UNIVERSITI PUTRA MALAYSIA

EVALUATION OF LOCALLY ISOLATED AQUATIC BACTERIA AS NANOFACTORIES FOR GREEN SYNTHESIS OF IRON OXIDE AND SILVER NANOPARTICLES

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PATRICIA JAYSHREE SAMUEL JACOB

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

…being confident of this very thing, that He who has begun a good work in you will complete it until the day of Jesus Christ.

Philippians 1 : 6.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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The Malaysian tropical aquatic environment embodies a vast ecological system of immense biodiversity. Its largely untapped microbial population represent a rich bioresource that could be exploited for commercial applications replacing unsustainable conventional processes that require high investment and release toxic residues. Iron oxide nanoparticles (IONP) and silver nanoparticles (AgNP) have demonstrated tremendous potential in a variety of applications. Unfortunately, high investment costs, low yield and failure for effective scale-up using current protocols calls for alternative eco-friendly, cost-effective solutions. Green synthesis mechanisms using microorganisms as nanofactories offer a facile, sustainable and environment-friendly approach. Thus, the objectives underscored in this thesis was to isolate Malaysian aquatic bacteria with the ability for extracellular, aerobic generation of IONP and AgNP, to optimise synthesis parameters including temperature, precursor and biomass concentration using Response Surface Methodology (RSM) for maximum NP yield and potential throughput scale up and to explore the application potential of biosynthesized AgNP for antimicrobial activity and IONP as a nanoadsorbent for industrial dyes.

As a result of this study, 24 prokaryote strains were isolated from the 15 tropical shallow riverbed sediment samples while 34 prokaryote strains were isolated from among 15 mangrove marine sediment sample isolates which demonstrated extracellular generation of IONP and AgNP respectively. Molecular characterization using 16S rRNA sequencing showed phylogenetic similarities of MS2 and MA6 to *Spectromyces sp*. and *Bacillus sp* respectively. HrTEM images of biosynthesized IONP (size range: 12.5–18.21 nm) and AgNP (size range: 9.81 – 23.81 nm) depicted monodispersed, spherical nanostructures with clear crystalline lattice fringes. EDX peaks of IONP showed Fe to O ratios of 3 :4. Statistical optimization of temperature, biomass concentration and precursor concentration showed highest IONP yield using 55.58% MS2 culture supernatant (CS) with 2.46 mM $FeCl_3.6H_2O$ at 55.75 ^oC while highest AgNP yield was predicted by incubating 80% MA6 CS with 2.0 mM $AgNO_3$ at 60 ^oC. MS2 generated IONP demonstrated nanoadsorbent potential with 62.87%, 57.4% and 12.25% removal of 10 mg/L Crystal Violet (CV), Methylene Blue (MB) and Methyl Orange (MO) from aqueous solutions respectively. MA6 generated AgNPs demonstrated antimicrobial activity for *Eshcericha coli* (inhibiton zone: 1.2 $\pm$ 0.05 cm) and *Salmonella typhi* (inhibition zone: 1.23 $\pm$ 0.11 cm). It is therefore concluded that MS2 and MA6 are valuable microbial nanofactories which could be developed as a sustainable alternative to existing IONP and AgNP synthesis methodology.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENILAIAN BAKTERIA YANG DIPENCILKAN DARI PERSEKITARAN AKUATIK TEMPATAN SEBAGAI PENGILANG NANO DALAM SINTESIS HIJAU NANOPARTIKEL BESI OKSIDA DAN PERAK

Oleh

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Persekitaran akuatik tropika Malaysia merangkumi sistem ekologi yang unik dengan sumber biodiversiti yang luas. Populasi mikroorganisma akuatik merupakan sumber kaya alam yang masih belum diterokai luas untuk menggantikan proses industri terkini yang tidak mampan, memerlukan pelaburan tinggi dan meninggalkan residu toksik. Partikel nano logam seperti partikel nano besi oksida (IONP) dan partikel nano perak (AgNP) berpotensi luas dalam pelbagai aplikasi. Akan tetapi, protocol penghasilan IONP dan AgNP terkini memerlukan kos pelaburan tinggi, menjanakan hasil yang rendah dan gagal menjalankan peningkatan skala secara berkesan. Kaedah yang mesra alam dengan kos pembiayaan yang rendah amat diperlukan. Mekanisme sintesis hijau menggunakan mikroorganisma sebagai pengilang nano berpotensi mengatasi kelemahan protokol terkini. Objektif tesis ini adalah untuk memencilkan bakteria akuatik tempatan yang berupaya menghasilkan IONP dan AgNP melalui proses aerobik di luar sel dan mengoptimakan suhu, kepekatan prekursor dan masa penuaian kultur bakteria menggunakan Kaedah Permukaan Tindak balas (Response Surface Response (RSM)) untuk menjanakan penghasilan maksimum.

Dalam kajian ini, 2 strain prokariot aerobik tempatan telah dipencilkan dari 24 sampel sedimen sungai dan 34 sampel marin paya bakau Pantai Barat Semenanjung Malaysia yang berpotensi mensintesiskan IONP dan AgNP di luar sel. Dengan menggunakan kaedah urutan gen 16S rRNA, strain penghasil IONP, MS2, menunjukkan keserupaan filogeni dengan genera *Streptomyces* sementara MA6, strain penghasil AgNP, diidentifikasi sebagai Bacillus. Pencirian awal IONP menggunakan FESEM dan TEM menunjukkan partikel sfera, dalam lingkungan saiz 12.5 nm – 18.21 nm dengan nisbah Fe kepada O sebagai 3:4 menggunakan EDX. Keputusan RSM memberi hasil IONP maksima pada suhu 55^{o}C dengan kepekatan kultur supernatan 55.58% dan kepekatan precursor

2.0 mM $FeCl_3.6H_2O$ manakala hasil AgNP maksima diperolehi pada suhu 60°C dengan kepekatan kultur supernatan 80% dan kepekatan precursor 2.0 mM $AgNO_3$. IONP menunjukkan 62.87%, penyingkirkan pewarna pencemar alam Kristal Ungu (CV), 57.4% bagi Metilena Biru (MB) dan 12.25%. bagi Metilena Jingga (MO). Potensi antibakteria AgNP menggunakan kaedah resapan cakera menunjukkan diameter perencatan (1.2 ± 0.05) cm untuk *E. coli* dan (1.23 ± 0.11) cm untuk *S. typhi.* Kesimpulannya, MS2 dan MA6 merupakan pengilang nano IONP dan AgNP yang mantap dan layak diperkembangkan untuk dalam penghasilan nano di peringkat industri.

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LIST OF ABBREVIATIONS

AgNP	Silver nanoparticles
Amp	Ampicillin
ANOVA	Analysis of Variance
BBD	Box-Behnken Design
CCD	Central Composite Design
CCFD	Central Composite Face Design
CCRD	Central Composite Rotatable Design
CS	Culture Supernatant
CV	Crystal Violet
dH	Hydrodynamic diameter
DLS	Dynamic Light Scattering
dNTP	Deoxynucleotide triphosphate
EDS	Energy Dispersed Spectroscopy
EDX	Energy-dispersive X-ray spectroscopy
Ery	Erythromycin
FESEM	Field Emission Scanning Electron Microscopy
FTIR	Fourier-Transform InfraRed Spectroscopy
HrTEM	High-resolution Transmission Electron Microscopy
IONP	Iron oxide nanoparticles
ISO	International Organization for Standardization
MA	Marine Agar
MB	Methylene Blue
MNP	Metallic Nanoparticles

MO	Methyl Orange
MRI	Magnetic Resonance Imaging
NA	Nutrient Agar
NB	Nutrient Broth
NP	Nanoparticles
OD	Optical density
OM	Organic Matter
OVAT	One-Variable-At a-Time
PCR	Polymerase chain reaction
PDi	Polydispersity index
RSM	Response Surface Methodology
SAED	Selected Area (electron) Diffraction
TEM	Transmission Electron Microscopy
UV-Vis	Ultraviolet–visible spectrophotometry
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background

The inception of nanotechnology brought by the construction of versatile nanoparticles (NP) with exceptional properties has led to new, far-reaching frontiers in biomedicine, miniaturized electronics, and environmental remediation (Strambeanu *et al*., 2015). Metal nanoparticles (MNP) display distinct optical, thermo-electrical and magnetic properties caused by the quantum confinement of electronic energy levels at nanoscale dimensions making it valuable in industrial-based applications (El-Sayed, 2001). Among these, silver nanoparticles (AgNP) has long drawn interest owing to its excellent optical properties (Gharibshahi *et al*., 2016) thermo-electrical conductivity (Natsuki, 2015) and antimicrobial activity (Le Ouay & Stellacci, 2015) allowing it to find widespread application as biosensors (McFarland & VanDuyne, 2003), microelectronic fillers (Pothukuchi *et al*., 2004) and antimicrobial agents in textiles, food packaging, catheters and wound dressings (Rai *et al*., 2009). Recently, iron oxide nanoparticles (IONP) have attracted keen attention due to its unique magnetic and catalytic properties, allowing it to find pertinent applications in magnetic recording devices, ferrofluids, as magnetic resonance imaging (MRI) contrast enhancers (Motte, 2012), in magnetic hyperthermia, cell targeted drug delivery (Benyettou *et al*., 2011) and environmental remediation (Xu *et al*., 2012). In lieu of their wide range application potential, it is apparent that the demand for economical, eco-friendly bulk preparations of AgNP and IONP are greater now than ever.

At present, the synthesis of well-defined IONP and AgNP rely heavily on chemical and physical protocols that can be modified to modulate size, composition, morphology and physiochemical characteristics. Physical methods such as high energy ball milling, photolithography, etching, evaporation- condensation and laser ablation pose high investment costs for specialized equipment and high energy requirements (Dhand *et al*., 2015). Chemical methods such as co-precipitation, hydrothermal synthesis, microemulsion, polyol synthesis, sol-gel method and vapour deposition use potent solvents, reducing and capping agents that not only pose a threat to the environment (Kitching *et al*., 2015) but require painstaking adherence to convoluted procedures and competence in wielding complex equipment which are impractical for industrial scale production (Shah *et al*., 2015).

The immense investment, toxic environmental discharge and inability to generate copious amounts of AgNP and IONP using current methods pose a barrier to high-throughput production and sustainable commercialization endeavors, as end-users are hesitant to procure and fully utilize nanomaterials due to its high costing issues and uncertainty of supply. Although these approaches can be re-invented to abide by the principles of green chemistry, an apprehension still exists in *in vivo* applications due to residual contaminants that persist on the NP surface. This has led to the exploration of biological synthesis platforms such as plants and microorganisms as alternative routes to IONP and AgNP generation. Biological systems thrive under ambient conditions and perform well at low temperatures, thereby reducing high energy requirements and corresponding costs, making it more sustainable process compared to competing methods (Das *et al*., 2017). Additionally, the recovery of purified nanoparticles in downstream bioprocesses do not require painstaking measures to remove toxic organometallic residues on the NP surface, making it more compatible in biomedical applications such as cell targeted drug delivery, tissue imaging and hyperthermic chemotherapy (Shah *et al*., 2015).

Aquatic microorganisms have emerged as competent nanofactories, capable of large scale generation of well-defined metallic nanostructures (Baker *et al*., 2013). Their innate survival mechanisms such as bioaccumulation, biomineralization or biosorption (Duran *et al*., 2011) to cope with the harsh conditions in their habitat, enables them to reduce and detoxify heavy metal ions and catalyze the nucleation of metallic nanoparticles (Pantidos & Horsfall, 2014). Furthermore, these microbial bioprocesses can be easily fine-tuned through optimization of external process parameters such as temperature, precursor concentration and pH to generate economically viable, size specific, bio-compatible nanostructures in large volumes (Ghashghaei & Emtiazi, 2015). Compared to chemical systems, microbial fermentation does not use toxic solvents, stabilisers or capping agents and demonstrates surface bio-compatibility for *in vivo* biomedical applications (Ahmad *et al*., 2019).

1.2 Statement of Research Problem

Despite the tremendous reports detailing the scrupulous design and efficient functioning of IONPs and AgNPs in pertinent applications, its widespread use is limited due to recurring energy costs, high end equipment investment, meticulous synthesis protocols, scale up deficiencies and heightened environmental concern for toxicant discharge. Aspirations for sustainable industrial practices as outlined in the principles of Green Chemistry seek to design and implement cost efficient industrial processes without causing harm to the environment (Anastas & Warner, 1998). Green nanotechnology seeks to embody these principles in the design, development and application of advanced nanoscale materials (McKenzie & Hutchinson, 2004).

The production of IONP and AgNP using microbial bioprocesses succumbs to the need to develop reliable green protocols for the synthesis of MNP. Microorganisms such as yeast, bacteria, fungi, actinomycetes and cyanobacteria have demonstrate the potential to be developed as competent nanofactories capable of intracellular and extracellular generation of IONPs and AgNP (Hulkoti & Taranath, 2014). While intracellular biomineralization generates morphologically uniform nanocrystals, it requires laborious downstream cell disruption and separation procedures (Rahn Lee & Komeili, 2013). Extracellular biosynthesis are preferred as the production rate could be modulated for optimum yield, NP harvesting and purification is simplified and the scaling-up is commercially viable (Roh *et al*., 2006). Anaerobic extracellular processes depict low production rates, form undesirable metabolic byproducts and pose a surmountable challenge in maintaining anaerobic conditions in industrial settings (Moon *et al*., 2010). Aerobic bioprocesses are preferred as it can be fine-tuned to generate geometrically defined MNP (Kumari *et al*., 2016), perform well under existing environmental conditions (Patra & Baek, 2014) and depict rapid production rate and high yield (Fatemi *et al*., 2018).

Despite being surrounded by a rich coastal marine environment and abundant freshwater reservoirs, extensive microbial bioprospection in these environments in Malaysia are unapparent. Tropical aquatic ecosystems represent a major reservoir of global microbial biodiversity which play a critical role in nutrient and mineral cycling, energy flow and organic decomposition (Cotner & Biddanda, 2002). Ecological dynamics such as current flow, temperature fluctuation, humidity, light penetration and heavy metal ions influx, force microbial metabolic alterations and emerging pathways for improved tolerance (Costa Silva *et al*., 2017). It is inferred that microbial bioprospection in these environments would lead to the discovery of novel strains capable of rapid, extracellular aerobic generation of IONPs and AgNPs. Thus, it is imperative not only to tap these resources for the discovery of novel IONP and AgNP nanofactories which display these promising attributes but to optimise the microbial bioprocess using statistical tools for further development to commercial production.

Statitstical optimization of bioprocess parameters using software tools such as RSM uses mathematical models to optimise process parameters such as temperature, precursor and biomass concentration to project maximum IONP or AgNP yield. The data accumulated through these studies would be invaluable in pilot plant set-up and in the design of a commercial bioprocess. Compared to conventional statistical methods, RSM is advantageous as it uses experimental designs such as the Box-Behnken Design (BBD) and the Central Composite Design (CCD) to derive mathematical models that describe relationships between selected variables and explains the statistical significance of the influence of each independent and interactive variable on IONP or AgNP yield (Panda *et al*., 2007).

1.3 Justification

Conventional preparation routes for IONPs and AgNPs suffer from multiple drawbacks including heavy energy costs, meticulous synthesis protocols, and environmental toxicant discharge. An intense need exists to develop cleaner processes to generate IONP and AgNP that do not necessitate the use of toxicant solvents, highly specialized costly equipment and tedious protocols but can be carried out in existing environmental conditions. Green synthesis protocols using biological systems are suitable alternative routes which can be carried out in existing ambient conditions and minimal nutrient requirements using facile, clean technology. Although work has been reported in the biosynthesis of IONP or AgNP using local plants cells, algae and fungi, the exploration of Malaysian marine and freshwater tropical aquatic bacteria as IONP and AgNP nanofactories via rapid, extracellular aerobic routes are lacking. Isolation and characterization of local aquatic strains capable of IONP and AgNP generation and optimization of crucial parameters such as incubation temperature, precursor and biomass concentration that influence the microbial bioprocess would therefore lay the groundwork for the development of an industrial bioprocess using homegrown aquatic bacteria strains.

1.4 Hypotheses

1. locally-isolated aquatic bacteria have the potential for rapid, extracellular, aerobic generation of IONP and AgNP;
2. process parameters such as temperature, culture supernatant (CS) and precursor concentration influence microbial generation of IONP and AgNP and can be optimized for maximum yield;
3. microbially generated IONP have the potential to remove anionic and cationic industrial dyes from aqueous solutions;
4. microbially generated AgNP demonstrate antimicrobial activity against Gram negative and Gram positive pathogenic bacteria.

1.5 Objectives of study

1. to isolate and identify bacteria from the riverbed soil sediments of Sungai Mahang, Negeri Sembilan and mangrove marine soil sediments of Bagan Lalang, Selangor which demonstrate facile, aerobic, extracellular synthesis of IONP or AgNP;
2. to perform physico-characterization of microbial-generated IONP and AgNP as a preliminary assessment of successful MNP formation;
3. to optimise incubation temperature, culture supernatant (CS) harvesting time and precursor concentration towards the microbial bioprocess to generate IONP and AgNP using RSM;
4. to evaluate the potential of microbial-generated IONP to remove environmental pollutants such as CV, MB and MO from aqueous solutions;
5. to determine the antimicrobial potential of microbial-generated AgNP against microorganisms such as *E. coli*, *S. aureus* and *S. typhi*.

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BIODATA OF STUDENT

Patricia Jayshree Samuel Jacob was born in 1970 in Johor Bahru, Malaysia. She completed her secondary education at SM St Mary's Secondary School, Kuala Lumpur and continued to Penang Free School to complete her Pre-University or STPM qualifications.

From May 1990 to August 1994, Patricia attended Universiti Sains Malaysia in Penang where she graduated with a Bachelor's in Applied Sciences (Hons) majoring in Biotechnology with a Management minor. During the pursuit of this undergraduate degree, she worked on her senior year research project under the supervision of Dr Nazalan Najimudiin in a project entitled Cloning of the promoter of *B. polymyxa* into E.coli where she was introduced to basic molecular and cloning techniques and acquired much skill in the microbiology laboratory.

After undergraduate school, the author spent 2 years in a research position in the Dept of Medical Microbiology, Universiti Malaya. During the 1997 Southeast Asian large-scale air quality disaster, she realized her passion for the environment and was intrigued to pursue a postgrad degree in Environmental Management in Universiti Malaya which led her to her Masters qualification in August 2000. During the pursuit of this course, she undertook several courses such as WasteWater Management, Environmental Technology and Environmental Chemistry together with a dissertation based on Environmental Health. She commenced her academic career as a lecturer in Kolej UNITEK Malaysia in January 1998 and has since been involved in tertiary education in the Biotech programme specializing in Environmental modules. She is currently employed at Nilai University where she has served as a Senior Lecturer for more than a decade. In September 2014, she entered the Ph.D. program at Universiti Putra Malaysia undertaking a project under the specialization of Nanobiotechnology, desiring to explore green methods in nanotechnology.

Patricia is married to Edmond Das since January 1997 and resides in Seremban with 3 beautiful children who are the joy of her life.

LIST OF PUBLICATIONS

Jacob, P.J., Masarudin, M.J., Hussein, M.Z. and Rahim, R.A., (2019). Optimization of process parameters influencing the sustainable construction of iron oxide nanoparticles by a novel tropical wetlands *Streptomyces spp.* Journal of Cleaner Production 232 (2019) : 193 – 202.

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