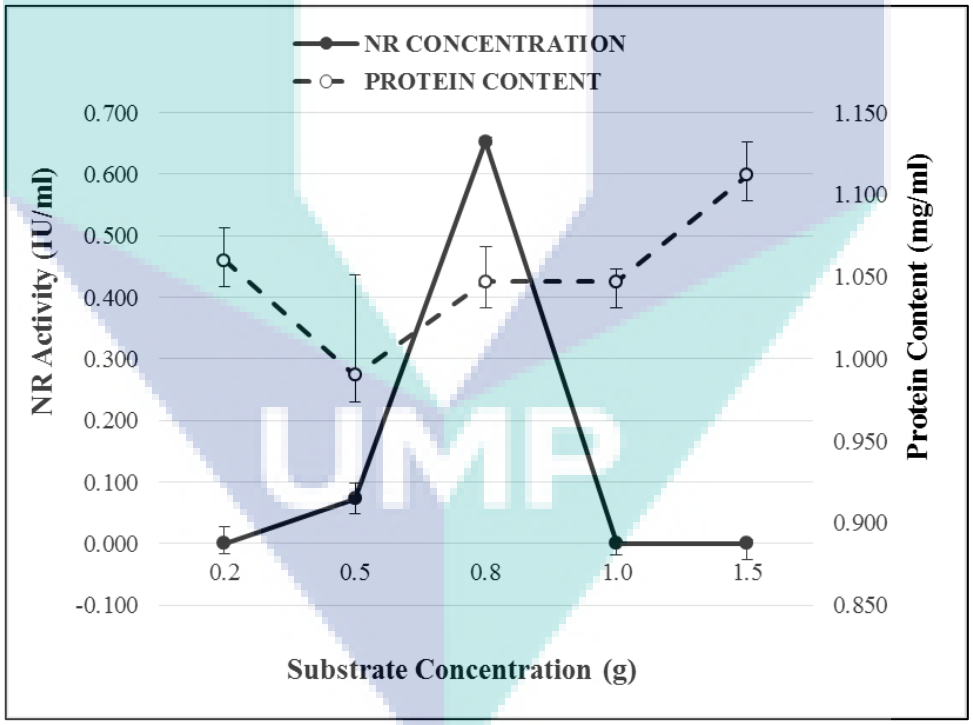

Figure 4.8 Values for NR activity and protein content in different substrate concentration for GL7

Next, the incubation periods of 10 days, 15 days and 20 days were chosen to study the optimum incubation period. The long period interval was chosen to incubate the culture because the substrate used which is disposed x-ray sheets were quite tough

compared to other substrate that frequently used by others such as silver nitrate ($AgNO_3$) (Ibrahim, 2015). The silver nanoparticles were synthesized for the first time by using disposed x-ray sheets because there are no previous studies yet reported the use of this substrates in order to produce silver nanoparticles and nitrate reductase enzyme.

However, Masebinu & Muzenda, (2014) studied how silver could be recovered from disposed x-ray sheets and found that silver can be recovered from the disposed x-ray sheets either through the hydrometallurgical or the pyro-metallurgical processes. The sheets were shredded and treated in acidic or caustic solution such as sodium hydroxide or hydrogen peroxide (Bas, Yazici, & Deveci, 2012) to release the silver from the sheets. According to Bankar, Joshi, Kumar, & Zinjarde (2010), when the reaction mixture was incubated for 15 days, the micro-aggregates of silver nanosized crystallites was observed using a scanning electron microscope (SEM).

According to the Figure 4.9, the result for the CL4C isolates revealed that, the NR activity was optimized at 15 days incubation period with protein content up to 0.765±0.010 mg/ml and the NR activity was up to 1.197±0.020 IU/ml. Meanwhile, for the GL7 (refer Figure 4.10) also NR activity was optimum at 15 days incubation periods. The protein content was 1.047±0.021 mg/ml and the NR activity was 0.653±0.003 IU/ml. But during 20 days incubation period, both isolates showed no NR activity because the culture was incubated too long.

The long interval period for incubation was normal in this studied by using tough substrate because at the end of the incubation period, the silver nanoparticles were successfully formed and the enzyme activity was successfully detected during optimum periods. In previous studied by Keskin, Kilic, Donmez, & Tekiney (2016), they were done the synthesis of silver nanoparticles by incubated the cyanobacteria strains for 15 days and the synthesis of nanoparticle was occurred outside the cell. However, Sithara, Selvakumar, Arun, Anandan, & Sivashanmugam (2017) studied suggested that the test of silver nanoparticle stability should be done because in their studied, the interval incubation time 0-30 days was conducted.

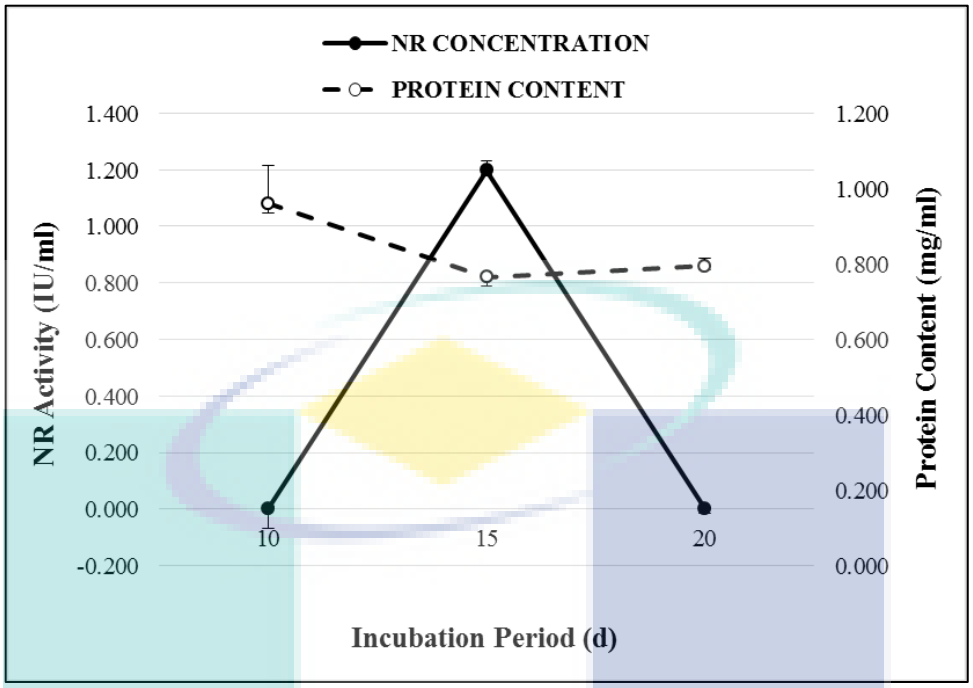

Figure 4.9 Values for NR activity and protein content in different incubation periods for CL4C

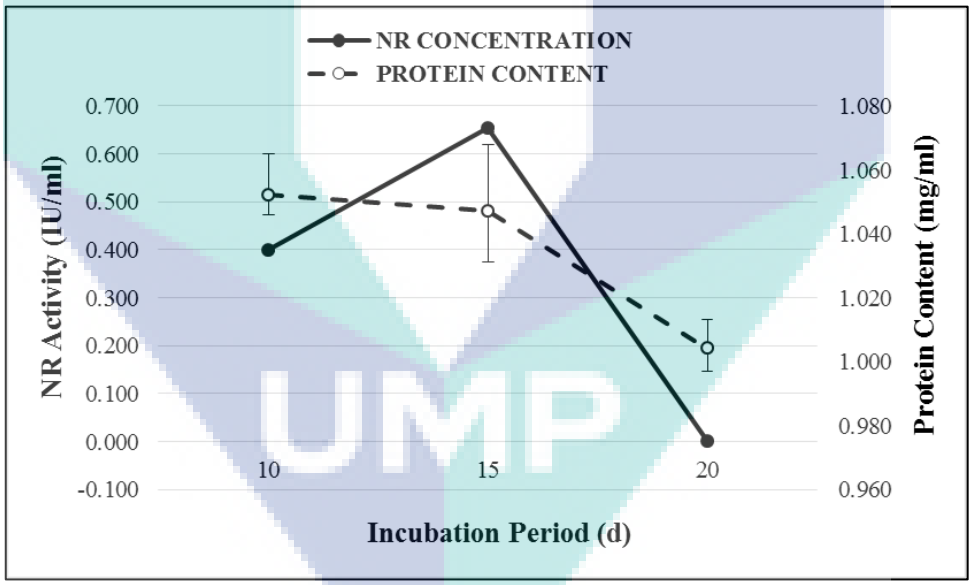

Figure 4.10 Values for NR activity and protein content in different incubation periods for GL7

4.4.3 Effect of Different Initial pH

Figure 4.11 show that the initial pH values for the CL4C sample reached its optimum at pH 7 with the protein content up to 0.730±0.014 mg/ml and NR activity up to 0.254±0.000 IU/ml. Figure 4.12 show that the isolates in GL produced a high NR activity at pH 8 which is 0.399±0.008 IU/ml with the protein content 0.990±0.008 mg/ml.

During this studied, the pH of modified minimal media (MM9) was adjusted by using 0.1N hydrochloric acid (HCl) and 1N sodium hydroxide (NaOH) instead of adjusted by using buffer. Generally, it is depends on the cell used and possible to adjust the pH without buffer as long as the HCl and NaOH in small concentration. Other that, the precaution was took during this preparation because if the NaOH was over added, the HCl should not be added to fix it (vice versa) because it will create salt in the media and may influence the bacteria growth. Many authors such as by UT Health San Antonio (2016) suggested using this protocol when prepared the modified minimal media.

According to the both data obtained, the change in the pH affected the enzymes activity and both isolates produced enzymes at different optimum pH as they were isolated from different sources. For CL4C isolate, it was isolated from the spoiled food which the microorganism grow best at pH 7 at normal temperature and GL7 was isolated from the water source which the normal range of pH is 6.5 to 8.5 (Water Research Center, 2014). In this regard, the optimum pH of the enzyme depends on where it normally works; the enzyme might be denatured by extreme pH as they are soluble proteins. The changes in pH may also affect the shape of the enzymes and substrate and these changes may decrease the substrates' ability to bind to their active site (Wong, 2016).

For CL4C isolate, the NR activity was low during pH 4 because of environment in culture media was acidic but showed very low activity in pH 5. This was the limitation in this studied. Next, it was gradually increased during pH 6 until reached the optimum at pH 7. After that, the NR activity was continued to slower again during pH 8 and 9. Also for the GL7 isolate, there was limitation when conducted the studied during

pH 7 because the result showed no different NR activity when compared to the control. Khan (2008) reported that the nitrate reductase activity was optimal at pH 7.0 and the activity maintained optimal and detectable from pH 5.0 to pH 9.0. This finding showed the enzyme has the ability to adapt to a broad range of pH and has a different role in the environment at different physiological pHs.

By comparing with previous research by Rogosa (2017), when the medium was treated with agar and yeast extract at the initial pH of 6.4, which is a suitable pH value for bacterial growth. Consequently, all the *Lactobacillus plantarum* strains failed to reduce the nitrates in aerobically nor anaerobically and the pH value was decreased to pH 3.4. According to Kamiloglu, Kaban, & Kaya (2016), a quick drop in pH will prevent the reduction of nitrate by microbial and the pH should not be decreased below 5.4 until enough amount of nitrate reductase achieved. However, the cultures were able to reduce the nitrates in the pH of 7.4. The *Micrococcus aureus* type 80 was able to reduce the nitrate at pH 7 and based on the accumulation of nitrogen gas, some organisms have consistently reduced their nitrate completely at pH 7.5 or above.

Many authors posited that the range of pH 7 to pH 8 as an optimal environment for nitrate reductase activity and suggested the incubation temperature between 20 °C and 25°C (Krywult & Bielec, 2013). In the denitrification process, the nitrate acts as an intermediate and they depending on the pH and temperature of the medium. By optimizing the pH, it can affect bacterial growth and their enzymatic activities (Estuardo, Marti, Huilinir, Lillo, & Von Bennewitz, 2008).

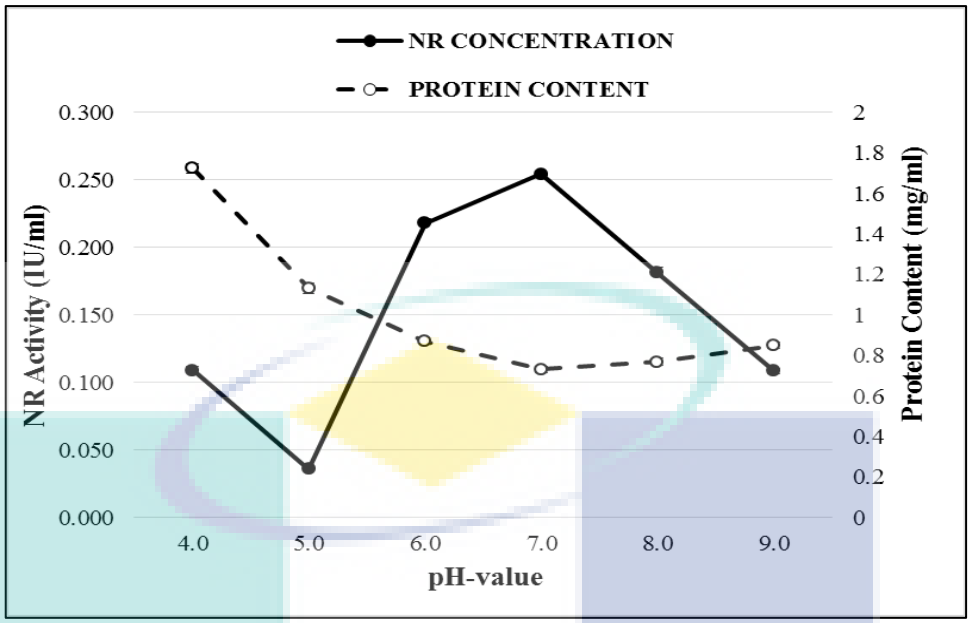

Figure 4.11 Values for NR activity and protein content in different initial pH for CL4C

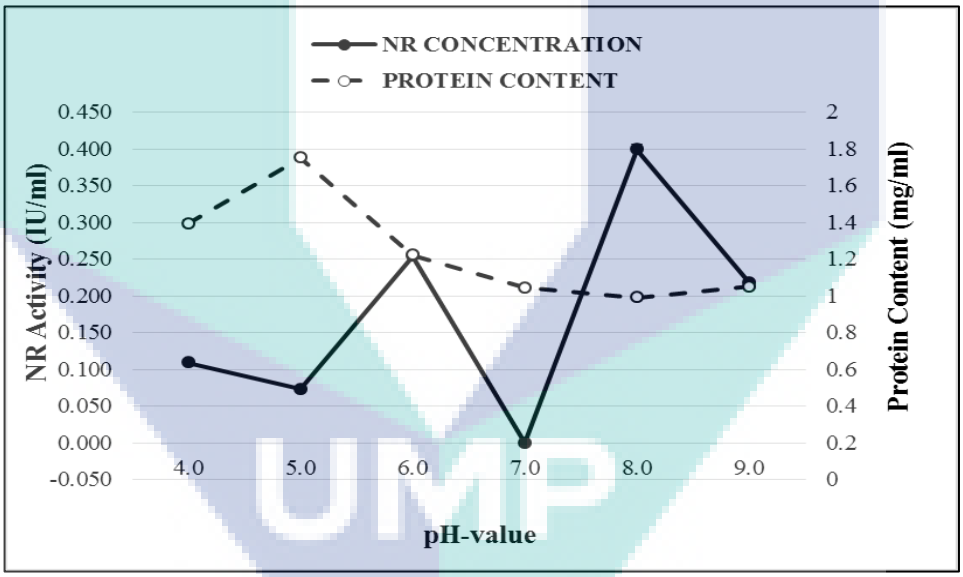

Figure 4.12 Values for NR activity and protein content in different initial pH for GL7

4.4.4 Effect of Different Nitrogen Source and Different Concentration

At the beginning, the effect of different nitrogen sources were studied which are yeast extract, malt extract, ammonium chloride, potassium nitrate and sodium nitrate. Based on Figure 4.13 and Figure 4.14, the result revealed that by using sodium nitrate treated in the production medium MM9, the nitrate was reduced and shown the highest enzyme activity compared to other nitrogen sources. For CL4C isolate, the NR activity

obtained was 98.361±0.223 IU/ml with the protein content 0.884±0.009 mg/ml. Whereas for GL7 isolate, the NR activity was 122.960±0.122 IU/ml with the protein content 1.047±0.007 mg/ml. Esen & Urek (2014) stated that the nitrate is an important element and incorporated in the most vital structural component like proteins. But the nitrate concentration may affect the enzymatic activities and nitrogen assimilation pathway (Perez-Garcia et al., 2011).

In both isolate, when the yeast and malt extract was added to the culture medium, the result showed no and very low NR activity for CL4C and GL7 isolate respectively. This can be concluded that, the yeast and malt extract become an inhibitor for NR enzyme. A previous research proved that when medium supplemented with yeast extract, the nitrate reduction could become weak or absent which is supported by the negative responses to total growth (Rogosa, 2017). Although the NR enzyme was being partially purified, the yeast also become inhibitor for NR activity (Provan, Aksland, Meyer, & Lillo, 2000).

Meanwhile, when ammonium chloride was added, the low NR activity was also detected. But with the presence of potassium nitrate, the higher NR activity was obtained compared to ammonium chloride, malt extract and yeast extract. Balotf, Niazi, Kavoosi, & Ramezani (2012) found that, both sodium and potassium nitrate increased the nitrite accumulation in dose dependent manner. Previous studied stated that when the medium was treated with ammonium chloride or potassium nitrate in cotton leaf discs, there was a 20 to 30 % decrease in nitrate reduction as early as 30 min after the incubation started. This is supported by Talon et al. (1999) who stipulated that when nitrate was present in a culture medium, the growth rate for staphylococci was improved during the incubating period.

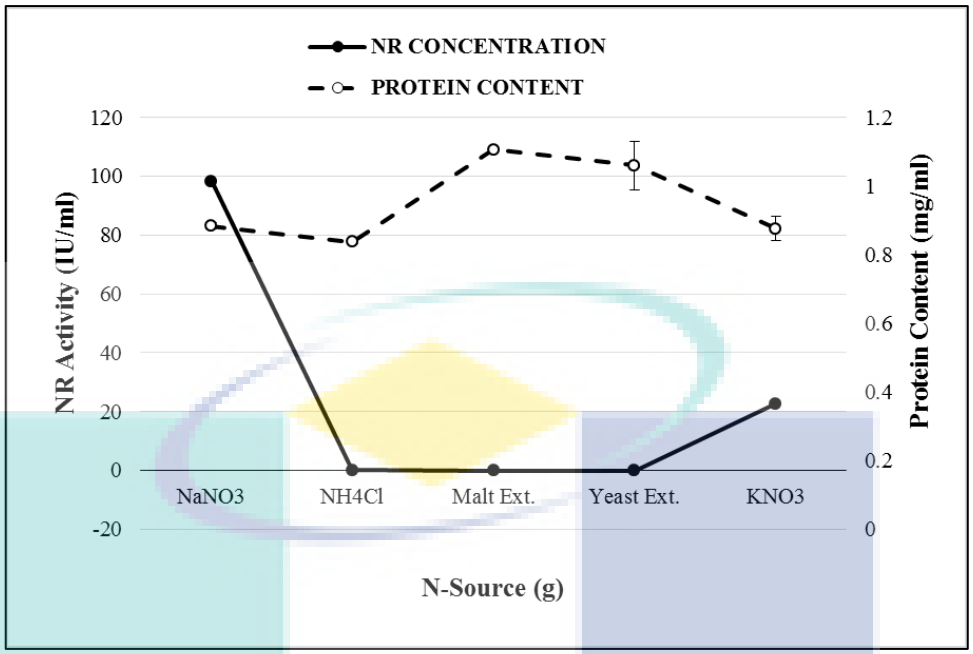

Figure 4.13 Values for NR activity and protein content in different N-source for CL4C

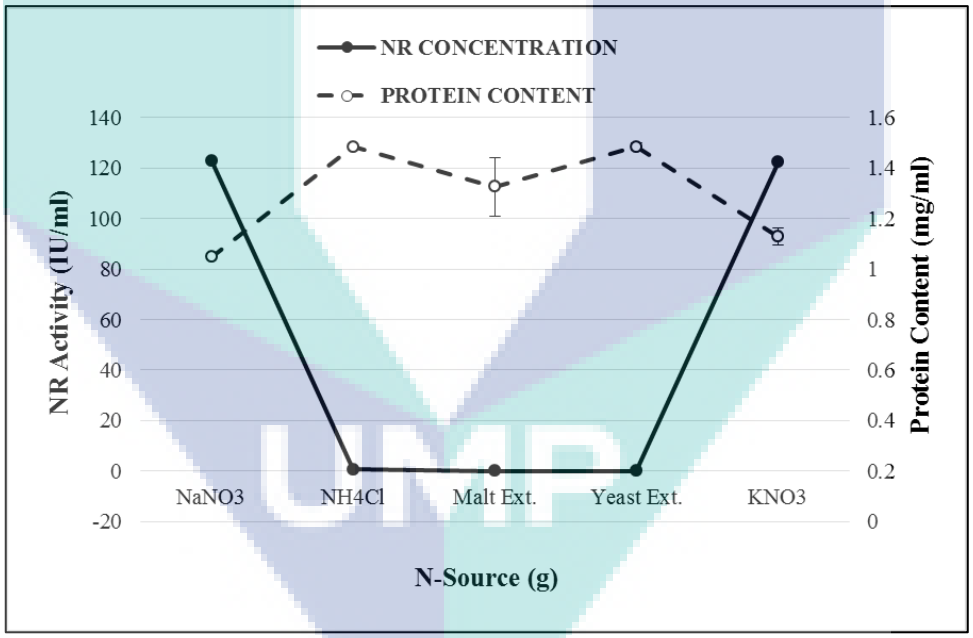

Figure 4.14 Values for NR activity and protein content in different N-source for GL7

After the optimum nitrogen source had been determined, the analysis on the effect of different nitrogen source concentration was carried out since the NR enzyme activity was depended also on the different concentration added. Figure 4.15 and Figure 4.16 show that both isolates reached their optimum activity at the concentration of 0.03g sodium nitrate ($NaNO_3$) compared to 0.00g, 0.01g, 0.02g, 0.04g and 0.05g.

For CL4C, the protein content was 0.922±0.010 mg/ml and the enzyme activity was 133.119±0.054 IU/ml. Meanwhile, for GL7, the result revealed that the protein content was 1.082±0.058 mg/ml and enzyme activity was 119.622±0.050 IU/ml. This shows that nitrogen source is required in bacterial growth as they used to form amino acids, DNA and RNA. The nitrate is a salt that can dissociate to give NO_3^- to the bacteria. At the beginning, the main purposed of this studied was to investigate whether in minimum usage of sodium nitrate, the most potent isolate was able to produce optimum enzyme activity or not. So, it can be concluded that, the NR activity was optimum depended on the ability of bacterial isolate itself whether they can optimize in low or high concentration of nitrogen source.

By comparing the studied by Esen & Urek (2014), the highest NR activity was obtained in the presence of 100 mM sodium nitrate. They conclude that the higher enzyme activity, the higher the nutritional value in culture. The nitrate supply promote the nitrate uptake, nitrate reduction, NR activity and nitrate expression at low concentration of sodium nitrate (Balotf et al., 2012). The sodium and potassium nitrate caused the significant increase in nitrate at low concentration, whereas, in high concentrations, the salts suppressed the expression of gene considerably. This is shown through the ability of sodium and potassium nitrates to restore the nitrate reductase activity in wheat leaves (Shahrokh et al., 2014).

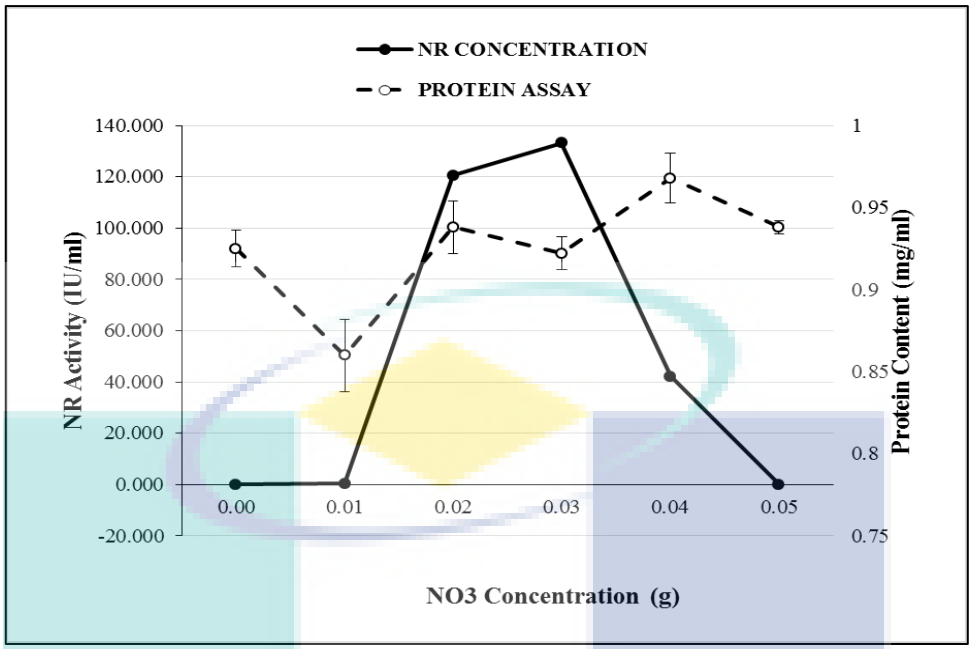

Figure 4.15 Values for NR activity and protein content in different $NaNO_3$ concentration for CL4C

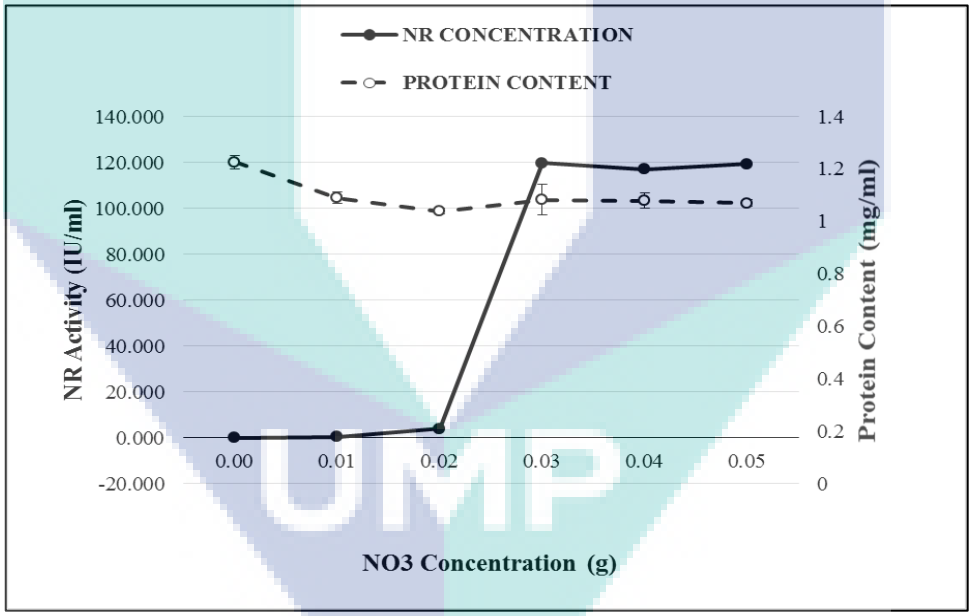

Figure 4.16 Values for NR activity and protein content in different $NaNO_3$ concentration for GL7

4.4.5 Finalized Data on the Optimum Parameters for CL4C and GL7 Isolates

After the optimum conditions for both isolates was revealed, the disposed x-ray sheets were pre-treated again with 4N NaOH and the complete production medium containing bacterial culture were prepared again with all the optimum parameters obtained in one flask for each isolate. After that, the supernatant obtained from last

batch had undergone a protein and nitrate reductase enzyme assay to obtain the final data.

Table 4.7 shows the finalized data for protein assay and NR activity for CL4C and GL7 isolates. Based on the average obtained, the optimum parameters for CL4C and GL7 was summarized as shown in Table 4.8. Both CL4C and GL7 were optimum in static incubation condition, 15 days incubation period and sodium nitrate as nitrogen source. But for substrate concentrations, the NR activity for CL4C isolate optimum at 0.5g and GL7 isolate optimum at 0.8g. For pH values, CL4C isolate optimum at pH 7 and GL7 isolate optimum at pH 8.

Table 4.7 Finalized data for quantification of protein and NR activity for CL4C and GL7 isolates

Isolate Code	NR Activity (IU/ml)	Protein Content (mg/ml)
CL4C	69.952 ± 0.090	1.139 ± 0.029
GL7	108.882 ± 0.354	1.454 ± 0.024

Table 4.8 Summary of optimum parameters for CL4C and GL7 isolates

No	Parameters	Isolate Code	
		CL4C	GL7
1	Incubation condition	Static	Static
2	Incubation period (day)	15	15
3	Substrate Concentration (g)	0.5	0.8
4	Nitrogen Source	$NaNO_3$	$NaNO_3$
5	pH values	7.0	8.0
	Enzyme Activity (IU/ml)	69.952 ± 0.090	108.882 ± 0.354
	Protein Content (mg/ml)	1.139 ± 0.029	1.454 ± 0.024

From the results achieved, it can be concluded that the enzyme activity in GL7 isolate produced more NR activity and protein content compared to CL4C isolate. The evidence of enzyme activity occured proved that the selected most potent microbial isolates were able to reduce the nitrate to nitrite. In the context of nitrogen source, an enzyme activity was the highest in the presence of nitrate. This signifies that the nitrification process was not taking place in the reducing processed as the nitrification is

the process where biological oxidation converts the ammonia or ammonium to nitrite followed by oxidation of nitrate to nitrite.

However, it can be assumed that the denitrification process was occurred due to the reduction of the nitrates to nitrogen gas. This was proved by conducted the nitrate reductase biochemical test by observed the color changed. After all of the data were collected, the samples underwent TEM, XRD, ICP-MS and Biolog Microbial Identification System analysis.

4.5 Analysis of Silver Nanoparticles

To confirm whether the silver nanoparticles formed was deposited in the bacterial cell or not, the pellets obtained was sent for TEM analysis. According to Narasimha, Praveen, Mallikarjuna, & Raju (2011), TEM technique was used to visualize the size and morphology of nanoparticles.

For this analysis, the samples were sent to UiTM, Puncak Alam for analysis by using TECNAI G^2 model transmission electron microscopy (TEM). According to the result obtained, unfortunately, the analysis failed to capture the silver nanoparticles image deposited in the both bacteria isolates. This probably can conclude that, the synthesis of silver nanoparticles was occurred outside a cell (extracellular) not in a cell (intracellular).

To confirm the synthesis of silver nanoparticles was occurred extracellular, the x-ray diffraction analysis (XRD) was done. The supernatant was freeze-dried to obtain the powder form. Once the freeze-drying process finished, the powder obtained was sent for XRD (Rigaku, Miniflex II) analysis at FIST Laboratory, Universiti Malaysia Pahang, Gambang. The metallic nature of the particles was analyzed through the XRD technique. Two samples were analyzed and Figure 4.17 shows the XRD pattern obtained for silver nanoparticles synthesized of CL4C isolate grown on disposed x-ray sheets at 37 °C in darkness.

According to the result, only 7 silver nitrite peaks were obtained with an average grain size about 19.53 nm. The diffraction peaks were at 22.32°, 29.45°, 33.85°, 45.32°,

52.17°, 57.94° and 71.2° (see the data in Appendix J). While, Figure 4.18 shows the XRD pattern obtained for silver nanoparticles synthesized of GL7 isolate grown on x-ray sheets at 37 ℃ for the same incubation period. According to the results, GL7 isolate exhibited only two silver nitrite peaks obtained with the average size of 52.35 nm. The diffraction peaks were observed at 31.18° and 32.93° (see the data in Appendix K).

From all the result obtained, CL4C samples obtained the smallest size of SNPs, compared to GL7 and the XRD pattern shows the peak in the whole spectrum of 2-theta values ranging from 10 to 120. The 2-theta values represent the face centered cubic lattice of silver. So this can be concluded that, the synthesis of silver nanoparticles was occurred outside a cell since the silver particles was detected in the supernatant, not in the bacterial cell.

It is important to know the exact nature of the silver particles formed and this can be deduced from the XRD spectrum of the sample. The pure crystalline silver structures were also shown clearly in the XRD pattern spectra (Narasimha et al., 2011). In general, the widening peaks in XRD patterns of solids are associated with particle size effect. The smaller nanoparticle size is signified by broader peaks and reflected the effects due to the growth of crystal nuclei and experimental conditions (Panigrahi, 2013).

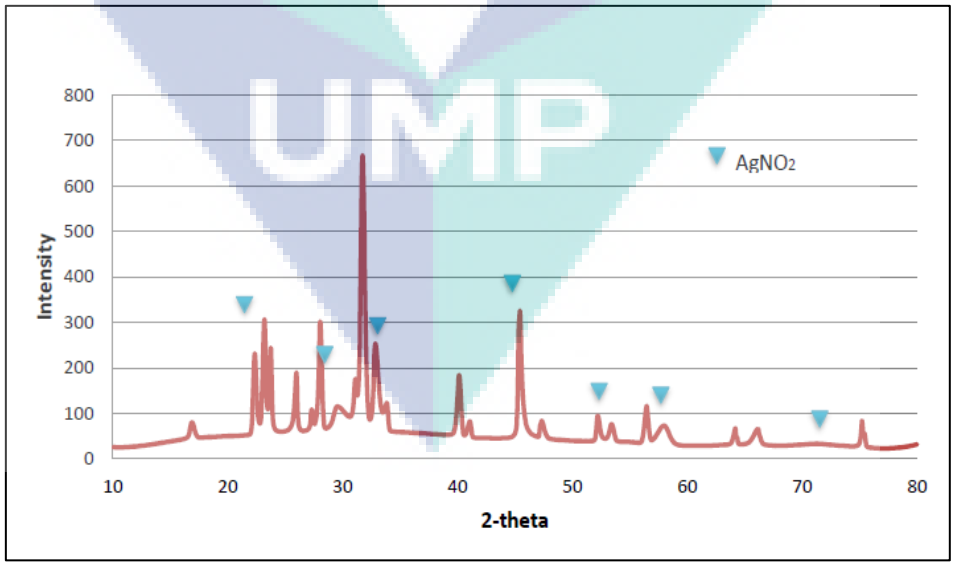

Figure 4.17 The silver nitrite peaks obtained at 22.32°, 29.45°, 33.85°, 45.32°, 52.17°, 57.94° and 71.2° for CL4C

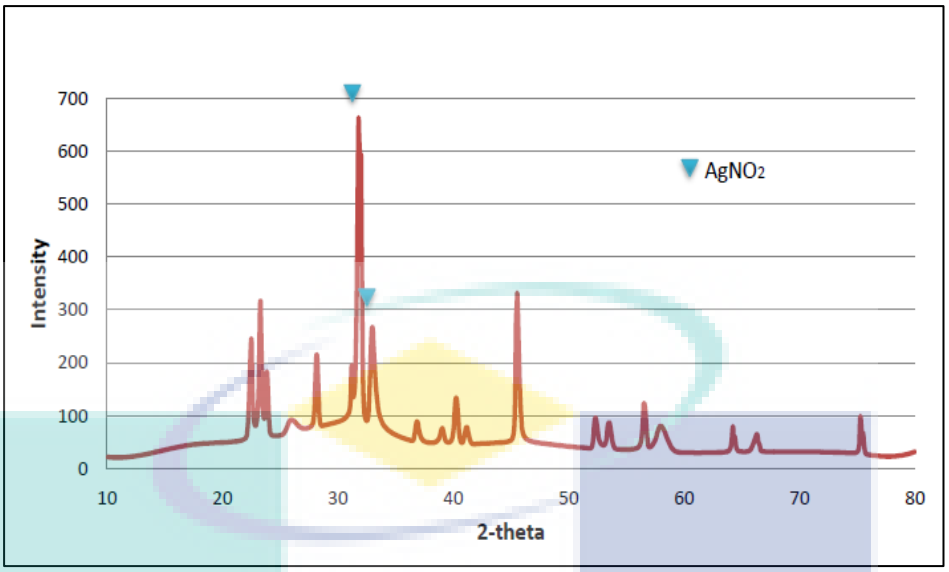

Figure 4.18 The silver nitrite peaks obtained at 31.18° and 32.93° for GL7

Based on the observation on color change of the production medium's MM9 after incubation period, the color of the CL4C samples turned to yellow while the control, which did not contain any microbial isolates was colorless, as shown in Figure 4.19. Meanwhile, the GL7 color changed from colorless to a paler shade of yellow compared to CL4C. In this light, the color changes affect the size of nanoparticles obtained. The formation of SNPs is able to produce the particular color in the reaction mixtures due to their specific properties (Kumar & Jayabalakrishnan, 2011). The appearance of yellowish-brown coloration in the silver nitrate treated culture supernatant suggests the formation of SNPs. A similar observation was made by Duran, Marcato, Alves, Souza, & Esposito (2005) in the extracellular biosynthesis of SNPs by using *Fusarium oxysporum* strain. The brown color of the medium could be due to excitation of surface Plasmon vibration exhibited by the nanoparticles (Narasimha et al., 2011).

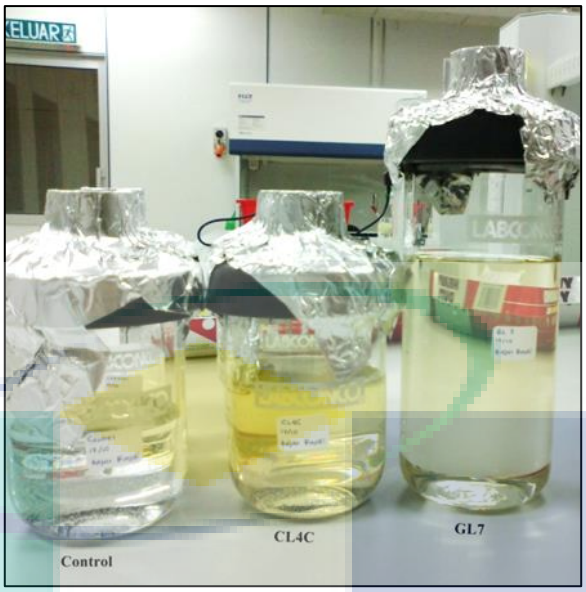

Figure 4.19 The different color of CL4C and GL7 samples compared to the control after incubation period (after centrifugation)

Then, to examine the concentration of selected elements in both samples as low as one part in 10^{15} (part per quadrillion, ppq), the same supernatant was sent to single particle ICP-MS analysis at Central Laboratory, Universiti Malaysia Pahang, Gambang. This analysis comprises of a mass spectrometry to detect metals and several non-metals on non-interfered low-background isotopes. The samples were ionized with inductively coupled plasma and achieved ions to be separated and quantified by a mass spectrometer. The single particle ICP-MS was carried out in order to determine the particle number concentration and the mass of metal in individual particles and size distribution. However, this analysis did not provide information on the shape of the particle.

The 500ml of supernatant from both samples were sent for analysis. Table 4.9 and Table 4.10 show the results for selected elements, which are Potassium (K^+), Calcium (Ca^{2+}), Copper (Cu^{2+}) and Silver (Ag^+). For both isolates, the concentration of silver elements was less than 0.5 ppb compared to other elements. The results shown in Table 4.9 and Table 4.10 revealed that the volume of the silver particles was too small (0.5 ppb for both microbial isolates). Both isolates contain the highest concentration of potassium because this element was used in the preparation of the MM9 production medium.

Table 4.9 ICP-MS result for CL4C isolate

No	Parameter	Concentration	Unit
1	K^+	916.3	ppm
2	Ca^{2+}	6.9	ppm
3	Cu^{2+}	115.2	ppb
4	Ag^+	Less than 0.5	ppb

Table 4.10 ICP-MS result for GL7 isolate

No	Parameter	Concentration	Unit
1	K^+	605.4	ppm
2	Ca^{2+}	152.2	ppm
3	Cu^{2+}	89.8	ppb
4	Ag^+	Less than 0.5	ppb

4.6 Identification of Microorganism

The CL4C and GL7 bacterial cells were analyzed by using the Biolog Microbial Identification System in the Central Laboratory, University Malaysia Pahang. Both isolates were found to be Gram-negative bacteria as shown in Table 4.11.

Table 4.11 Biolog Microbial Identification System results for CL4C and GL7 isolates

No	Isolate Code	Result		Test Method
		Name	Probability	
1	GL7	*Enterobacter aerogenes*	50.30%	Gen III Microplate IFB (Protocol B)
2	CL4C	*Morganella morganii*	70.00%	Biolog Microbial Identification System

The data were confirmed by conducting a Gram staining and observed by bright field microscope (100 x magnifications). It showed that the bacterial isolates were identified to be Gram-negative for both isolates because the blue stain turned to pink after several Gram staining processes. Figure 4.20 shows the Gram staining for CL4C isolate predicted as *Morganella morganii* and Figure 4.21 shows the result for GL7 isolate predicted as *Enterobacter aerogenes.*

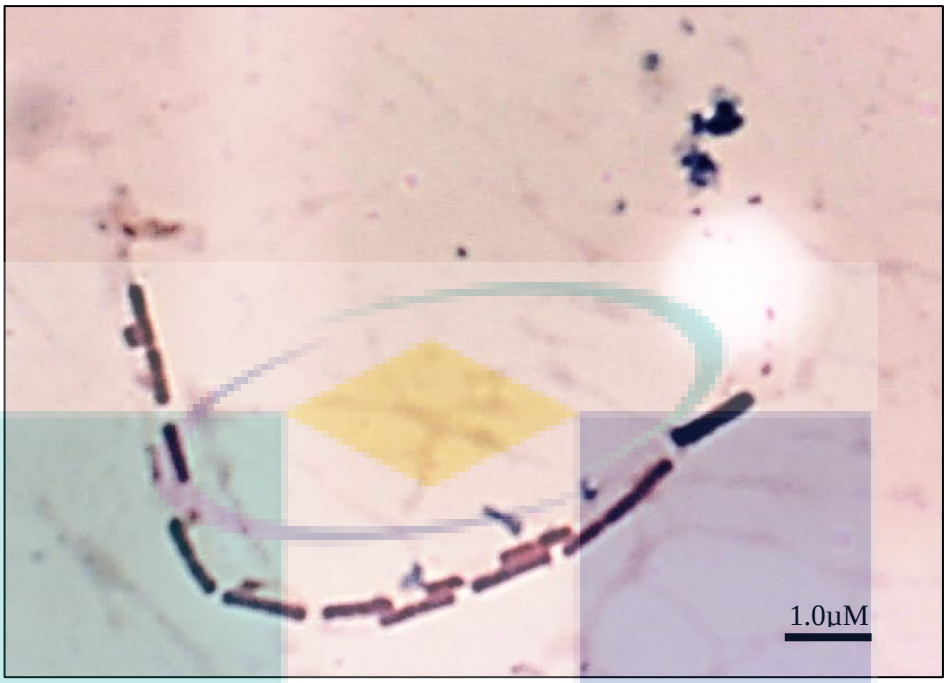

Figure 4.20 CL4C isolate predicted to be *Morganella morganii*

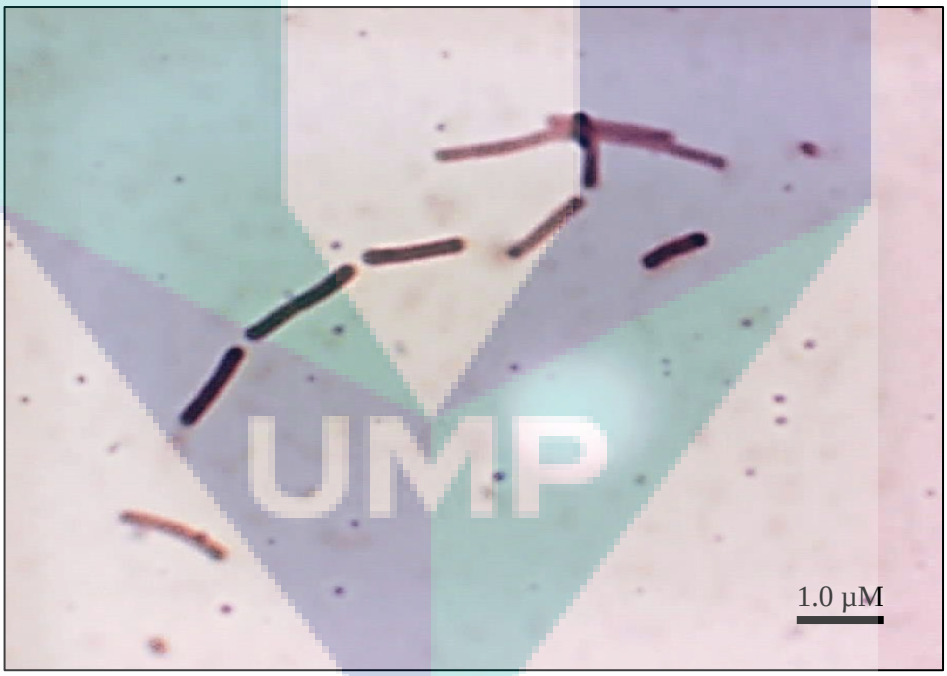

Figure 4.21 GL7 isolate predicted to be *Enterobacter aerogenes*

Numerous studies on nitrate reductase have been carried out on various plants, and found that other microorganisms are also able to reduce the nitrates to nitrites. According to Pugazhenthiran et al. (2009), Gram-positive bacteria such as *Bacillus* sp, Gram-negative bacteria can also reduce the nitrates to nitrites. Furthermore, the

Rhizobium sp. and *Azotobacter* sp. isolates were found to reduce nitrate aerobically in their optimum conditions (Shahrokh et al., 2014), and the *Escherichia coli* was also studied by the researcher to determine its function as a nitrate reductase, while *Enterobacter aerogenes* showed positive result in the biochemical test for nitrate reduction as reported in this study.

Enterobacter aerogenes is a rod-shaped bacterium which is found to be oxidase negative, catalase positive, citrate positive and indole negative. But during this studied, it can be concluded that, this bacteria has high potential to be nitrate positive also. This bacteria can cause opportunistic infections and is sensitive to antibiotics. It is generally found in the human gastrointestinal tract and found to live in various wastes but does not cause any disease in healthy individuals.

On the other hand, studies have proven the microbial power of *Morganella morganii* to reduce the nitrate. *Morganella morganii* is a straight rod bacterium which can produce enzyme catalase. The bacterium is able to convert hydrogen peroxide (H_2O_2) to water and oxygen ($H_2O + O_2$). During the biochemical test, this bacterium showed positive results in the indole test and the methyl red test. It can be found in the normal flora in the intestinal tract of humans, mammals and reptiles. On the other hand, it is resistant to some antibiotics such as penicillin, ampicillin, oxacillin, first-generation and second-generation cephalosporins, macrolides, lincosamides, fosfomycin, colistin, and polymyxin B (Chen & Wang, 2015).

A previous research by Parikh et al. (2011) found that all the members of the *Morganella* genus, including the *Morganella morganii* strain facilitate the extracellular synthesis of silver nanoparticles. This is confirmed by the clear presence of silver (Ag) suface plasmon resonance characteristic (between 400 and 500 nm) in all the samples. The *Morganella morganii* was able to synthesize the extracellular silver nanoparticles in the urine samples of patients with Catheter Associated Urinary Tract Infections (CAUTI). This bacterial strain was able to synthesize silver nanoparticles as early as 1 h reaction time and the yield of SNPs increased as the reaction progressed over time (Abd et al., 2013).

According to Karthik & Radha (2012), *Enterobacter aerogenes* can be used in the extracellular synthesis of silver nanoparticles by using TEM, UV-visible spectroscopy, Energy Dispersive X-ray Spectroscopy (EDX) and Scanning Electron Microscopy (SEM). During the surface plasmon resonance analysis, the absorption spectra showed the progressive 410 - 420 nm wavelengths that corresponded to the silver. The size of SNPs ranged between 25 – 35 nm and they were spherical shaped.

In addition to *Morganella morganii* and *Enterobacter aerogenes*, there are many Gram-negative bacteria which are able to synthesis of silver nanoparticles including *Aeromonas* sp., *Acinetobacter calcoaceticus*, *Geobacter sulfurreducens*, *Idiomarina* sp., *Bordetella* sp., *Gluconobacter roseus*, *Proteus mirabilis*, *Klebsiella pneumonia*, *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris*, *Shewanella oneidensis*,*Stenotrophomonas maltophilia*, *Xanthomonas oryzae*, and *Yersinia enterocolitica* (Singh, Shedbalkar, Wadhwani, & Chopade, 2015)

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Introduction

The purpose of this chapter is to provide the summary of the study's result and to conclude the research conducted. This chapter also presents recommendations for future research.

5.2 Conclusion

In conclusion, the objectives of this research were achieved. The various unknown bacterial isolates were successfully isolated from 3 different sources which are soil, water and spoiled food in order to find the most potent isolates. Next, they was purified and predicted as *Enterobacter aerogenes* for GL7 and *Morganella morganii* for CL4C by using Biolog Microbial Identification System analysis. Then, it was confirmed as Gram-negative bacteria by Gram staining.

Parameter controlling which are incubation condition, incubation periods, substrate concentration, initial pH values and nitrogen source was conducted after the primary and secondary screening. The CL4C most potent isolate predicted as *Morganella morganii* was found to be optimum at static incubation condition, 15 days incubation period with a 0.5g substrate concentration. $NaNO_3$ was used as a nitrogen source and showed optimum enzyme activity at pH 7.0 with 69.952±0.090 IU/ml.

Whereas, the GL7 isolate predicted as *Enterobacter aerogenes* isolate code was optimum at static incubation condition, 15 days incubation period, 0.8g substrate

concentration with the use of $NaNO_3$ as a nitrogen source. Optimum enzyme activity was observed at pH 8.0 with 108.882±0.354 IU/ml.

The size of SNPs was successfully determined by using XRD analysis and for CL4C isolate was 19.53 nm while the size for GL7 isolate was 52.35 nm. Lastly, the volume of the silver particles detected by ICP-MS was 0.5 ppb for each microbial isolates.

5.3 Recommendation

The study on bioremediation using disposed x-ray sheets are recommend as there is a lack of studies on this substrate. Consequently disposed x-ray sheets can be reused instead of being wasted. The study of nature disposed x-ray sheets and effect of each component on the microbial production are recommend. The growth curve for every parameter chosen also is recommended to study.

It is also recommends to carry out purification process in order to harvest high NR enzyme and produce silver nanoparticles as large-scale production is very important for SNPs commercialization. Next, the studies about the effect of SNPs on antimicrobial activity also need to be focus.

In the term of most potent bacteria chosen, it is recommend studying also the ability of fungi and other microorganism to use disposed x-ray sheets as substrate in order to produce silver nanoparticles.

REFERENCES

Abd, F. G., Al-Kawaz, A. J. A. H., & Al-Dahmoshi, H. O. M. (2013). Phenotypic and genotypic investigation on silver nanoparticles of *Morganella Morganii* Recovered from (Cauti), Iraq. *International Journal of Medicine and Pharmaceutical Sciences*, *3*(3), 29–38.

Acharya, T. (2015). Nitrate Reduction Test: principle, procedure and results [online]. Retrieved from https://microbeonline.com/nitrate-reduction-test-principle-procedure-results/

Adikaram, C. P., Perera, J., & Wijesundera, S. S. (2012). The manual mycobacteria growth indicator tube and the nitrate reductase assay for the rapid detection of rifampicin resistance of *M . Tuberculosis* in low resource settings. *BMC Infectious Disease*, *12*(326), 1–6.

Al-Marhaby, F. A., & Seoudi, R. (2016). Preparation and characterization of silver nanoparticles and their use in catalytic reduction of 4-nitrophenol. *World Journal of Nano Science and Engineering*, *6*(3), 29–37.

Amin, M., Anwar, F., Janjua, M. R. S. A., Iqbal, M. A., & Rashid, U. (2012). Green synthesis of silver nanoparticles through reduction with Solanum xanthocarpum L. berry extract: Characterization, antimicrobial and urease inhibitory activities against *Helicobacter pylori*. *International Journal of Molecular Sciences*, *13*(8), 9923–9941.

Ayangbenro, A. S., & Babalola, O. O. (2017). A new strategy for heavy metal polluted environments : a review of microbial biosorbents. *International Journal of Environmental Research and Public Health*, *14*(1), 94.

Balotf, S., Niazi, A., Kavoosi, G., & Ramezani, A. (2012). Differential expression of nitrate reductase in response to potassium and sodium nitrate : Realtime PCR analysis. *Australian Journal of Crop Science*, *6*(1), 130–134.

Bankar, A., Joshi, B., Kumar, R. A., & Zinjarde, S. (2010). Banana peel extract mediated novel route for the synthesis of silver nanoparticles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *368*(1–3), 58–63.

Bas, A. D., Yazici, E. Y., & Deveci, H. (2012). Recovery of silver from x-ray film processing effluents by hydrogen peroxide treatment. *Hydrometallurgy*, *121–124*(August 2017), 22–27.

Bhatia, S. (2016). *Nanoparticles Types , Classifi cation , Characterization , Fabrication Methods and Drug Delivery Applications. Natural Polymer Drug Delivery Systems.* Springer International Publishing Switzerland 2016.

Bobade, S. N., & Khyade, V. B. (2012). Influence of inorganic nutrients on the activity of enzyme, nitrate reductase in the leaves of mulberry, *Morus alba* (L) (M-5 variety). *Research Journal of Recent Sciences 1*(5), 14–21.

Burow, K. R., Nolan, B. T., Rupert, M. G., & Dubrovsky, N. M. (2010). Nitrate in groundwater of the United States, 1991-2003. *Environmental Science and Technology, 44*(13), 4988–4997.

California Code of Regulations. (2013). Defining hazardous waste [online]. Retrieved from http://www.dtsc.ca.gov/LawsRegsPolicies/index.cfm%5CnClick

Campbell, E. (2015). Enzyme-Based Analytical Chemistry [Online]. Retrieved from https://www.epa.gov/sites/production/files/2015-11/documents/c15-campbell-p1.pdf

Campbell, W. H. (1999). Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. *Annu. Rev. Plant Physiol. Plant Mol. Biol, 50*, 277–303.

Chen, Y., & Wang, F. (2015). Insights on nitrate respiration by *Shewanella*. *Frontiers In Marine Science, 1*(January), 1–9.

Chow, F., Capociama, F. V, Faria, R., & Oliveira, M. C. D. E. (2007). Characterization of nitrate reductase activity in vitro in *Gracilaria caudata J . Agardh* (Rhodophyta , Gracilariales). *Revista Brasil. Bot., 30*(2), 123–129.

Dash, L. (2013). *Biological Synthesis and Characterization of Silver Nanoparticles using Bacillus thuringiensis*. Master Thesis. National Institute of Technology, India.

Deljou, A., & Goudarzi, S. (2016). Green extracellular synthesis of the silver nanoparticles using thermophilic *Bacillus Sp* . AZ1 and its antimicrobial activity against several human pathogenetic bacteria. *Iran J Biotechnol 14*(2), 25-32.

Demoling, F., Daniela, F., & Baath, E. (2015). Comparison of factors limiting bacterial growth in. *Soil Biology & Biochemistry, 39*(October 2007).

Dental Microbiology Lab. (1995). General microbiological techniques [Online]. Retrieved from http://www.medschool.lsuhsc.edu/Microbiology/DMIP/dmex05.htm

Diantoro, M., Fitrianingsih, R., Mufti, N., & Fuad, A., (2014). Synthesis of silver nanoparticles by chemical reduction at various fraction of MSA and their structure characterization. AIP Conferece Proceedings. Universitas Negeri Malang (UM).

Duran, N., Marcato, P. D., Alves, O. L., Souza, G. I. H. De, & Esposito, E. (2005). Mechanistic aspects of biosynthesis of silver nanoparticles by several *Fusarium oxysporum* strains. *Journal of Nanobiotechnology*, *3*, 8.

Elahmohammad, A. T. D., & Adeebauniesshareef, P. T. (2014). Biosynthesis of silver nanoparticles using culture supernatant of *Pseudomonas aeruginosa* and antibacterial activity. *International Journal of Enhanced Research in Science Technology & Engineering*, *3*(12), 174–180.

Esen, M., & Urek, R. O. (2014). Nitrate and iron nutrition effects on some nitrate assimilation enzymes and metabolites in *Spirulina platensis*. *Turkish Journal of Biology*, *38*, 690–700.

Estuardo, C., Marti, M. C., Huilinir, C., Lillo, E. A., & Von Bennewitz, M. R. (2008). Improvement of nitrate and nitrite reduction rates prediction. *Electronic Journal of Biotechnology*, *11*(3).

Feldstein, S., & Sloan, B. (2015). Actual and stereotyped speech tempos of extraverts and introverts. *Journal of Personality*, *52*(2), 188-204.

Firdhouse, M. J., & Lalitha, P. (2015). Biosynthesis of silver nanoparticles and its applications. *Journal of Nanotechnology*, *2015* (September 2014), 18.

Gandhi, H., & Khan, S. (2016). Nanomedicine & nanotechnology biological synthesis of silver nanoparticles and its antibacterial activity. *Journal of Nanomedicine & Nanotechnology*, *7*(2), 2–4.

Ganesh, K. V, Inbakandan, D., Radhika, R. S. R., Stanley, A. L., Manoharan, N., Govindaraju, K., & Singaravelu, G. (2008). Biological synthesis and applications of gold and silver nanoparticles - a review biological synthesis and applications of gold and silver nanoparticles. *International Journal on Applied Bioengineering*, *2*(January).

Gudikandula, K., & Maringanti, S. C. (2017). Synthesis of silver nanoparticles by chemical and biological methods and their antimicrobial properties. *Journal of Experimental Nanoscience*, *11*(9), 714–721.

Gupta, R., Mukherjee, K., & Singh, S. (2014). In silico characterization of nitrate reductase involved in gold nanoparticles synthesis in *Pseudomonas* denitrificans. *International Journal of Pharma and Bio Sciences*, *5*(3), 376–388.

Houston, F. S. A. M. (2007). X-Ray Film Processing - MilitaryNewbie.com [Online]. Retrieved from http://militarynewbie.com/wp-content/uploads/2013/10/US-Army-medical-course-X-ray-Film-Processing-MD0954.pdf

Ibrahim, H. M. M. (2015). ScienceDirect green synthesis and characterization of silver nanoparticles using banana peel extract and their antimicrobial activity against representative microorganisms. *Journal of Radiation Research and Applied Sciences*, *8*(3), 265–275.

Iravani, S., Korbekandi, H., Mirmohammadi, S. V, & Zolfaghari, B. (2014). Synthesis of silver nanoparticles : chemical, physical and biological methods. *Research in Pharmaceutical Sciences*, *9*(6), 385-406.

Jain, P., & Pradeep, T. (2005). Potential of silver nanoparticle-coated polyurethane foam as an antibacterial water filter. *Biotechnology and Bioengineering*, *90*(1), 59–63.

Jawaad, R. S., Sultan, K. F., & Al-Hamadani, A. H. (2014). Synthesis of silver nanoparticles. *ARPN Journal of Engineering and Applied Science*, *9*(4), 586–592.

Karki, G. (2017). Factors affecting microbial bacterial growth [Online]. Retrieved from http://www.onlinebiologynotes.com/factor-affecting-bacterial-growth/

Kaiser, G. (2017). Factors that influence bacterial growth [Online]. Retrieved from file:///C:/Users/acer/Desktop/ref July onwards/17.2_ Factors that Influence Bacterial Growth - Biology LibreTexts.html

Kamiloglu, A., Kaban, G., & Kaya, M. (2016). Contribution of catalase positive cocci on flavour formation in fermented sausages. *British Journal of Applied Science & Technology*, *17*(1), 1–8.

Karthik, C., & Radha, K. V. (2012). Biosynthesis and characterization of silver nanoparticles using *Enterobacter Aerogenes*: a kinetic approach. *Digest Journal of Nanomaterials and Biostructures*, *7*(3), 1007–1014.

Keerthirathne, T. P., Ross, K., Fallowfield, H., & Whiley, H. (2016). A review of temperature, ph , and other factors that influence the survival of *Salmonella* in mayonnaise and other raw egg products. *Pathogens*, *5*(63).

Keskin, N. O. S., Kilic, N. K., Donmez, G., & Tekiney, T. (2016). Green synthesis of silver nanoparticles using cyanobacteria and evaluation of their photocatalytic and antimicrobial activity green synthesis of silver nanoparticles using cyanobacteria and evaluation of their photocatalytic and antimicrobial activity. *Journal of Nano Research*, *40*(June), 120–127.

Khan, A. (2008). *Isolation, Purification and Partial Characterization of Respiratory Nitrate Reductase (NarGHJI) from M. tuberculosis H37Ra.* Ph.D. Thesis. University of Pune, India.

Krywult, M., & Bielec, D. (2013). Method of measurement of nitrate reductase activity in field conditions. *Journal of Ecological Engineering*, *14*(1), 7–11.

Kumar, K. V., & Jayabalakrishnan, R. M. (2011). Extracellular synthesis of silver nanoparticles using leaf extract of *Cassia Auriculata*. *Digest Journal of Nanomaterials and Biostructures*, *6*(1), 279–283.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265–275.

Mandal, D., Bolander, M. E., Mukhopadhyay, D., Sarkar, G., & Mukherjee, P. (2006). The use of microorganisms for the formation of metal nanoparticles and their application. *Appl Microbiol Biotechnol*, *69*(5), 485–492.

Masebinu, S. O., & Muzenda, E. (2014, 22 to 24 October). Review of silver recovery techniques from radiographic effluent and x-ray film waste. Proceedings of the World Congress on Engineering and Computer Science (Vol. II, pp. 22–24). San Francisco.

Mazid, M., Khan, T. A., & Mohammad, F. (2012). Role of nitrate reductase in nitrogen fixation under photosynthetic regulation. *World J of Pharmaceutical Research*, *1*(3), 386–414.

McFarland, A. D., & Duyne, R. P. V. (2003). Single silver nanoparticles as real-time optical sensors with zeptomole sensitivity. *Nano Letters*, *3*(8), 1057–1062.

Minnesota Pollution Control Agency. (2011). Managing photographic and x-ray waste (Vol. 4194) [Online]. Retrieved from https://www.pca.state.mn.us/sites/default/files/w-hw4-46.pdf

Mohammadian, A., Shojaosadati, S. A., & Rezaee, M. H. (2007). *Fusarium oxysporum* mediates photogeneration of silver nanoparticles. *Sci. Iran,14*, 323-326.

Morones, J. R., Elechiguerra, J. L., Camacho, A., Holt, K., Kouri, J. B., Ramírez, J. T., & Yacaman, M. J. (2005). The bactericidal effect of silver nanoparticles. *Nanotechnology*, *16*(10), 2346–2353.

Nair, B., & Pradeep, T. (2002). Coalescence of nanoclusters and formation of submicron crystallites assisted by *Lactobacillus* strains. *Crystal Growth and Design*, *2*(4), 293–298.

Narasimha, G., Praveen, B., Mallikarjuna, K., & Raju, B. D. P. (2011). Mushrooms (*Agaricus bisporus)* mediated biosynthesis of sliver nanoparticles , characterization and their antimicrobial activity. *International Journal of Nano Dimension*, *2*(1), 29–36.

Natarajan, K., Selvaraj, S., & Murty, V. R. (2010). Microbial production of silver nanoparticles. *Digest Journal of Nanomaterials and Biostructures*, *5*(1), 135–140.

Natsuki, J., Natsuki, T., & Hashimoto, Y. (2015). A Review of silver nanoparticles : synthesis methods, properties and applications. *International Journal of Materials Science and Applications*, *4*(5), 325–332.

Olson, J. S. C., & Markwell, J. (2007). Assays for determination of protein concentration. *Current Protocols in Protein Science*, *48*(1), 3.4.1-3.4.29

Panigrahi, T. (2013). *Synthesis And Characterization Of Silver Nanoparticles Using Leaf Extract Of Azadirachta Indica*. Master Thesis. National Institute of Technology Rourkela, India.

Parikh, R. Y., Ramanathan, R., Coloe, P. J., Bhargava, S. K., Patole, M. S., Shouche, Y. S., & Bansal, V. (2011). Genus-wide physicochemical evidence of extracellular crystalline silver nanoparticles biosynthesis by *Morganella spp*. *Plos One*, *6*(6), 1–7.

Particle Sciences. (2012). Glossary of drug nanotechnology (Vol. 9) [Online]. Retrieved from http://www.particlesciences.com/docs/technical_briefs/TB_2012_9-Nanotechnology.pdf

Perez-Garcia, O., Escalante, F. M., de-Bashan, L. E., Bashan, Y., (2011). Heterotrophic cultures of microalgae: metabolism and potential products. *Water Research 45*(1), 11-36.

Provan, F., Aksland, L., Meyer, C., & Lillo, C. (2000). Deletion of the Nitrate reductase n-terminal domain still allows binding of 14-3-3 proteins but affects their inhibitory properties. *American Society of Plant Biologists*, *123, 757-764.*

Puckett, L. J., Tesoriero, A. J., & Dubrovsky, N. M. (2011). Nitrogen contamination of surficial aquifers is a growing legacy. *Environmental Science & Technology*, *45*(3), 839–844.

Pugazhenthiran, N., Anandan, S., Kathiravan, G., Prakash, N. K. U., Crawford, S., & Ashokkumar, M. (2009). Microbial synthesis of silver nanoparticles by *Bacillus sp*. *Journal of Nanoparticle Research*, *11*(7), 1811–1815.

Redinbaugh, M. G., & Campbell, W. H. (1985). Quaternary structure and composition of squash NADH:nitrate reductase. *Journal of Biological Chemistry*, *260*(6), 3380–3385.

Reynolds Jackie. (2011). Nitrate test handbook [Online]. Retrieved from http://delrio.dcccd.edu/jreynolds/microbiology/2420/files/nitrate.pdf

Rogosa, M. (2017). Strains of the genus *Lactobacillus*. *J. Gen. Microbiol*, *24*, 401–408.

Saifuddin, N., Wong, C. W., & Yasumira, A. A. N. (2009). Rapid biosynthesis of silver nanoparticles using culture supernatant of bacteria with microwave irradiation. *E-Journal of Chemistry*, *6*(1), 61–70.

Sandu, M., Lupascu, T., Tarita, A., Goreacioc, T., Turcan, S., & Mosanu, E. (2014). Method for nitrate determination in water in the presence of nitrate. *Chemistry Journal of Moldova*, *9*(2), 8–13.

Sastry, M., Ahmad, A., Islam Khan, M., & Kumar, R. (2003). Biosynthesis of metal nanoparticles using fungi and actinomycete. *Current Science*, *85*(2), 162–170.

Scheurwater, I., Koren, M., Lambers, H., & Atkin, O. K. (2002). The contribution of roots and shoots to whole plant nitrate reduction in fast-and slow-growing grass species. *Journal of Experimental Botany*, *53*(374), 1653–1642.

Shah, M., Fawcett, D., Sharma, S., & Tripathy, S. K. (2015). Green synthesis of metallic nanoparticles via biological entities. *Materials (Basel)*, *8*(11), 7278-7308.

Shahrokh, S., Hosseinkhani, B., & Emtiazi, G. (2014). The impact of silver nanoparticles on bacterial aerobic nitrate reduction process. *J Bioproces Biotechniq*, *4*(3), 1-4.

Silver, S. (2003). Bacterial silver resistance: Molecular biology and uses and misuses of silver compounds. *FEMS Microbiology Reviews*, 27(2-3), 341-353.

Singh, R., Shedbalkar, U. U., Wadhwani, S. A., & Chopade, B. A. (2015). Bacteriagenic silver nanoparticles : synthesis, mechanism, and applications. *Appl Microbiol Biotechnol*, 99(11), 4579-4593.

Sithara, R., Selvakumar, P., Arun, C., Anandan, S., & Sivashanmugam, P. (2017). Economical synthesis of silver nanoparticles using leaf extract of *Acalypha hispida* and its application in the detection of Mn (II) ions. *Journal of Advanced Research*, *8*(6), 561–568.

Sohail, M., & Adeloju, S. B. (2016). Nitrate biosensors and biological methods for nitrate determination. *Talanta*, *153*(March), 83–98.

Sohaskey, C. D., & Modesti, L. (2009). Differences in nitrate reduction between *Mycobacterium tuberculosis* and *Mycobacterium bovis* are due to differential expression of both narGHJI and narK2. *FEMS Microbiol Lett*, *290*, 129–134.

Srikar, S. K., Giri, D. D., Pal, D. B., Mishra, P. K., & Upadhyay, S. N. (2016). Green synthesis of silver nanoparticles : a review. *Green and Sustainable Chemistry*, *6*(February), 34–56.

Syed, A., Saraswati, S., Kundu, G. C., & Ahmad, A. (2013). Spectrochimica acta part a : molecular and biomolecular spectroscopy biological synthesis of silver nanoparticles using the fungus *Humicola sp*. and evaluation of their cytoxicity using normal and cancer cell lines. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *114*, 144–147.

Talon, R., Walter, D., Chartier, S., Barriere, C., & Montel, M. C. (1999). Effect of nitrate and incubation conditions on the production of catalase and nitrate reductase by *Staphylococci*. *International Journal of Food Microbiology*, *52*(1–2), 47–56.

The Water Planet Company. (2009). Nitrification & denitrification [Online]. Retrieved from https://www.yumpu.com/en/document/view/11509528/nitrification-denitrification-the-water-planet-company

The World Count, (2017). Discover meaningful facts [Online]. Retrieved from http://www.theworldcounts.com/

Tiwari, M., Narayanan, K., Thakar, M. B., Jagani, H. V, & Rao, J. V. (2013). Biosynthesis and wound healing activity of copper nanoparticles. *IET Nanobiotechnology*, *10*(October 2013), 1–8.

UT Health San Antonio (2016). Minimal salts media [Online]. Retrieved from http://nmr.uthscsa.edu/html/education/m9.shtml

Vaidyanathan, R., Gopalram, S., Kalishwaralal, K., Deepak, V., Pandian, S. R. K., & Gurunathan, S. (2010). Enhanced silver nanoparticle synthesis by optimization of nitrate reductase activity. *Colloids and Surfaces B: Biointerfaces*, 75(1), 335–341.

Water Research Center, 2014. The pH of water [Online]. Retrieved from https://www.water-research.net/index.php/ph

White, R., & Heckenberg, D. (2011). Waste and what makes it hazardous? [Online]. Retrieved from http://www.academia.edu/2309023/White_R._and_Heckenberg_D._2011_What_is_Hazardous_Waste_and_What_Makes_It_Hazardous_-_Briefing_Paper_2_Criminology_Research_Unit_University_of_Tasmania_Hobart

Wong, D. (2016). Enzymes [Online]. Retrieved from http://studylib.net/doc/8306128/enzymes

World Health Organization. (2017). *Guidelines for Drinking-water Quality* (Fourth Edi). WHO Library Cataloguing-in-Publication.

Zhang, X. F., Liu, Z. G., Shen, W., & Gurunathan, S. (2016). Silver nanoparticles: synthesis, characterization, properties, applications, and therapeutic approaches. *International Journal of Molecular Sciences*, *17*(9), 1534.

Zhang, X., Yan, S., Tyagi, R. D., & Surampalli, R. Y. (2011). Synthesis of nanoparticles by microorganisms and their application in enhancing microbiological reaction rates. *Chemosphere*, *82*(4), 489–494.

APPENDIX A

Manual for Minimal Media (M9) Preparation

GIBCO
invitrogen cell culture

M9 Minimal Salts (2X)

Gibco® M9 Minimal Salts (2X) solution is used in the preparation of M9 minimal media. The composition of the M9 Minimal Salts include buffering agents, a nitrogen source and necessary ions critical to the completion of M9 minimal media. M9 minimal media also requires a carbon source to support microbial growth. As a complete media, M9 minimal media is typically used to define the nutritional needs of bacteria such as *Escherichia coli* (*E. coli*). Auxotrophs, organisms having unique nutritional requirements due to mutation(s), will not be able to grow in M9 minimal media without additional supplements such as amino acids or other required nutrients.

Description	Cat. No.	Size
M9 Minimal Salts (2X)	**A13744-01**	**1000 mL**

Intended Use

For research use only. CAUTION: Not intended for human or animal diagnostic or therapeutic uses.

Storage and Handling

15°C to 30°C

Shelf Life

12 months

Medium Preparation

M9 Minimal Salts (2X) solution (see formulation below) is not a complete medium and requires dilution and supplementation of a carbon source and other nutrients to propagate microbial growth.

Disodium Phosphate Heptahydrate.....25.6 g/L
Monopotassium Phosphate.....6 g/L
Sodium Chloride.....1 g/L
Ammonium Chloride.....2 g/L
Final pH (2X solution): 6.6-7.0 at 25°C

M9 Minimal Complete Medium Preparation: The following preparation is a guide for use. Additional supplementation may be required depending on the nutritional needs of the microbe.

1. Aseptically add 500 mL/L M9 Minimal Salts (2X) medium to a sterile container.
2. Aseptically add the following sterile solutions to the container:
   a. 20 mL of 20% D-Glucose solution
   b. 2 mL of 1.0 M $MgSO_4$ solution
   c. 0.1 mL of 1.0 M $CaCl_2$ solution
3. Adjust to volume to 1000mL with sterile H_2O and mix until homogeneous.

Note: Additional supplements may include: casamino acids, unnatural amino acids, heavy isotope labeled amino acids, trace metals, thiamine, antibiotics, etc.

Note: Different carbon sources and pH adjustment can also be used to complete M9 minimal media. If necessary, sterile filtration may be used to sterilize the final formulation.

Use

Procedure
Consult appropriate references for recommended test procedures. ***(See References Section)***

Expected Results

Growth should be evident by the appearance of turbidity.

User Quality Control

Prepare complete M9 minimal medium as described in the **Medium Preparation** section. Inoculate and incubate at 33 to 37°C for 18 to 48 hours.

Organism	Inoculum CFU	Recovery
E. coli (BL21 (DE3))	30-300	Good to excellent

Related Products

LB Broth, (10855)

Terrific Broth, (A13743)

Technical Support

For additional product and technical information, such as Material Safety Data Sheets (MSDS), Certificate of Analysis, etc, please visit our website at www.invitrogen.com. For further assistance, please email our Technical Support team at Techsupport@Invitrogen.com.

References

1. Davis, L.G., M. D. Dibner and J.F. Battey. 1986. Basic methods in molecular biology. Elsevier, New York, NY.
2. Davis, R.W., et. Al. 1980. Manual for genetic engineering: advanced bacterial genetics:204. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
3. Sambrook, J., E. F. Fritsch and T. Maniatis.. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

APPENDIX B

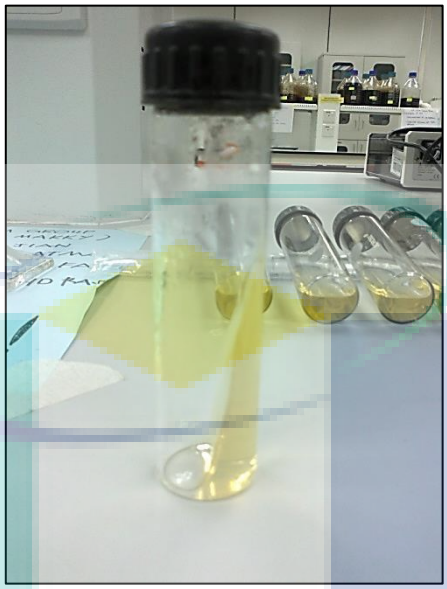

Figure 3.4 Agar slant preparation

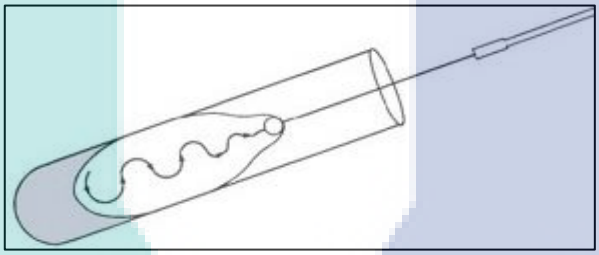

Figure 3.5 Bacterial isolates spread technique for agar slants

APPENDIX C

Figure 3.6 NB added in 100 ml conical flask for inoculumn preparation

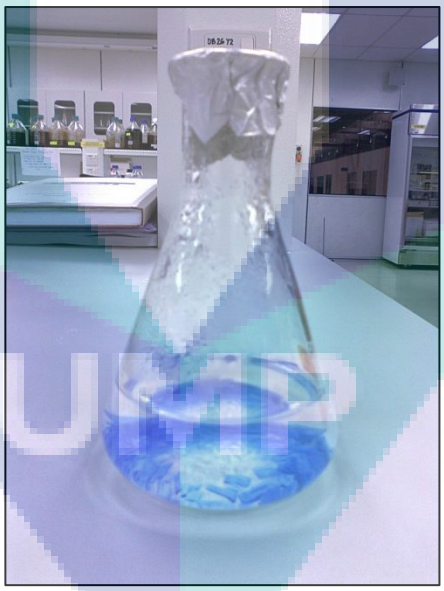

Figure 3.9 The flask of the production media containing 0.5 g pre-treated x-ray sheet pieces as substrate.

APPENDIX D

Preparation of 4M NaOH

About 160 g of NaOH powder was dissolved in one liter of distilled water. This mixture was used for the preparation of 4M NaOH solution according to Eq. (3.1).

Equation 3.1 The calculation for preparation 4M NaOH solution

$$g = \frac{N \times \text{equivalent weight} \times \text{volume}}{1000} \quad (3.1)$$

$$= \frac{4N \times 40.0\ g/mol \times 1000\ ml}{1000}$$

$$= 160\ g$$

Equation 3.2 The calculation for preparation $MgSO_4$ and $CaCl_2$ solution

$MgSO_4$ Solution:

$$g = \frac{M \times Mwt \times volume}{1000} \quad (3.2)$$

$$= \frac{1.0\ M \times 246.48 \times 100\ ml}{1000}$$

$$= 24.648\ g \text{ for } 1\ M/100\ ml\ MgSO_4$$

$CaCl_2$ Solution:

$$g = \frac{1.0\ M \times 110.98 \times 50\ ml}{1000}$$

$$= 5.5\ g \text{ for } 1\ M/50\ ml\ CaCl_2$$

APPENDIX E

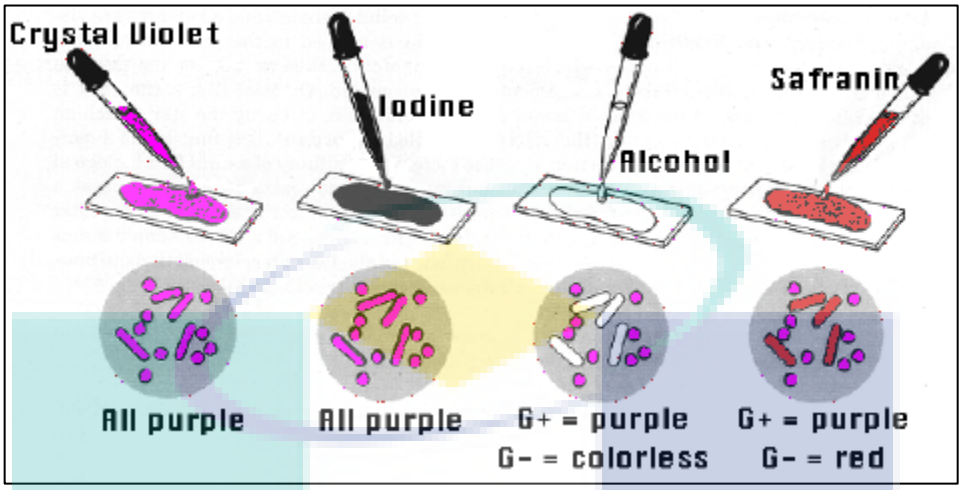

Figure 3.10 The procedure for Gram staining

Source: Dental Microbiology Lab (1995)

APPENDIX F

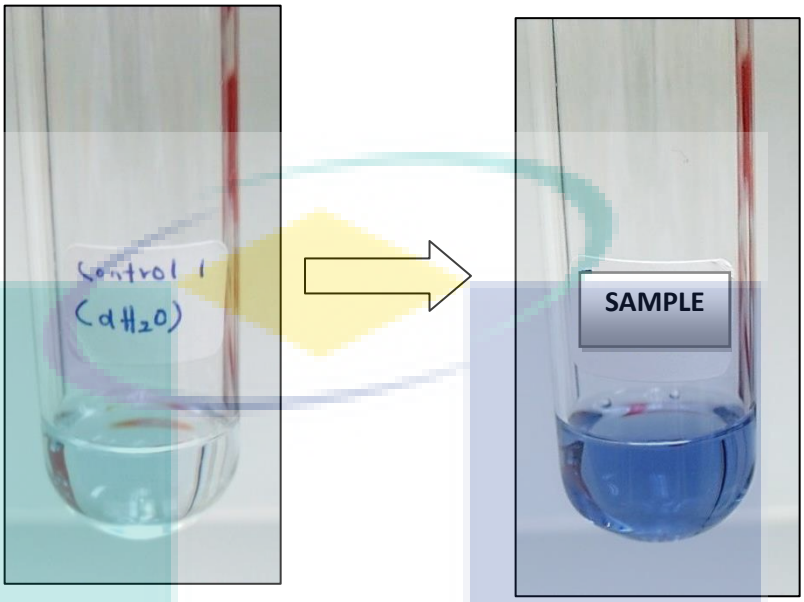

Figure 4.1 The color of protein reagent mixture was change from colorless to blue

Table 4.6 BSA Protein standard curve

Tube	BSA (ml)	d.H_2O (ml)	BSA concentration (mg/ml)	Protein Content (Abs at 700 nm) Mean ± SD
1	0.0	2.0	0	0.000
2	0.1	1.9	1	0.593 ± 0.069
3	0.2	1.8	2	0.845 ± 0.017
4	0.3	1.7	3	1.462 ± 0.110
5	0.4	1.6	4	1.697 ± 0.331
6	0.5	1.5	5	2.111 ± 0.494
7	0.6	1.4	6	2.406 ± 0.237
8	0.7	1.3	7	2.538 ± 0.152
9	0.8	1.2	8	2.996 ± 0.482
10	0.9	1.1	9	3.223 ± 0.482
11	1.0	1.0	10	3.277 ± 0.338

APPENDIX G

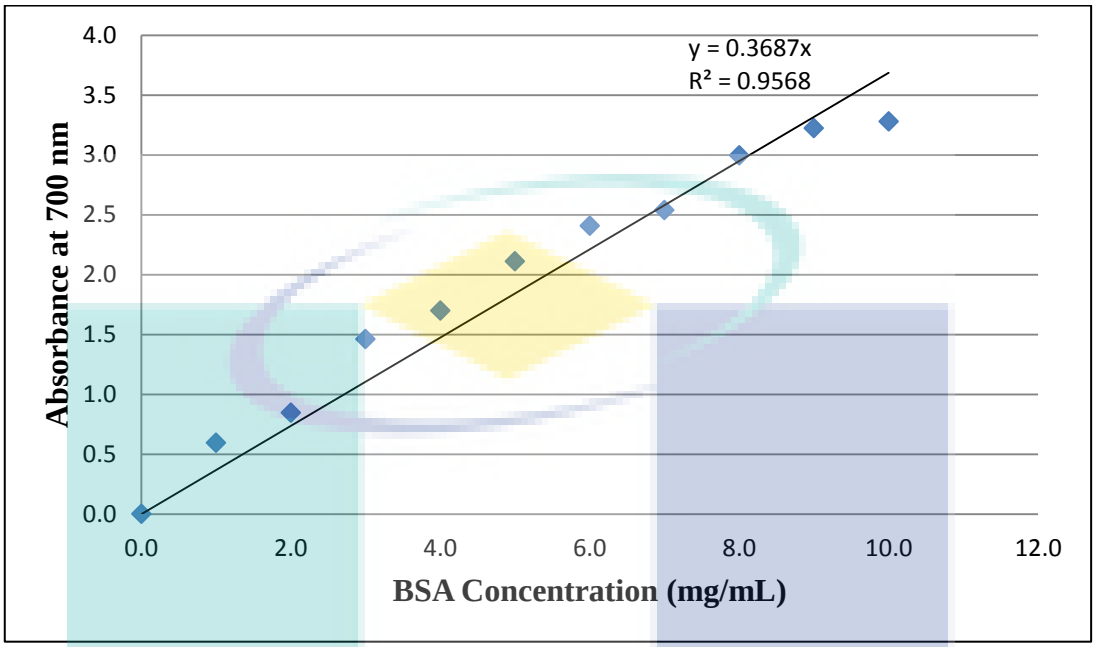

Figure 4.2 The BSA protein standard curve (mg/ml)

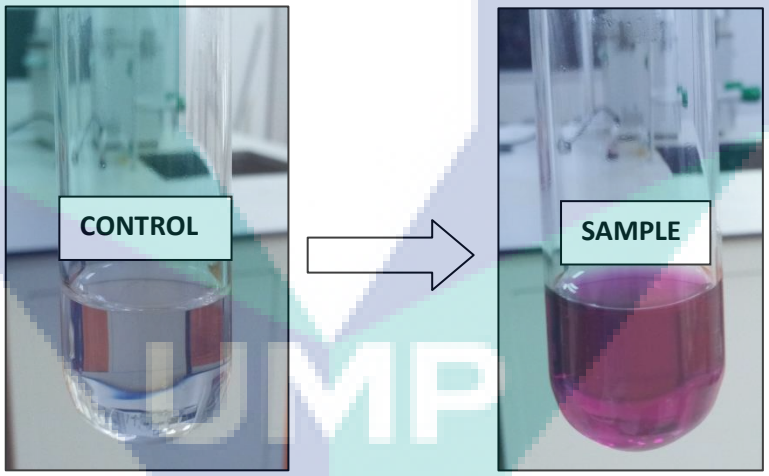

Figure 4.4 The color of enzyme mixture was change from colorless to pink

APPENDIX H

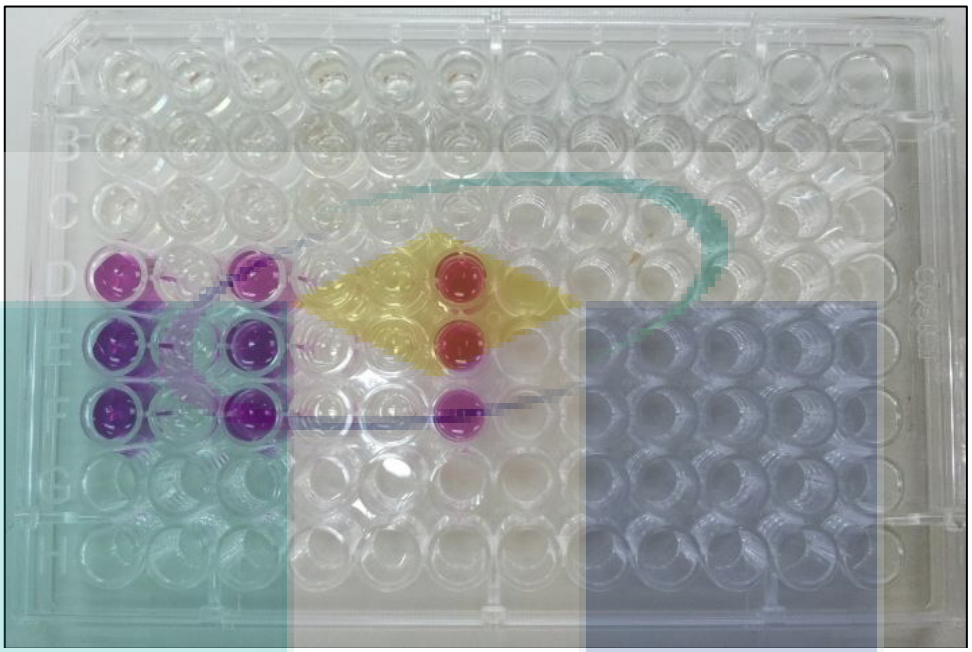

Figure 4.5 The samples were loaded in appropriate wells with pink color produced

APPENDIX I

Nitrite Standard Curve

After the parameters were determined in all selected samples, the nitrate standard curve was conducted. The data recorded in Table 4 ` ` ``

.12 shows the nitrite standard curve conducted with the final concentration of stock was 200 µg/ml.

Table 4.12 Nitrite standard curve

Tube	Nitrite (ml)	d.H_2O (ml)	Nitrite concentration (µg/ml)
1	0.0	10.0	0
2	0.4	9.6	20
3	0.8	9.2	40
4	1.2	8.8	60
5	1.6	8.4	80
6	2.0	8.0	100
7	2.4	7.6	120
8	2.8	7.2	140
9	3.2	6.8	160
10	3.6	6.4	180
11	4.0	6.0	200

A nitrite standard curve must be performed in order to quantitate the total of nitrate and nitrite concentration in the samples. They were prepared by dissolving 250 mg of $NaNO_3$ in 500ml of distilled water which produced 500µg/ml of stock solution. Then, 400 µl of the stock solution was diluted in 9.6 ml distilled water to produce the concentration of 20µg/ml.

After reading the absorbance of the standards at 540 nm by using the UV-vis spectrophotometer, the calibration curve was plotted based on values of absorbance vs. the nitrite concentration as shown in Table 4.13. The graph was plotted as shown in Figure 4.22. The purpose of this standard curve is to compare and measure the unknown concentration of nitrite in the samples with the known concentration. Both enzyme assay and enzyme standards used the same method which combines the unknown or

known samples with assay reagent as mentioned in Saifuddin et al. (2009) and the colorimetric techniques used to measure absorbance.

After the graph was plotted, the linear regression or straight line was obtained by assuming the overall relationship between concentration and absorbance. The equation for this line is:

$$y = mx + c \tag{3.4}$$

$$Absorbance = 0.022x + (y\ intercept)$$

$$X\ (\mu g/ml) = \frac{Absorbance}{0.022}$$

The enzyme concentration (µg/ml) of the previous parameter controlling part in the most potent isolate samples were calculated based on this equation. The absorbance in the equation was the absorbance reading obtained from the measurement of selected samples at 540 nm by using UV-Vis spectrophotometer. This enzyme standard curve was obtained after parameter controlling to make sure the standard curve was done in an expected range.

Table 4.13 Data for nitrite concentration (µg/ml) and absorbance at 540nm

Tube	Nitrite concentration (µg/ml)	Enzyme concentration (Abs at 540 nm) Mean ± SD
1	0	0.000
2	20	1.103 ± 0.069
3	40	1.384 ± 0.017
4	60	1.543 ± 0.110
5	80	1.942 ± 0.331
6	100	2.399 ± 0.494
7	120	2.610 ± 0.237
8	140	3.040 ± 0.152
9	160	3.340 ± 0.482
10	180	3.980 ± 0.482
11	200	4.430 ± 0.338

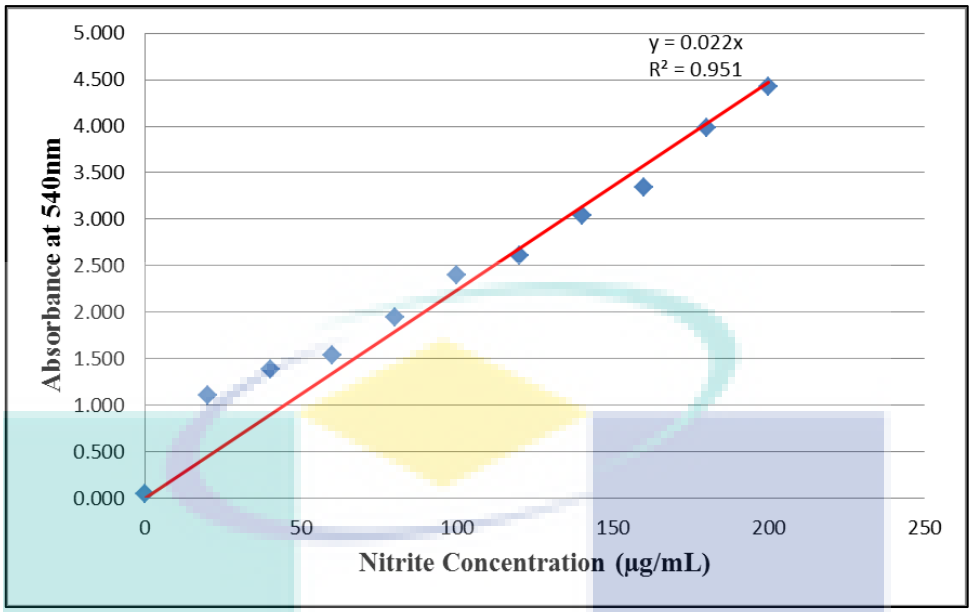

Figure 4.22 The nitrite standard curve (µg/ml)

APPENDICES J

XRD data analysis for CL4C

2-theta (deg)	d (ang.)	Height (cps)	Int. I(cps¥deg)	FWHM(deg)	Size	Phase name
16.84(6)	5.260(19)	24(5)	12.3(15)	0.43(6)	197(27)	Unknown,
22.323(13)	3.979(2)	122(11)	49.1(14)	0.301(14)	281(13)	Silver Nitrite, (0,1,1)
23.166(11)	3.8363(17)	170(13)	62.7(18)	0.285(11)	298(12)	Unknown,
23.747(9)	3.7438(14)	124(11)	30.1(17)	0.198(13)	428(28)	Unknown,
25.95(3)	3.430(3)	92(10)	33.3(13)	0.23(3)	371(43)	Unknown,
27.27(3)	3.267(4)	31(6)	7.6(11)	0.19(4)	451(86)	Unknown,
28.013(12)	3.1826(13)	169(13)	61.8(18)	0.282(12)	303(13)	Unknown,
29.45(6)	3.030(6)	36(6)	55(4)	1.27(11)	68(6)	Silver Nitrite, (1,1,0)
31.05(3)	2.877(3)	57(8)	17(2)	0.22(4)	387(62)	Unknown,
31.672(10)	2.8227(8)	417(20)	203(4)	0.386(8)	223(5)	Unknown,
32.774(17)	2.7303(13)	127(11)	71(3)	0.45(2)	191(10)	Unknown,
33.85(4)	2.646(3)	42(6)	27(5)	0.46(9)	189(37)	Silver Nitrite, (0,0,2)
40.11(2)	2.2460(13)	95(10)	38(2)	0.349(18)	253(13)	Unknown,
41.06(6)	2.197(3)	25(5)	9.2(13)	0.32(6)	277(51)	Unknown,
45.318(8)	1.9995(3)	222(15)	93(2)	0.269(11)	334(14)	Silver Nitrite, (1,1,2)
47.20(4)	1.9239(17)	27(5)	10.8(10)	0.36(4)	248(29)	Unknown,
52.17(4)	1.7519(11)	47(7)	15.9(11)	0.24(5)	380(75)	Silver Nitrite, (2,0,0)
53.36(5)	1.7154(16)	27(5)	13.2(12)	0.39(5)	237(28)	Unknown,
56.44(3)	1.6290(9)	60(8)	21.7(12)	0.33(3)	289(22)	Unknown,
57.94(7)	1.5902(17)	28(5)	35.5(18)	1.12(7)	85(5)	Silver Nitrite, (2,1,1)

Continued

2-theta (deg)	d (ang.)	Height (cps)	Int. I(cps¥deg)	FWHM(deg)	Size	Phase name
64.18(3)	1.4500(5)	28(5)	8.5(8)	0.23(4)	430(79)	Unknown,
66.14(6)	1.4117(11)	25(5)	19.5(14)	0.59(7)	167(20)	Unknown,
71.2(6)	1.323(10)	3.4(19)	16(3)	3.4(8)	30(7)	Silver Nitrite, (1,4,1)
75.20(2)	1.2624(3)	55(7)	14.0(8)	0.18(2)	582(65)	Unknown,

APPENDICES K

XRD data analysis for GL7

2-theta (deg)	d (ang.)	Height (cps)	Int. I(cps¥deg)	FWHM(deg)	Size	Phase name
22.474(18)	3.953(3)	129(11)	43.8(15)	0.259(16)	327(21)	Unknown,
23.239(13)	3.824(2)	180(13)	57(2)	0.231(14)	367(22)	Unknown,
23.821(18)	3.732(3)	86(9)	27.3(17)	0.23(2)	370(35)	Unknown,
25.89(17)	3.44(2)	17(4)	18(4)	0.94(16)	90(15)	Unknown,
28.13(3)	3.170(3)	96(10)	30(2)	0.27(2)	316(27)	Unknown,
31.183(17)	2.8659(15)	69(8)	9.9(16)	0.10(3)	851(234)	Silver Nitrite, (0,2,0)
31.765(14)	2.8147(12)	234(15)	54(3)	0.181(16)	476(41)	Unknown,
31.992(13)	2.7952(11)	343(19)	145(4)	0.300(15)	288(15)	Unknown,
32.930(17)	2.7177(14)	131(11)	80(3)	0.441(19)	196(9)	Silver Nitrite, (1,0,1)
36.81(6)	2.440(4)	27(5)	12.7(13)	0.35(7)	247(49)	Unknown,
39.03(12)	2.306(7)	21(5)	11.7(15)	0.35(11)	252(78)	Unknown,
40.20(3)	2.2412(16)	63(8)	25.3(17)	0.34(2)	262(19)	Unknown,
41.09(5)	2.195(3)	23(5)	10.0(10)	0.37(5)	242(36)	Unknown,
45.43(2)	1.9946(8)	219(15)	78(3)	0.27(2)	331(27)	Unknown,
52.26(5)	1.7489(15)	45(7)	18.7(13)	0.31(5)	301(53)	Unknown,
53.41(5)	1.7140(15)	37(6)	15.7(11)	0.35(5)	263(35)	Unknown,
56.50(2)	1.6275(6)	73(9)	23.7(13)	0.25(3)	372(39)	Unknown,
57.88(6)	1.5918(15)	33(6)	42.3(18)	1.00(6)	94(6)	Unknown,
64.22(3)	1.4492(6)	44(7)	11.7(9)	0.17(3)	578(108)	Unknown,
66.33(6)	1.4081(11)	24(5)	12.0(14)	0.43(6)	232(34)	Unknown,
75.273(17)	1.2614(2)	69(8)	15.3(10)	0.172(17)	609(61)	Unknown,

ACHIEVEMENTS

International Award

1. Gold prize in Seoul International Invention Fair 2014 (SIIF-2014) organized by the Korea Invention Promotion Association in Seoul, Korea (November 28th – December 1st 2014). Title: Nanoparticles Based Microbial Nitrate Reductase From Disposed X-Ray Film.

National Award

1. Gold medal in Bio-Malaysia 2013, Johor Bharu, Malaysia. Title: Biotechnology of Hazardous Waste Management for Nanoparticle Production. 21-23 October 2013.

Internal Award

1. Silver medal from Citrex 2013. 27 – 28 March 2013, University Malaysia Pahang, Malaysia.
2. Participated in Science Exhibition during ez-SciMat Carnival 2012. 2-4 November 2012. FIST, UMP, Malaysia.

Publications

1. Noor Afifah Fauzi, Siti Hajar Mohd Rasdi, Essam A. Makky & Mohd Hasbi Ab Rahim, (2016) Bioremediation of Disposed X-Ray Film for Enzymes Production, *Global Journal of Advance Research*, Volume 3, Issue 2, pp. 101-106.
2. Essam A. Makky, Siti H. Mohd Rasdi, J.B. Al-Dabbagh, G.F. Najmuldeen and Ab Rahim Mohd Hasbi, (2014) Bioremediation of hazardous waste for silver nanoparticles production, *International Journal of Current Microbiology and Applied Sciences*, Volume 3 Number 12, pp. 364-371.
3. Essam A. Makky, Siti H. Mohd Rasdi, J.B. Al-Dabbagh, G.F. Najmuldeen, (2014). Microbial Biotechnology for Silver Nanoparticles Production from Disposed X-Ray Film. Proceeding Book of International Conference on Innovative Trends in Multidisciplinary Academic Research (ITMAR – 2014). 20-21 October 2014, Istanbul, Turkey, pp. 60.