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ชื่อโครงการ การศึกษาปริมาณความเข้มข้นของสารละลายซิลเวอร์ไนเตรตที่มีผลต่อการสังเคราะห์อนุภาคเงินนาโนของเชื้อรา *Aspergillus niger* และประสิทธิภาพในการยับยั้งเชื้อแบคทีเรีย

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ปีการศึกษา 2561

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

หัวข้อโครงงาน	การศึกษาปริมาณความเข้มข้นของสารละลายซิลเวอร์ไนเตรตที่มีผลต่อการสังเคราะห์อนุภาคนาโนเงินของเชื้อรา *Aspergillus niger* และประสิทธิภาพในการยับยั้งเชื้อแบคทีเรีย
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ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย อนุมัติให้นับโครงงานฉบับนี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาบัณฑิต ในรายวิชา 2312499 โครงงานวิทยาศาสตร์

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THE EFFECT OF SILVER NITRATE CONCENTRATION ON BIOLOGICAL SYNTHESIS OF SILVER NANOPARTICLES BY *Aspergillus niger* AND THE EVALUATION OF ITS ANTIBACTERIAL PROPERTIES

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Project Title: The Effect of Silver Nitrate Concentration on Biological Synthesis of Silver Nanoparticles by *Aspergillus niger* and the Evaluation of Its Antibacterial Properties

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Abstract

Recently, biosynthesis of nanoparticles has attracted scientist's attention due to its cost-effective production and less toxic wastes during the process of synthesis compared to chemical and physical methods. Moreover, many studies have reported that biosynthesized nanoparticles, especially silver nanoparticles (AgNPs), demonstrated a broad-spectrum antibacterial activity. Therefore, this synthesis method could be a good alternative way to develop environmental-friendly nanotechnology. In this study we investigated the biosynthesis of AgNPs using cell free extract (CFE) from *Aspergillus niger* and enhanced the product yields by the optimization of silver nitrate ($AgNO_3$) concentrations. These AgNPs were characterized through UV-visible spectrophotometry, transmission electron microscopy (TEM) and dynamic light scattering (DLS) measurement. The 5-80 nm of spherical AgNPs were visualized using TEM. A peak at 420 nm was observed by UV-visible spectrophotometry, and the result showed that the maximum synthesis of AgNPs was obtained at 2 mM of $AgNO_3$ concentration under the condition parameters of 30°C incubation temperature at pH of 7 and the use of 25 grams of fungal biomass. The antibacterial activities of the AgNPs were investigated against Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria *(Escherichia coli*) by determination of inhibition zones using agar-well diffusion method. The various doses of AgNPs (1000, 500 and 250 μg/mL) exhibited antibacterial activity; the inhibition zones of *S. aureus* were in the range of 9.3-12.7 mm, whereas *E. coli* showed unclear inhibition zones. To sum up, our study demonstrated a successful biosynthesis method of AgNPs using *A. niger* and the application of this nanomaterial as an antibacterial agent.

ชื่อโครงการ: การศึกษาปริมาณความเข้มข้นของสารละลายซิลเวอร์ไนเตรตที่มีผลต่อการสังเคราะห์อนุภาคเงินนาโนของเชื้อรา *Aspergillus niger* และประสิทธิภาพในการยับยั้งเชื้อแบคทีเรีย

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อ.ที่ปรึกษาโครงการ: อาจารย์ ดร.สริสา ณ ป้อมเพ็ชร์

ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปีการศึกษา 2561

ในปัจจุบันการสังเคราะห์อนุภาคนาโนด้วยวิธีการทางชีวภาพเป็นที่สนใจอย่างมากในวงการวิทยาศาสตร์เนื่องจากการผลิตอนุภาคนาโนด้วยวิธีดังกล่าวมีต้นทุนไม่สูง และไม่ก่อให้เกิดของเสียที่เป็นพิษในกระบวนการผลิตเมื่อเปรียบเทียบกับวิธีการผลิตทางเคมีและกายภาพ นอกจากนี้ยังมีรายงานการศึกษาที่พบว่าอนุภาคนาโนที่สังเคราะห์ได้จากวิธีการทางชีวภาพ โดยเฉพาะอนุภาคเงินนาโนมีฤทธิ์ในการยับยั้งหรือฆ่าเชื้อจุลินทรีย์ครอบคลุมทั้งเชื้อแกรมบวกและเชื้อแกรมลบอีกด้วย ดังนั้นวิธีการสังเคราะห์ทางชีวภาพนี้อาจเป็นทางเลือกที่ดีในการพัฒนานาโนเทคโนโลยีที่เป็นมิตรกับสิ่งแวดล้อม ในการศึกษานี้ได้ทำการสังเคราะห์อนุภาคเงินนาโนโดยใช้สารสกัดจาก *Aspergillus niger* และตรวจสอบโดยทำการวัดค่าการดูดกลืนแสงด้วยเครื่อง UV-visible spectrophotometer จากนั้นทำการส่องดูลักษณะและขนาดของอนุภาคภายใต้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน (TEM) และทำการวิเคราะห์ขนาดและการกระจายตัวของอนุภาคด้วยวิธี dynamic light scattering (DLS) โดยใช้เครื่อง Zetasizer Nano range นอกจากนี้ยังศึกษาผลของสารละลายซิลเวอร์ไนเตรตความเข้มข้นต่างๆ ในการผลิตอนุภาคเงินนาโนอีกด้วย ผลจากการพิสูจน์อัตลักษณ์ของอนุภาคเงินนาโนพบว่ามีลักษณะเป็นทรงกลมขนาด 5 ถึง 80 นาโนเมตร และพบว่ามีการแสดงผลของค่าการดูดกลืนแสงที่ความยาวคลื่น 420 นาโนเมตร ผลจากการศึกษาการสังเคราะห์อนุภาคเงินนาโนด้วยวิธีทางชีวภาพพบว่าสภาวะที่เหมาะสมคือใช้น้ำหนักเปียกของรา 25 กรัม ค่าความเป็นกรด-ด่างเท่ากับ 7 ความเข้มข้นของสารละลายซิลเวอร์ไนเตรตเท่ากับ 2 มิลลิโมลาร์ และบ่มที่อุณหภูมิ 30 องศาเซลเซียส ในการตรวจสอบฤทธิ์การต้านเชื้อแบคทีเรียแกรมบวก (*Staphylococcus aureus*) และแบคทีเรียแกรมลบ (*Escherichia coli*) ด้วยวิธี agar-well diffusion โดยใช้อนุภาคเงินนาโนที่กระจายตัวอยู่ในน้ำที่ความเข้มข้น 1000, 500 และ 250 ไมโครกรัมต่อมิลลิลิตรมาทำการทดสอบ พบว่าสารดังกล่าวให้ค่าโซนการยับยั้ง *S. aureus* ในช่วง 9.3 ถึง 12.7 มิลลิเมตร ในขณะที่ใน *E. coli* พบโซนของการยับยั้งไม่ชัดเจน จากผลการศึกษาที่กล่าวมาผู้วิจัยได้รายงานถึงความสามารถในการใช้เชื้อรา *A. niger* เพื่อสังเคราะห์อนุภาคเงินนาโน และความสามารถในการยับยั้งเชื้อแบคทีเรียของอนุภาคเงินนาโนที่สังเคราะห์ได้จากวิธีทางชีวภาพซึ่งจะเป็นประโยชน์ต่อการพัฒนาวัสดุนาโนต่อไป

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Thanakorn Takkrathok

April 2019

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List of Abbreviations

AgNPs	Silver nanoparticles
$AgNO_3$	Silver nitrate
CFU	Colony-forming unit
CDB	Czapex Dox broth
DLS	Dynamic Light Scattering
LB	Luria-Bertani
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
NPs	Nanoparticles
PDA	Potato dextrose agar
PDB	Potato dextrose broth
ROS	Reactive oxygen species
TEM	Transmission electron microscopy

Chapter 1

Introduction

1.1 Background and significance of the study

According to World Health Organization (WHO), antibiotic resistance is one of the biggest problems to global health. In recent decades, numerous bacteria have shown less sensitivity to antibiotic treatment. The recovery of multidrug-resistant bacteria is a public healthcare problem and requires multiple broad-spectrum antibiotic treatments, which is related to toxicity and high cost. People who are infected with antibiotic-resistant bacteria usually spend more time in hospitals and require several different antibiotics to treat these infections. In 2009, the cases of antibiotic resistance in Thailand were estimated to cause 38,481 deaths with an economic loss of 2,539 to 6,084 million baht. (Pumart et al. 2012) Regarding to aforementioned impacts, the antibiotic resistance problem has been aware as one of the most healthcare problems in Thailand.

Table 1.1 Nosocomial and antibiotic-resistant bacteria infections in Thailand in 2009. (Pumart et al. 2012)

Type of hospital	**Nosocomial infections (times)**	**% of infection in the hospital**	**Drug-resistant infections**
University hospital	24,480	7.60	7,997
Central hospital	155,725	5.34	50,870
Community hospital	64,143	2.07	20,953
Private hospital	24,280	4.90	7,932
Total	268,628	3.93	87,751

Table 1.2 Antibiotic-resistant bacteria infections, classified by types and infection sites. (Pumart et al. 2012)

Site of infection	**Drug-resistant bacteria infections (Times)**				
	A. baumannii	***S. aureus***	***K. pneumoniae***	***E. coli***	***P. aeruginosa***
Lower respiratory tract	29,672	13,683	8,930	1,728	4,897
Urinary tract	1,152	288	1,728	2,449	169
Surgical Wound	432	864	115	346	94
Bloodstream	864	2,017	288	144	94
Etc.	4,433	1,872	4,177	6,450	864
Total	36,553	18,725	15,239	11,116	6,118

Table 1.3 Death of patients with antibiotic-resistant infections in Thailand in 2009. (Pumart et al. 2012)

Type of bacteria	**Number of deaths from infections**	**Number of deaths from drug-resistant infections**	**% of deaths from drug-resistant infections**
A. baumannii	22,567	19,071	84.51
S. aureus	9,698	9,079	93.62
K. pneumoniae	7,855	5,080	64.67
P. aeruginosa	10,791	3,496	32.40
E. coli	3,104	1,755	56.54
Total	54,014	38,481	71.24

Nanoparticles (NPs) are clusters of atoms in the size range from 1 to 100 nanometers leading to their unique properties that are different from bulk materials. Bulk materials generally display constant physical properties regardless of their size whereas properties of nanoparticles depend on their sizes and percentages of their surfaces.

In the past such as in Rome era as well as in Mesopotamia, nanoparticles were accidentally used by artisans to create a glittering surface of pots. For example, the artisans were not aware that they were using gold nanoparticles to produce Lycurgus cup. The size and shape of gold nanoparticles residing in the Lycurgus cup are responsible for the change of color from green to red-purple when the cup is illuminated inside (Figure 1.1). This is a result of an optical property of gold nanoparticles when they are reflected, scattered, or absorbed the light.

Figure 1.1 The Lycurgus cup appears green when illuminated from the outside and purple-red when illuminated from the inside.

Source: Marcio Loos, in Carbon Nanotube Reinforced Composites, 2015

Technology and engineering of metal nanoparticles are useful for a variety of applications such as catalysts, electronics, optics, biosensors, magnetics, as well as medical tools (Figure 1.2). Silver nanoparticles are considered as one of the most widely used nanomaterials. Silver nanoparticles are also one of nanomaterials which have been increasingly used for antimicrobial activity, and they have less toxicity on mammalian cells. (Mekkawy et al. 2017)

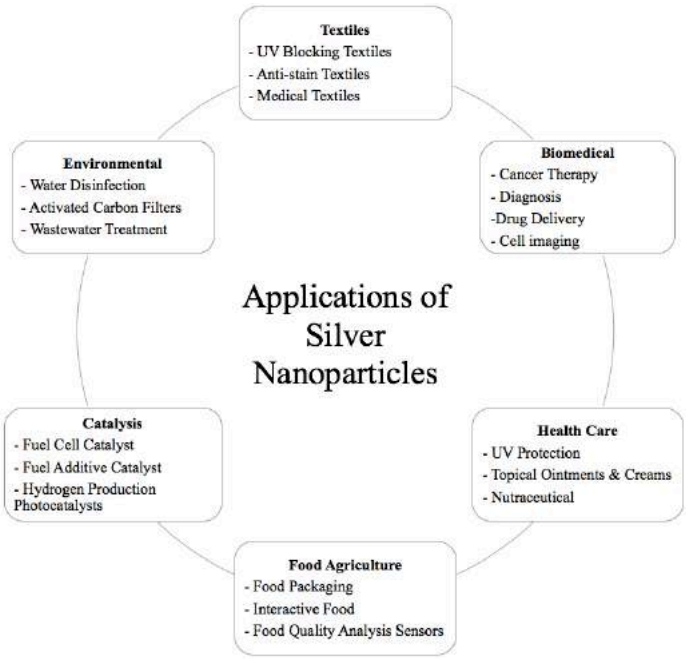


Figure 1.2 Application of silver nanoparticles. (Keat et al. 2015)

Historically, silver was known in 4,000 B.C.E. as the third metal used by ancients after gold and copper (Alexander 2009). Silver was one of the most important antimicrobial agents that was available before the introduction of antibiotics (Barras, Aussel, and Ezraty 2018). Among the many tests of metallic nanoparticles, the silver nanoparticle (AgNP) was found to be the most effective agent against Gram-positive and Gram-negative bacteria including antibiotic-resistant bacteria, so it can be used as an effective broad-spectrum antibacterial treatment (Wang, Hu, and Shao 2017). The four main cytotoxic mechanisms of AgNPs against microorganisms are 1) adhesion of AgNPs to the bacterial cell membrane resulting in a damage of the membrane and alteration of the transportation across the membrane, 2) penetration of AgNPs inside the bacterial cell leading to the interaction of AgNPs with numerous cellular organelles and biomolecules that causes the interruption of cellular function activities, 3) generation of reactive oxygen species (ROS) inside the cell leading to a cell damage, and 4) induction of genotoxicity (Lee and Jun 2019). These mechanisms are shown in Figure 1.3.

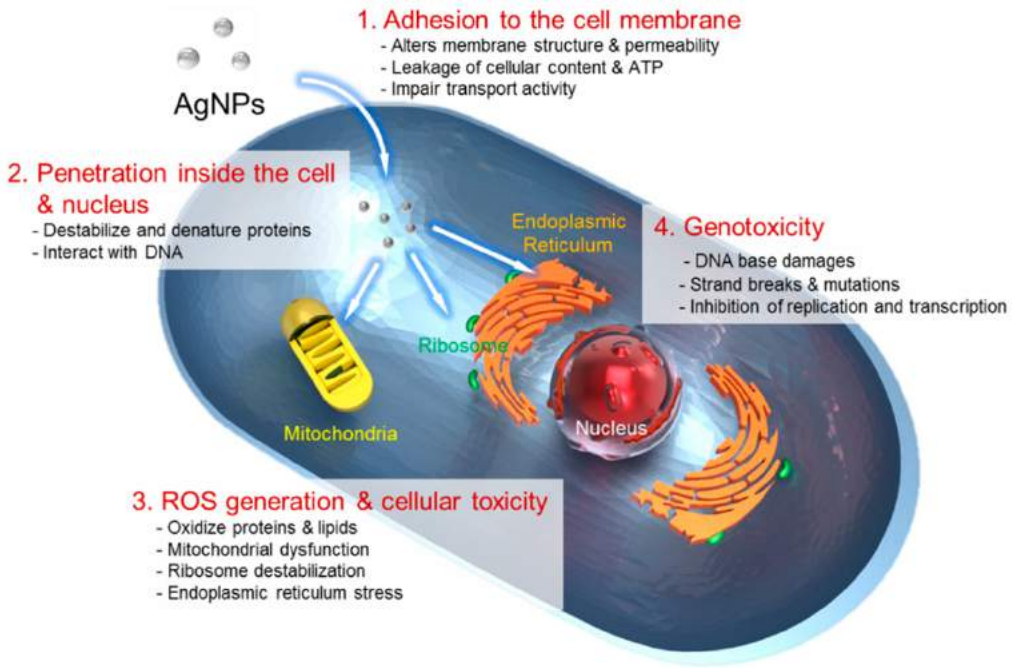


Figure 1.3 Mode of action of silver nanoparticles. (Lee and Jun 2019)

The synthesis methods of silver nanoparticles are mainly divided into top-down and bottom-up approaches as shown in Figure 1.4. Top-down approaches are methods that minimize bulk materials to generate nanostructures, while bottom-up approaches involve an assembly of atoms or molecules to create larger nanostructures. Alternatively, the synthetic approaches can

also be categorized into physical, chemical, and biological synthesis. The physical and chemical synthesis tend to be more expensive and hazardous compared to the biological synthesis of AgNPs (Lee and Jun 2019). The biological process is cost-effective that gives a high yield, safe and environmentally acceptable procedure involving in using bacteria, fungi and even plants (Iravani et al. 2014). Fungi have been known to secrete higher amounts of extracellular bioactive substances than bacteria, which make fungi more suitable for large-scale production of nanoparticles. (Li et al. 2012)

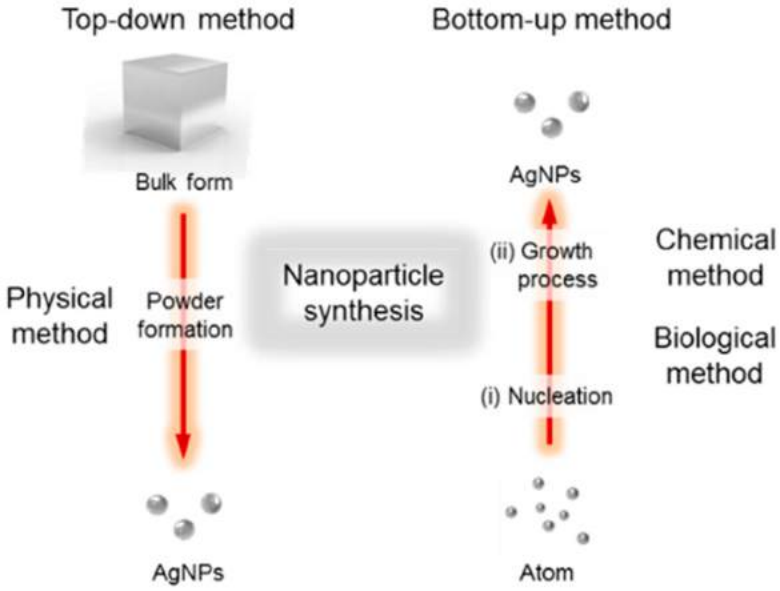


Figure 1.4 General types of synthesis of metal nanoparticles. (Lee and Jun 2019)

Aspergillus niger (Figure 1.5) is a fungus that can be a good candidate in the synthesis of AgNPs. This fungus is known to secrete large amounts of biomolecules into cell culture medium. Some of those secreted molecules can be reducing agents that reduce silver ions into silver nanoparticles during the extracellular process of metal nanoparticle synthesis (Figure 1.6). Briefly, the AgNPs could be synthesized by the reduction of silver ions in aqueous silver nitrate solution ($AgNO_3$) after the incubation with cell free fungal medium for 3-5 days. The characterization of biosynthesized AgNPs are normally performed by UV-visible spectrophotometry, transmission electron microscopy (TEM), and zetasizer nano range etc. (Zomorodian et al. 2016).

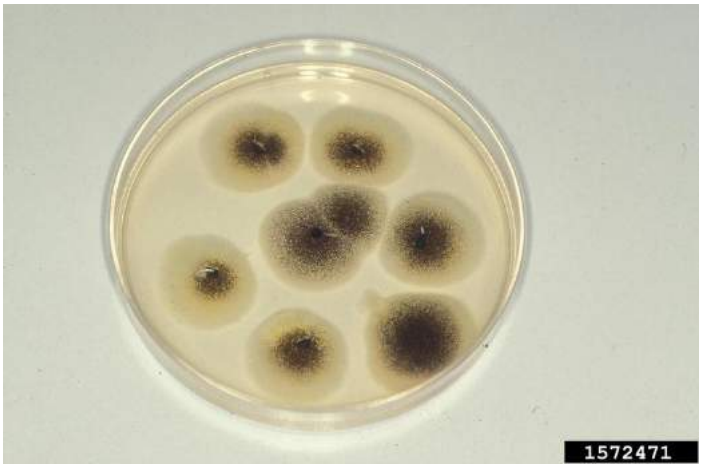


Figure 1.5 *Aspergillus niger* on Potato Dextrose Agar (PDA) medium.
Source: Gerald Holmes, California Polytechnic State University at San Luis Obispo, Bugwood.org

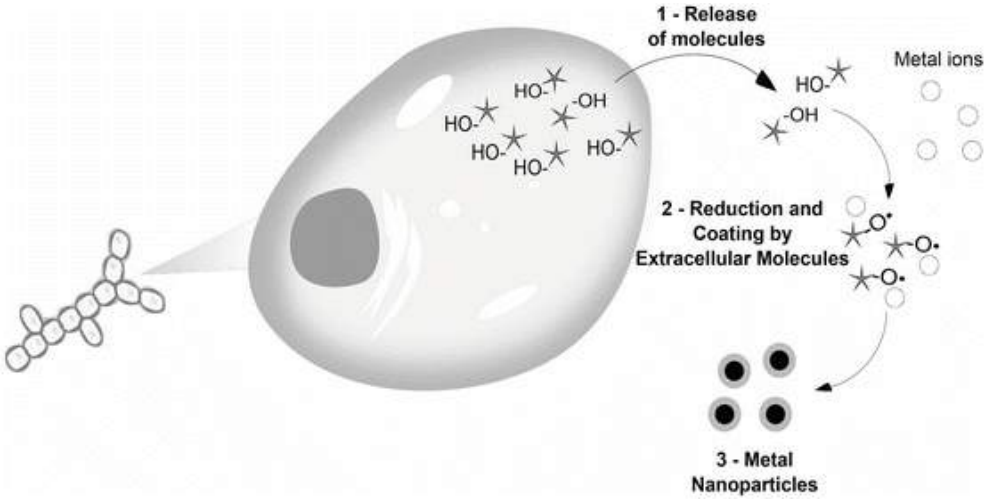


Figure 1.6 The extracellular green synthesis of metal nanoparticles by fungi. (Silva, Bonatto, and Polez 2016)

In recent study, the optimization of parameters including media, fungal biomass, pH, temperature and $AgNO_3$ concentration could accelerate the production of AgNPs. Under different cultural conditions, fungi might secret different kind of metabolites and proteins, which could affect the reduction of aqueous silver ions in biosynthesis of AgNPs. (Saxena et al. 2016)

In agreement with previous reports, the optimization of $AgNO_3$ concentration could increase the AgNPs production. Herein, we investigated the biosynthesis of AgNPs at different concentrations of $AgNO_3$ by using *A. niger* as a source of reducing agents. Then at the different $AgNO_3$ concentrations, the formations of AgNPs were observed by UV-visible spectrophotometry. The biosynthesized AgNPs were also measured hydrodynamic diameters and size distributions by Dynamic Light Scattering (DLS) technique and characterized for shapes and sizes by transmission electron microscopy (TEM). The antibacterial efficacy of AgNPs was evaluated against Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria *(Escherichia coli*) by agar-well diffusion method. The result suggested that the biosynthesized AgNP could be a potential antibacterial agent.

1.2 Objectives

1. To develop an environmental-friendly technology for the synthesis of silver nanoparticles using *Aspergillus niger*.
2. To investigate the optimum of silver nitrate concentration in biosynthesis of silver nanoparticles using *Aspergillus niger*.
3. To evaluate the antibacterial efficacy of the biosynthesized silver nanoparticles against Gram-positive and Gram-negative bacteria.

1.3 Practical application

1. Decreasing of the use of chemical-reducing agents and capping agents for the AgNPs production.
2. Developing of cost-effective technologies for AgNPs synthesis.
3. Increasing of production yields of biosynthesized AgNPs.
4. Producing of safe AgNPs which can be used in antimicrobial applications against bacteria.

Chapter 2
Materials

2.1 Equipment

1) Filtering Funnel
2) Cylinder 100 mL
3) Cylinder 500 mL
4) Spreader
5) Erlenmeyer Flask 250 mL from ISOLAB Laborgeräte GmbH, Germany
6) Laboratory Bottle 500 mL from DWK Life Sciences, Germany
7) Refrigerated Incubator Shaker Innova 4330 from New Brunswick scientific, USA
8) Digital Weighting Scale PG 2002-s from Mettler Toledo
9) Digital Kitchen Scale from Camry
10) ES-215 from TOMY Seiko, Japan
11) Autoclave ES-315 from TOMY Seiko, Japan
12) Micro Refrigerated Centrifuge KUBOTA 3700 from KUBOTA, Japan
13) Heat Plate MS-H280-Pro from Scilogex, USA
14) Hemocytometer from LW Scientific, USA
15) Light microscopy CH30RF200 from Olympus, Japan
16) Spectrophotometer Genesys 20 from Thermo Spectronic, USA
17) Spectrophotometer Nanodrop 2000c from Thermo Scientific, USA
18) Biological Safety Cabinet from LABMICROTECH, THAILAN
19) Ultra-Low Temperature Freezer MDF-U71V from Sanyo Electric, Japan
20) Hot Air Oven Contherm Digital Series
21) Incubator 37°C from Memmert, Germany
22) Micropipette 10 µL from Capp Bravo, Germany
23) Micropipette 100 µL from Capp Bravo, Germany
24) Micropipette 1000 µL from Capp Bravo, Germany
25) Hand Tally Counter 4 digit Manual
26) Pipette Controller Levo Plus from Dlab

27) Vortex mixer K-550 GE from Scientific Industries, USA

28) Elmasonic E Ultrasonic Cleaning, E30H from Germany

29) Transmission Electron Microscopy JEM-2100 from Jeol, Japan (Scientific and Technological Research Equipment Center, Chulalongkorn University)

30) Zetarsizer nano ZS from Malvern Instruments, UK (Scientific and Technological Research Equipment Center, Chulalongkorn University)

2.2 Materials

1) Plastic Petri Plates from Bioscan
2) Pipette Tips from Promega and Kirgen
3) Conical Tubes 50 mL from Thermo Scientific
4) Eppendorf Tubes 1.5 mL from Kirgen
5) Color-Fixed Indicator Strips from Machrey-nagel
6) Filter paper Grade 1 diameter 125 mm Whatman from GE Healthcare
7) Gauze
8) Plastic Pipette 5 mL from SPL LifeScience
9) Plastic Pipette 10 mL from SPL LifeScience
10) Plastic Pipette 25 mL from SPL LifeScience
11) Pasteur Pipette Outer Diameter 6 mm from Volac
12) Filter 0.2 μm from GE Healthcare
13) Syringe from Nipro, Thailand

2.3 Agents

1) Glycerol 85% from Himedia
2) Potato Dextrose Broth from Himedia
3) Czapex dox broth
4) Nutrient Broth from Himedia
5) Muller-Hinton Broth from Himedia
6) Agar from Himedia
7) Tween 20 from Life science
8) Silver Nitrate ($AgNO_3$) from POCH, Poland

9) Streptomycin sulphate from Himedia

10) Purified water type I

11) Purified water type II

2.4 Bacterial and fungal strains

1) *Escherichia coli* MSCU 0349 from MSCU microbial culture collection, Department of Microbiology, Faculty of Science, Chulalongkorn University

2) *Staphylococcus aureus* MSCU 0353 from MSCU microbial culture collection, Department of Microbiology, Faculty of Science, Chulalongkorn University

3) *Aspergillus niger* MSCU 0361 from MSCU microbial culture collection, Department of Microbiology, Faculty of Science, Chulalongkorn University

Chapter 3

Method

3.1 Biological synthesis of AgNPs

3.1.1 Spore collection

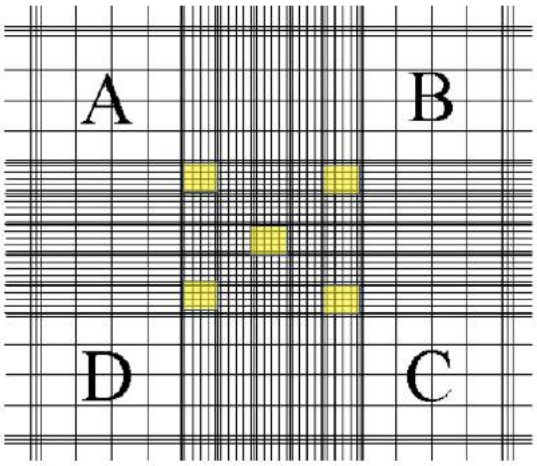
Aspergillus niger was inoculated by wire loop method on potato dextrose agar (PDA) and incubated at room temperature for 72 hours, then 2 mL of 0.2% Tween-20 was added onto the PDA plate containing of the sporulating fungal colonies. Then a sterile spreader was used to scrap fungal spores. Spore suspension were pipetted into Cryotube and mixed with glycerol to obtain 20% glycerol final concentration. Thus, the fungal spores were kept at -80°C.

3.1.2 *Aspergillus niger* culturing

A. niger was inoculated by wire loop method on potato dextrose agar (PDA) and incubated at room temperature for 72 hours. The fungus was identified on the basis of morphological characteristics such as color of the colony, texture of the mycelia.

3.1.3 Fungal spore collection and counting

2 mL of 0.2% Tween-20 was added onto the PDA plate containing of the fungus. The fungal spores were collected by scrapping the sporulating colonies with a sterile spreader and pipetting the spore suspension into a microcentrifuge tube. The spore suspension was carefully mixed and added 10 μL to each side of the hemocytometer. The spores inside the 5 yellow zones (the bellowed figure) of both sides of the hemocytometer were counted and recorded for the calculation of an average spore number per a milliliter.



* If a spore falls on the left or bottom line **DO NOT** count it.

* If spore falls on the right or top line **DO** count it.

3.1.4 Biomass production

A. niger was grown in 100 mL of potato dextrose broth (PDB) and Czapex Dox broth (CDB) and incubated at 30°C on a rotary shaker (200 rpm) for 96 hours. The biomass was harvested by filtration using Whatman® filter paper Number 1, followed by washing with 50 mL of sterile distilled water to remove any components of the medium for 3 times. The 25 grams wet weight of fungal biomass was placed in individual flasks containing 50 mL of sterile deionized water and incubated at 30°C on a rotary shaker (200 rpm) for 96 hours. The biomass was removed by filtration using Whatman® filter paper Number 1, and the filtrate was collected and used for biosynthesis of AgNPs.

3.1.5 Biosynthesis of AgNPs

For biosynthesis of AgNPs, 5, 10, 20, 30, and 40 mL of 10 mM $AgNO_3$ solution was added into 45, 40, 30, 20, and 10 mL of the fungal filtrate in an Erlenmeyer flask respectively to create a total silver ion (Ag^+) concentration of 1, 2, 4, 6 and 8 mM. The reaction mixture without $AgNO_3$ was used as a control. The prepared solutions were incubated at 30°C on a rotary shaker (200 rpm) for 96 hours. All solutions were kept in dark to avoid any photochemical reactions during the experiment. The AgNPs were purified by centrifugation at 14,000 rpm for 10 minutes to separate the particles out of the liquid parts. The centrifugation was repeated several times to concentrate the AgNPs. The concentrated samples were collected for further experiments.

Table 3.1 Final concentration of $AgNO_3$ solution and ratio between fungal filtrate and $AgNO_3$ solution in biosynthesis of AgNPs

Final conc. $AgNO_3$ Total (50 mL)	Fungal filtrate	Ratio	10 mM $AgNO_3$ solution
1 mM	45	9:1	5
2 mM	40	8:2	10
4 mM	30	6:4	20
6 mM	20	4:6	30
8 mM	10	2:8	40

3.2 Characterization of the synthesized AgNPs

The preliminary characterization of AgNPs was done by visual observation of color change in the solutions containing the fungal filtrate and $AgNO_3$. The formation of AgNPs could be observed by the change of solutions' color from pale brown to dark brown.

3.2.1 UV-visible spectrophotometry

The bioreduction of $AgNO_3$ solution and the formation of AgNPs was confirmed by measuring absorbances across wavelengths of 200-800 nm using a UV-Vis spectrophotometer. The specific peak of AgNPs could be found between 400-430 nm.

3.2.2 Transmission electron microscopy (TEM)

A drop of the biologically synthesized AgNPs was placed on a carbon-coated copper grid and dried at room temperature. TEM micrographs of the samples were taken using the TEM. The size and shape of AgNPs were studied.

3.2.3 Dynamic light scattering (DLS) measurement

The mean diameters and size distribution (polydispersity index, PDI) of nanoparticles in water were measured using a Zetasizer Nano range instrument (Malvern Instruments, Malvern). The CONTIN program was used to extract size distribution.

3.3 Antibacterial activity assay

3.3.1 Preparation of AgNPs dispersions

AgNPs suspension was centrifuged at 14,000 rpm for 10 minutes, then slowly pipetted the liquid out and kept the particles at the bottom of the microtubes. The microtubes containing of AgNPs were dried by leaving open air at room temperature. The dried AgNPs were weighed. After the measurement of AgNPs mass, the sterile DI water was added into the microtube to generate the stock of 10 mM AgNPs dispersion. The stock was diluted to give final concentrations of 1000, 500 and 250 μg/mL AgNPs dispersion.

3.3.2 Calibration curve of bacterial culture

First, the bacteria from -80°C stocks were inoculated on nutrient agar (NA) media overnight. After that, the pure bacterial strains were cultured in 100 mL NB broth. The serial dilutions of 1/2, 1/4, 1/8 and 1/16 of bacteria cultures were performed. Each of dilution tubes was measure the OD600 and subjected to the colony-counting method (3 replicates). After incubating the plates for 24 hours, the bacterial colonies were counted, and the numbers of bacterial CFU per milliliter were calculated using the bellowed equation.

$$Number\ of\ bacteria\ (CFUs/mL) = \frac{Number\ of\ colonies\ (CFUs) \times Dilution\ factor}{Amount\ plated}$$

The data were grouped in Excel to determine the formula based on the linear equation Y = mx + b slope.

3.3.3 Preparation of bacterial culture

S. aureus and *E. coli* were cultured on sterile LB agar medium and incubated at 37°C for 24 hours. After that, five single isolated colonies of each bacterial strain were picked from the culturing plates and suspended in 10 mL sterile Mueller-Hinton broth. The strains were grown on Mueller-Hinton broth at 37°C for 3-5 hours to reach the mid-log phase (O.D. 600 ~ 0.5). Then the bacterial cultures were diluted to give the final concentration of 1.5 x 10^8 CFU/mL (equal to 0.5 McFarland standard).

3.3.4 Preparation of plate inoculation

The Mueller-Hinton agar (MHA) plates were allowed to solidify at room temperature so any excess moisture will be absorbed into the medium. The bacterial suspension (concentration of 1.5 x 10^8 CFU/mL) was inoculated on entire plates of MHA using a sterile cotton-tipped swab.

3.3.5 Agar-well diffusion method

After the preparation of inoculated plates, wells were made on the agar using a pasteur pipette of 6 mm in diameter size. The wells were filled with 50 μL of the tested AgNPs dispersions (concentrations of 1000, 500 and 250 μg/mL). The plates were incubated at 37°C for 24 hours and measured the diameters of inhibition zone without subtracting the well diameter from the total inhibition zone diameter. Streptomycin and DI water were used as a positive and negative controls, respectively.

Chapter 4
Results

4.1 Biological synthesis of AgNPs

4.1.1 Color change

After the incubation of *A. niger* filtrate with $AgNO_3$ in the dark, the synthesis of AgNPs by the fungal filtrate was investigated. After the addition of $AgNO_3$ to cell free filtrate, the color of the mixture changed from pale brown to intense brown after 3 days of incubation. Figure 4.1 showed the color of *A. niger* (MSCU 0361) filtrate in aqueous solution of $AgNO_3$ at the beginning of the reaction (a) and after 3 days of the reaction (b). The control without $AgNO_3$ showed no color change when incubated in the same conditions. The negative control ($AgNO_3$ solution) also did not display the change in color. Thus, these results indicated that the formation of AgNPs occurred only in the conditions of the fungal filtrate with $AgNO_3$.

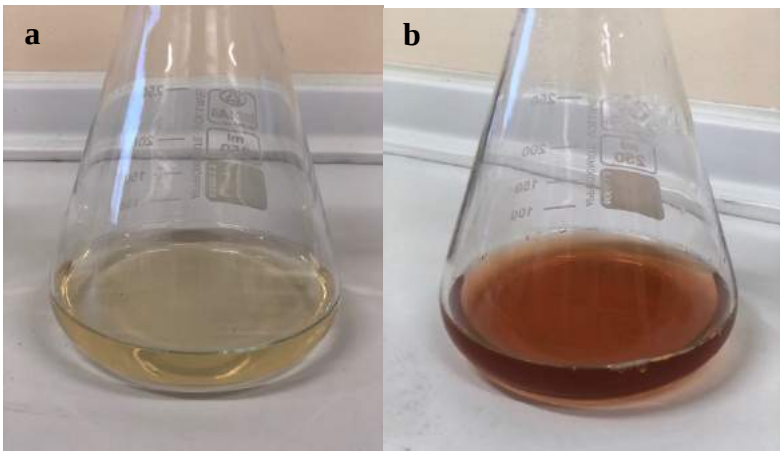


Figure 4.1 Biosynthesis of silver nanoparticles using 1mM $AgNO_3$ – color change during the reaction (a) beginning of the reaction (b) after 3 days of the reaction.

The figure 4.2 and 4.4 showed the color of filtrate from fungal biomass grown on PDB and CDB media in different aqueous solution of $AgNO_3$ at the beginning of the reaction and figure 4.3 and 4.5 showed the different color intensity of the mixture changed from pale brown to intense brown after 3 days of incubation. The results indicated that 2 mM $AgNO_3$-synthesized AgNPs gave a highest color intensity whereas 8 mM $AgNO_3$-synthesized AgNPs gave a lowest color intensity.

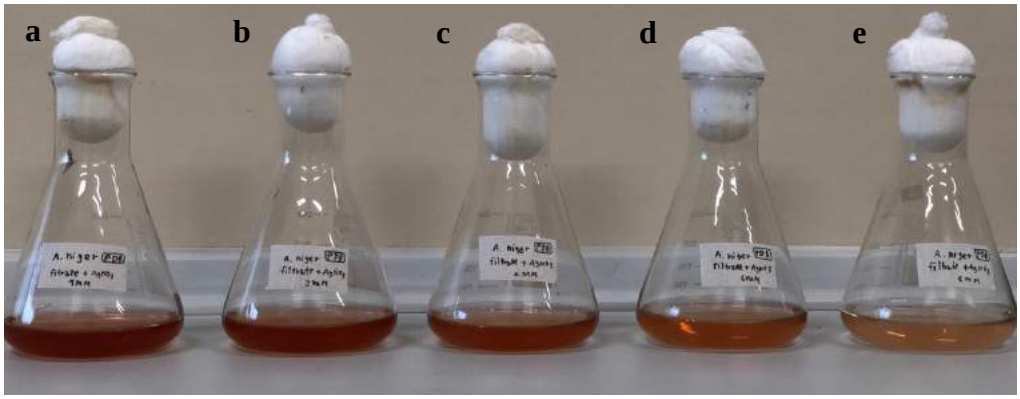


Figure 4.2 The beginning of biosynthesis of AgNPs synthesized by *A. niger* grown on potato dextrose broth (PDB) media at a different of $AgNO_3$ concentrations. (a) 1 mM $AgNO_3$, (b) 2 mM $AgNO_3$, (c) 4 mM $AgNO_3$, (d) 6 mM $AgNO_3$, and (e) 8 mM $AgNO_3$ with cell free fungal medium.

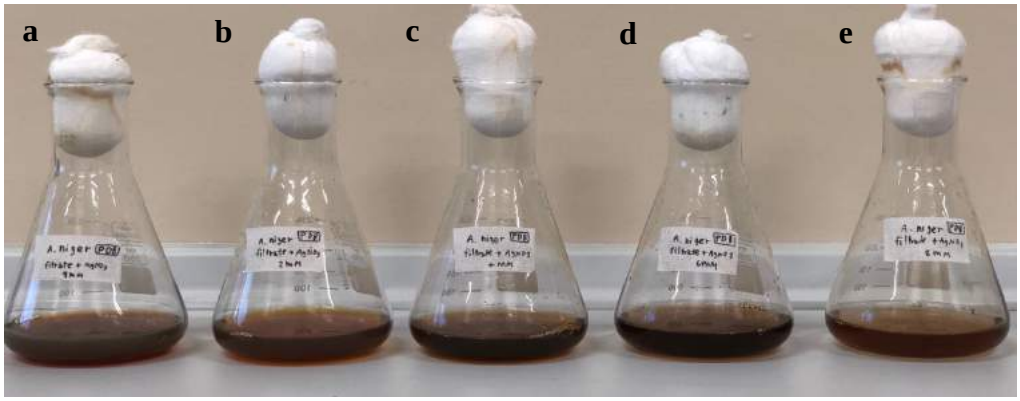


Figure 4.3 Biosynthesis of AgNPs synthesized by *A. niger* grown on potato dextrose broth (PDB) media at a different of $AgNO_3$ concentrations. (a) 1 mM $AgNO_3$-synthesized AgNPs, (b) 2 mM $AgNO_3$-synthesized AgNPs, (c) 4 mM $AgNO_3$-synthesized AgNPs, (d) 6 mM $AgNO_3$-synthesized AgNPs, (e) 8 mM $AgNO_3$-synthesized AgNPs.

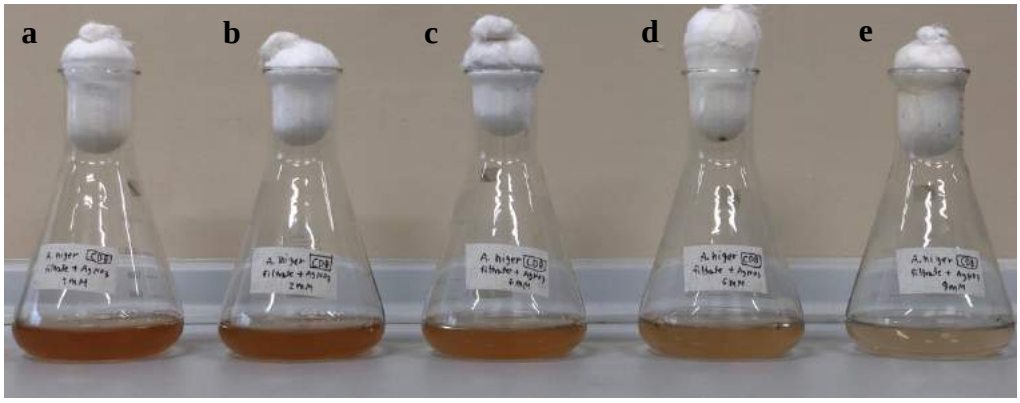


Figure 4.4 The beginning of biosynthesis of AgNPs synthesized by *A. niger* grown on Czapex Dox broth (CDB) media at a different of $AgNO_3$ concentrations. (a) 1 mM $AgNO_3$, (b) 2 mM $AgNO_3$, (c) 4 mM $AgNO_3$, (d) 6 mM $AgNO_3$, and (e) 8 mM $AgNO_3$ with cell free fungal medium.

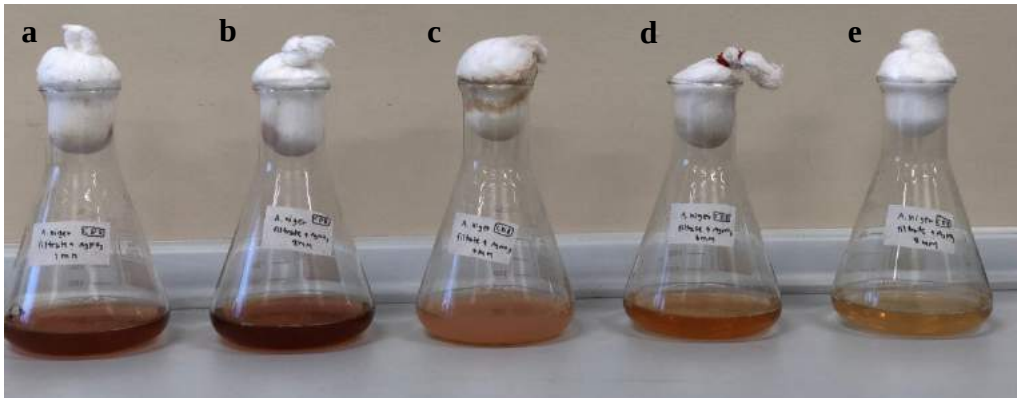


Figure 4.5 Biosynthesis of AgNPs synthesized by *A. niger* grown on Czapek Dox broth (CDB) media at a different of $AgNO_3$ concentrations. (a) 1 mM $AgNO_3$-synthesized AgNPs, (b) 2 mM $AgNO_3$-synthesized AgNPs, (c) 4 mM $AgNO_3$-synthesized AgNPs, (d) 6 mM $AgNO_3$-synthesized AgNPs, (e) 8 mM $AgNO_3$-synthesized AgNPs.

4.2 Characterization of the synthesized AgNPs

4.2.1 UV-visible spectrophotometry

The formation of AgNPs was investigated by UV-vis spectral analysis. The UV-Vis absorption peak at specific wavelength of 420 nm indicated that the solutions contained AgNPs. Among all of the samples incubated with different concentration of $AgNO_3$, the highest absorbance value was obtained at 2 mM of $AgNO_3$ concentration. The AgNPs synthesized by

the fungal filtrate biomass grown on PDB (Figure 4.6) gave a higher absorbance value than the one grown on CDB (Figure 4.7). The absorbances of the fungal filtrate alone as well as pure $AgNO_3$ solution (controls) were zero at the wavelength of 420 nm.

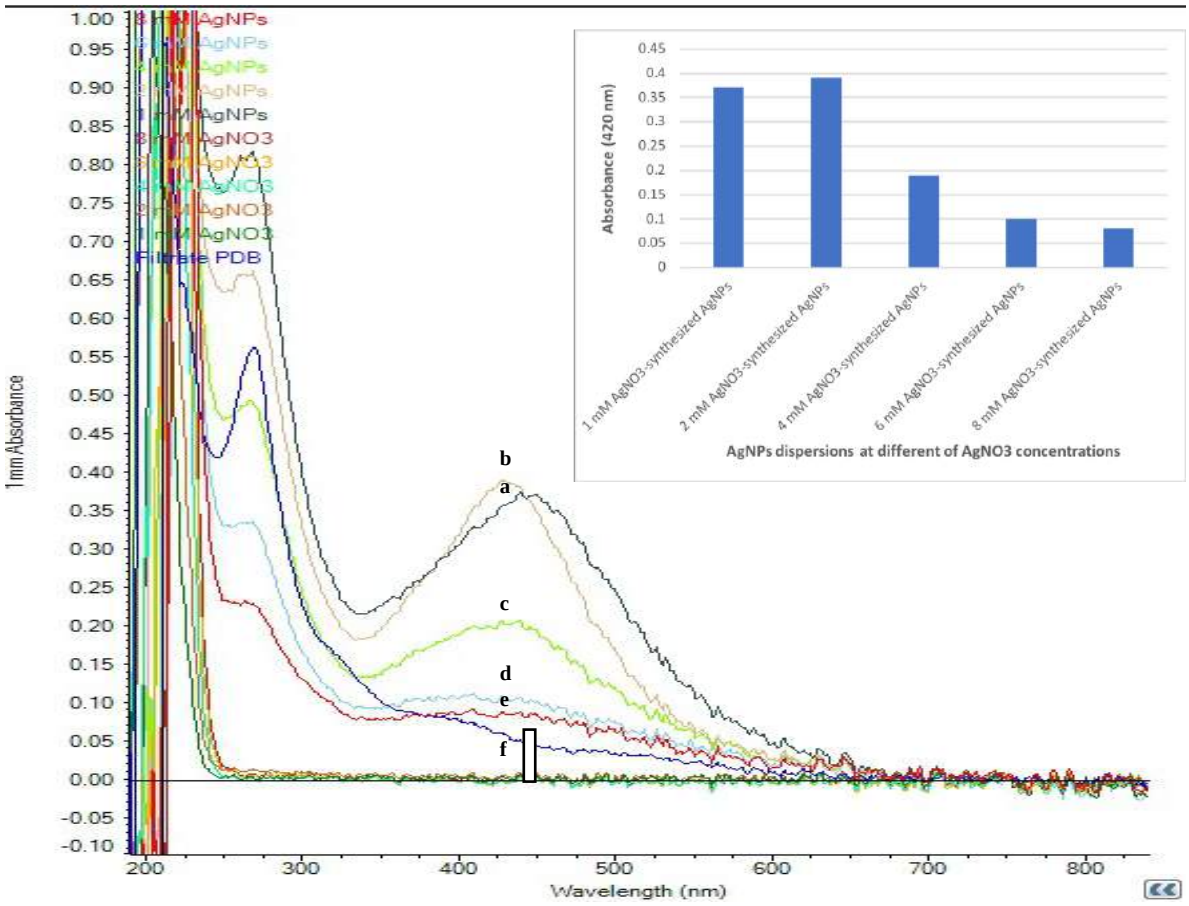


Figure 4.6 The UV-Vis absorption spectra and a histogram of extracellularly synthesized AgNPs by *A. niger* grown on potato dextrose broth (PDB) media. At 420 nm of wavelength, the specific peak of AgNPs appeared in these following conditions: (a) 1 mM $AgNO_3$-synthesized AgNPs, (b) 2 mM $AgNO_3$-synthesized AgNPs, (c) 4 mM $AgNO_3$-synthesized AgNPs, (d) 6 mM $AgNO_3$-synthesized AgNPs, (e) 8 mM $AgNO_3$-synthesized AgNPs. (f) The controls, both the fungal filtrate alone and $AgNO_3$ solutions at different concentration showed no unique peak of AgNPs.

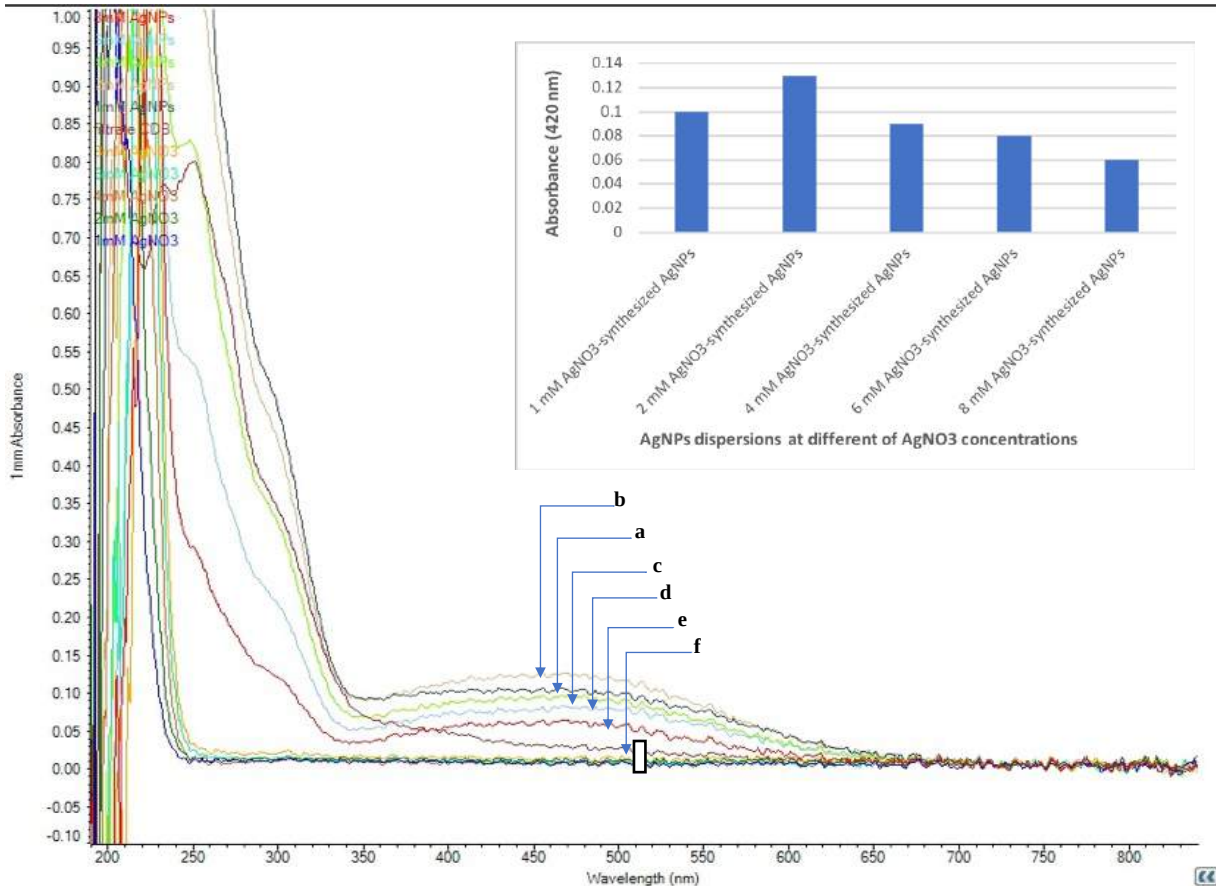


Figure 4.7 The UV-Vis absorption spectra and a histogram of extracellularly synthesized AgNPs by *A. niger* grown on Czapex Dox broth (CDB) media. At 420 nm of wavelength, the specific peak of AgNPs appeared in these following conditions: (a) 1 mM $AgNO_3$-synthesized AgNPs, (b) 2 mM $AgNO_3$-synthesized AgNPs, (c) 4 mM $AgNO_3$-synthesized AgNPs, (d) 6 mM $AgNO_3$-synthesized AgNPs, (e) 8 mM $AgNO_3$-synthesized AgNPs. (f) The controls, both the fungal filtrate alone and $AgNO_3$ solutions at different concentration showed no unique peak of AgNPs.

4.2.2 Transmission electron microscopy (TEM)

The TEM micrographs of AgNPs synthesized from 2 mM and 6 mM of $AgNO_3$ in both PDB and CDB conditions were shown in Figure 4.8, 4.9, 4.10 and 4.11 repectively. Most of the AgNPs were in spherical shape and the average particle size was 18.6 ± 16.8, 20.8 ± 16.6, 22.6 ± 11.8 and 19.5 ± 14.8 nm for 2 mM $AgNO_3$-synthesized AgNPs (PDB), 6 mM $AgNO_3$-synthesized AgNPs (PDB), 2 mM $AgNO_3$-synthesized AgNPs (CDB) and 6 mM $AgNO_3$-synthesized AgNPs (CDB) respectively (Table 4.1). Histograms (right-hand of the figures) indicate the AgNPs size distribution.

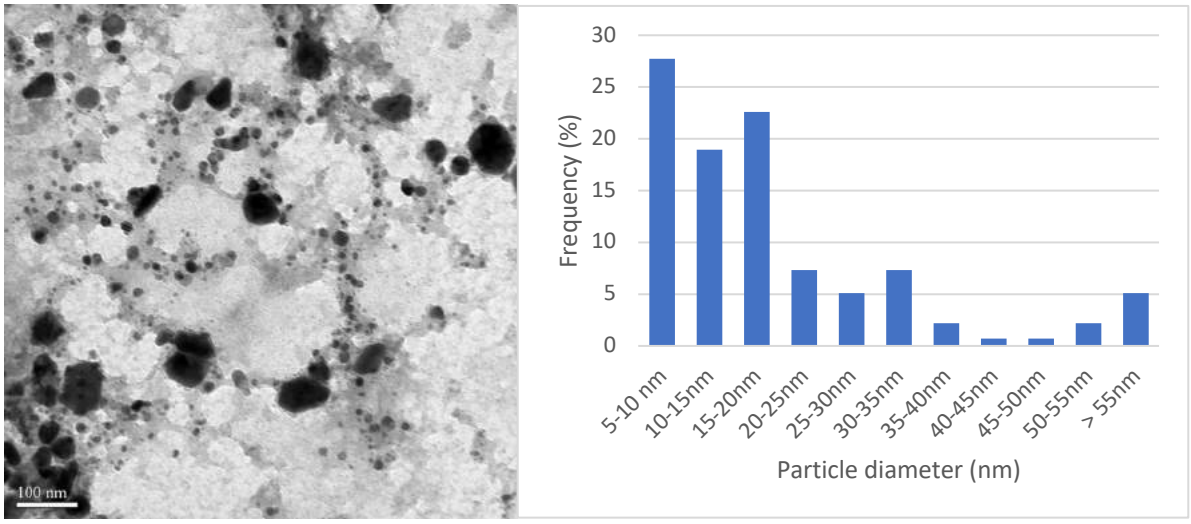


Figure 4.8 A representative TEM micrograph and a histogram for the 2 mM $AgNO_3$-synthesized AgNPs using the fungal filtrate of *A. niger* grown on PDB medium.

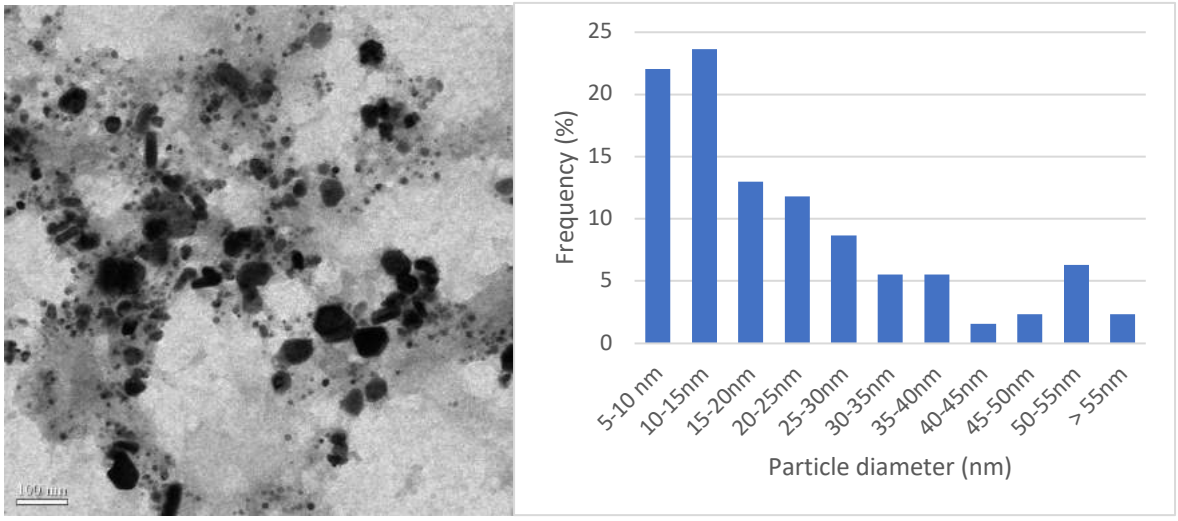


Figure 4.9 A representative TEM micrograph and a histogram for the 6 mM $AgNO_3$-synthesized AgNPs using the fungal filtrate of *A. niger* grown on PDB medium.

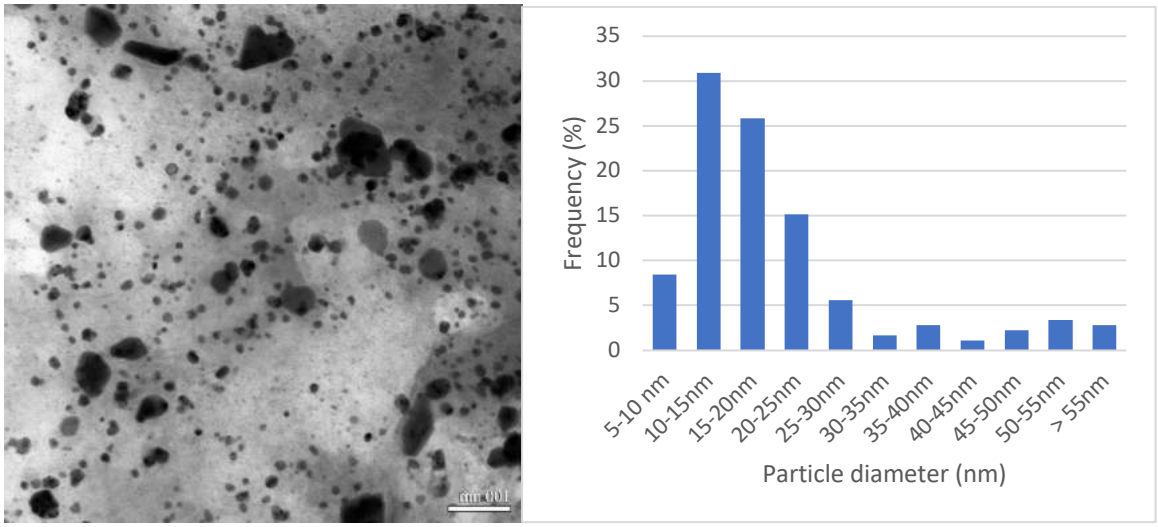


Figure 4.10 A representative TEM micrograph and a histogram for the 2 mM $AgNO_3$-synthesized AgNPs using the fungal filtrate of *A. niger* grown on CDB medium.

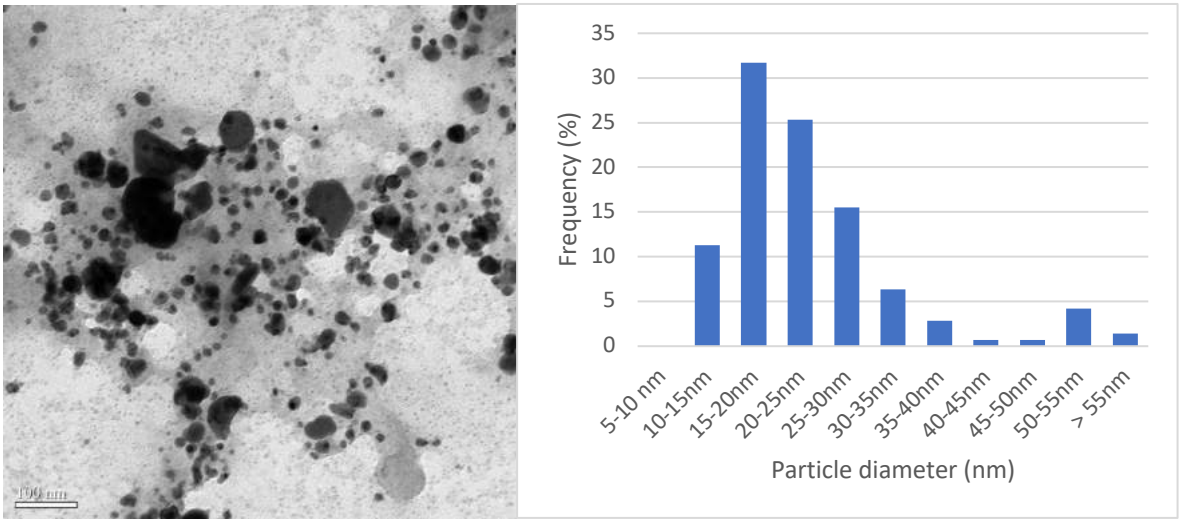


Figure 4.11 A representative TEM micrograph and a histogram for the 6 mM $AgNO_3$-synthesized AgNPs using the fungal filtrate of *A. niger* grown on CDB medium.

4.2.3 Dynamic light scattering (DLS) measurement

DLS technique was used to measure the hydrodynamic size in water dispersions. The particle sizes were measured using Zetasizer Nano range. The result showed that the sizes of AgNPs from DLS was bigger than the particle sizes obtained from TEM micrographs. Additionally, 2 mM $AgNO_3$-synthesized AgNPs was larger than 6 mM $AgNO_3$-synthesized AgNPs. PDIs of all the AgNPs dispersions were <0.5. The data was shown in Table 4.1.

Table 4.1 Characterizations of the AgNPs dispersions in term of size and PDI. Size was measured by using DLS and TEM**.**

AgNPs dispersion	**DLS: average Size ± SD (nm)**	**DLS: average PDI ± SD**	**TEM: average Size ± SD (nm)**
2 mM $AgNO_3$-synthesized AgNPs (PDB)	119.8 ± 2.4	0.4 ± 0.1	18.6 ± 16.8
6 mM $AgNO_3$-synthesized AgNPs (PDB)	152.7 ± 1.3	0.3 ± 0.0	20.8 ± 16.6
2 mM $AgNO_3$-synthesized AgNPs (CDB)	80.7 ± 2.4	0.4 ± 0.0	22.6 ± 11.8
6 mM $AgNO_3$-synthesized AgNPs (CDB)	116.0 ± 1.7	0.4 ± 0.1	19.5 ± 14.8

4.3 Antibacterial activity assay

4.3.1 Agar-well diffusion method

The effect of biosynthesized AgNPs against *S. aureus* and *E. coli* was studied using agar-well diffusion method (Table 4.2 and 4.3). In the tests with *S. aureus*, the 2 mM $AgNO_3$-synthesized AgNPs of both PDB and CDB conditions showed higher inhibition zone diameter than the 6 mM $AgNO_3$-synthesized AgNPs. The concentration of AgNPs that gave the best antibacterial activity was 1000 µg/mL. In the study of *E. coli*, AgNPs dispersions showed unclear inhibition zone, so we scored the results as zero for the zone of inhibition. Streptomycin and DI water was used as a positive and negative control respectively.

Table 4.2 Determination of inhibition zones for different AgNPs dispersions against *S. aureus* and *E. coli* using agar-well diffusion method. (These AgNPs dispersions were synthesized using the filtrate from *A. niger* grown on PDB medium.)

Dispersion (50 µL)	**Zone of inhibition ± SD (mm) (n=3)**			
	2 mM $AgNO_3$-synthesized AgNPs		**6 mM $AgNO_3$-synthesized AgNPs**	
	S. aureus	***E. coli***	***S. aureus***	***E. coli***
1000 µg/mL	12.7 ± 0.6	0.0 ± 0.0	11.3 ± 0.6	0.0 ± 0.0
500 µg/mL	11.3 ± 0.6	0.0 ± 0.0	11.3 ± 0.6	0.0 ± 0.0
250 µg/mL	9.7 ± 0.6	0.0 ± 0.0	10.7 ± 0.6	0.0 ± 0.0
Streptomycin (50 µg/mL)	10.7 ± 0.6	10.3 ± 0.6	10.7 ± 0.6	10.3 ± 0.6
DI water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 4.3 Determination of inhibition zones for different AgNPs dispersions against *S. aureus* and *E. coli* using agar-well diffusion method. (These AgNPs dispersions were synthesized using the filtrate from *A. niger* grown on CDB medium.)

Dispersion (50 µL)	**Zone of inhibition ± SD (nm) (n=3)**			
	2 mM $AgNO_3$-synthesized AgNPs		**6 mM $AgNO_3$-synthesized AgNPs**	
	S. aureus	***E. coli***	***S. aureus***	***E. coli***
1000 µg/mL	12.3 ± 0.6	0.0 ± 0.0	11.0 ± 0.0	0.0 ± 0.0
500 µg/mL	11.3 ± 0.6	0.0 ± 0.0	10.3 ± 0.6	0.0 ± 0.0
250 µg/mL	9.3 ± 0.6	0.0 ± 0.0	10.0 ± 0.0	0.0 ± 0.0
Streptomycin (50 µg/mL)	10.7 ± 0.6	10.3 ± 0.6	10.7 ± 0.6	10.3 ± 0.6
DI water	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

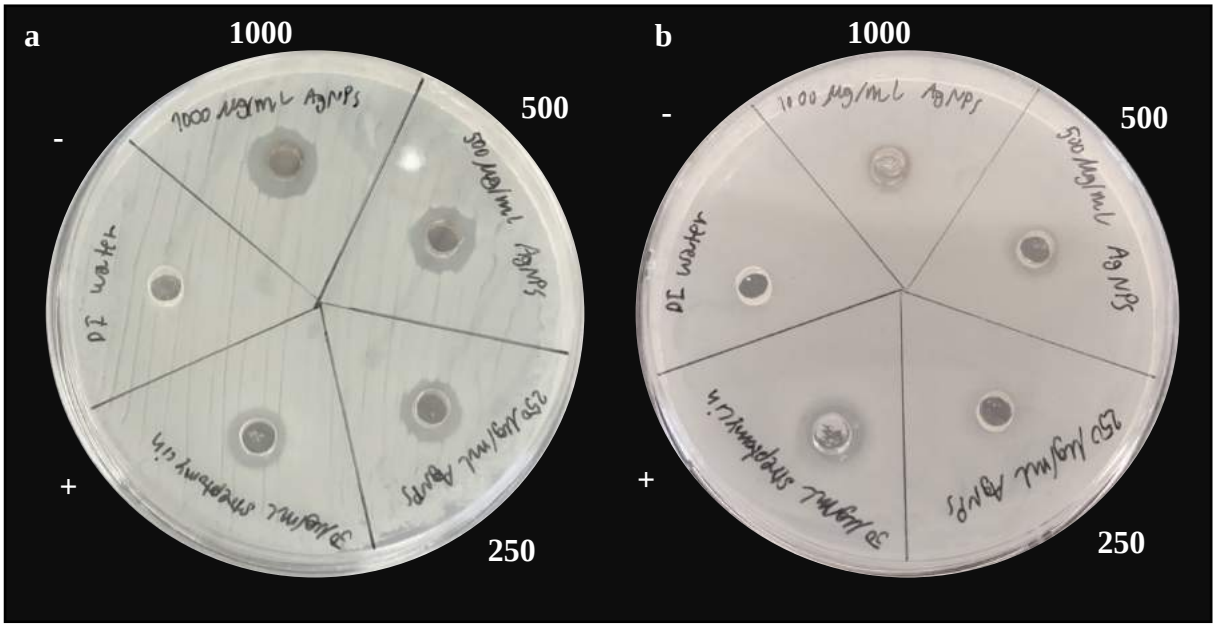


Figure 4.12 Antimicrobial activity of the 2 mM $AgNO_3$-synthesized AgNPs (concentrations of 1000, 500 and 250 µg/mL) by agar-well diffusion method: (a) *S. aureus* plate, (b) *E. coli* plate (The AgNPs dispersions used in this study were synthesized from the filtrate of *A. niger* grown on PDB medium.) Streptomycin and DI water was used as a positive and negative control respectively.

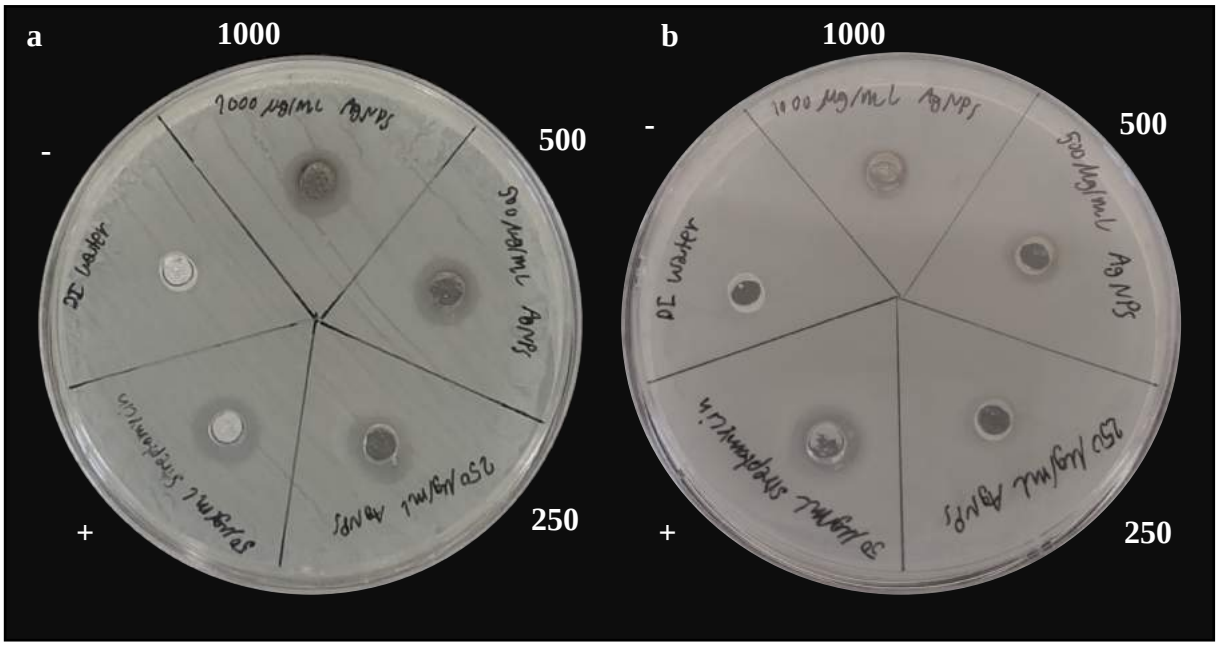


Figure 4.13 Antimicrobial activity of the 6 mM $AgNO_3$-synthesized AgNPs (concentrations of 1000, 500 and 250 µg/mL) by agar-well diffusion method: (a) *S. aureus* plate, (b) *E. coli* plate (The AgNPs dispersions used in this study were synthesized from the filtrate of *A. niger* grown on PDB medium.) Streptomycin and DI water was used as a positive and negative control respectively.

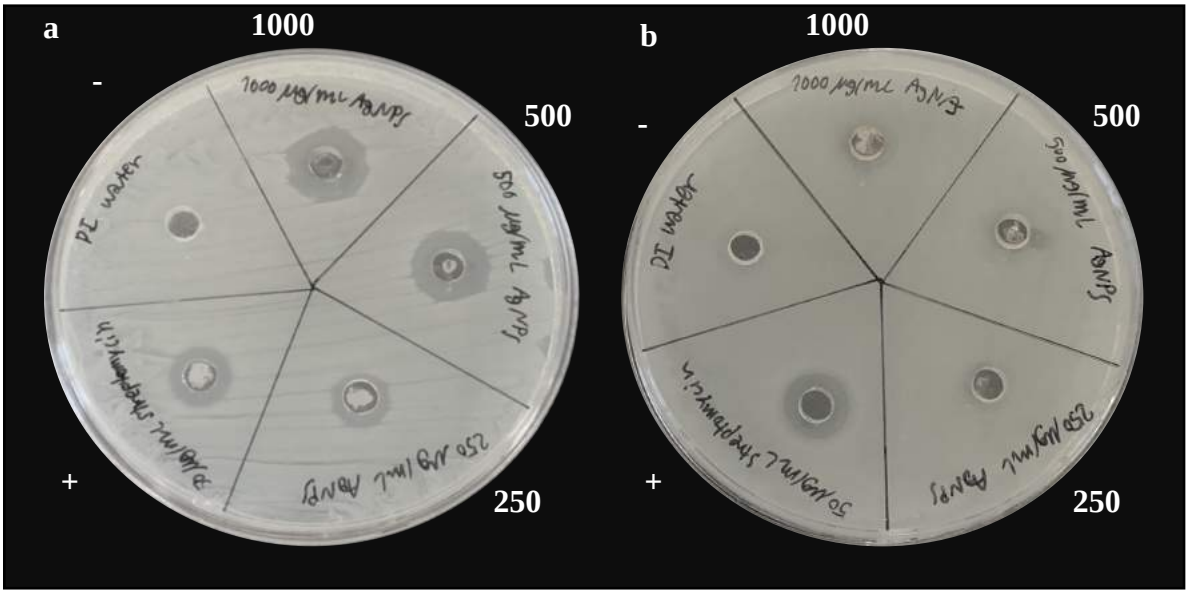


Figure 4.14 Antimicrobial activity of the 2 mM $AgNO_3$-synthesized AgNPs (concentrations of 1000, 500 and 250 µg/mL) by agar-well diffusion method: (a) *S. aureus* plate, (b) *E. coli* plate (The AgNPs dispersions used in this study were synthesized from the filtrate of *A. niger* grown on CDB medium.) Streptomycin and DI water was used as a positive and negative control respectively.

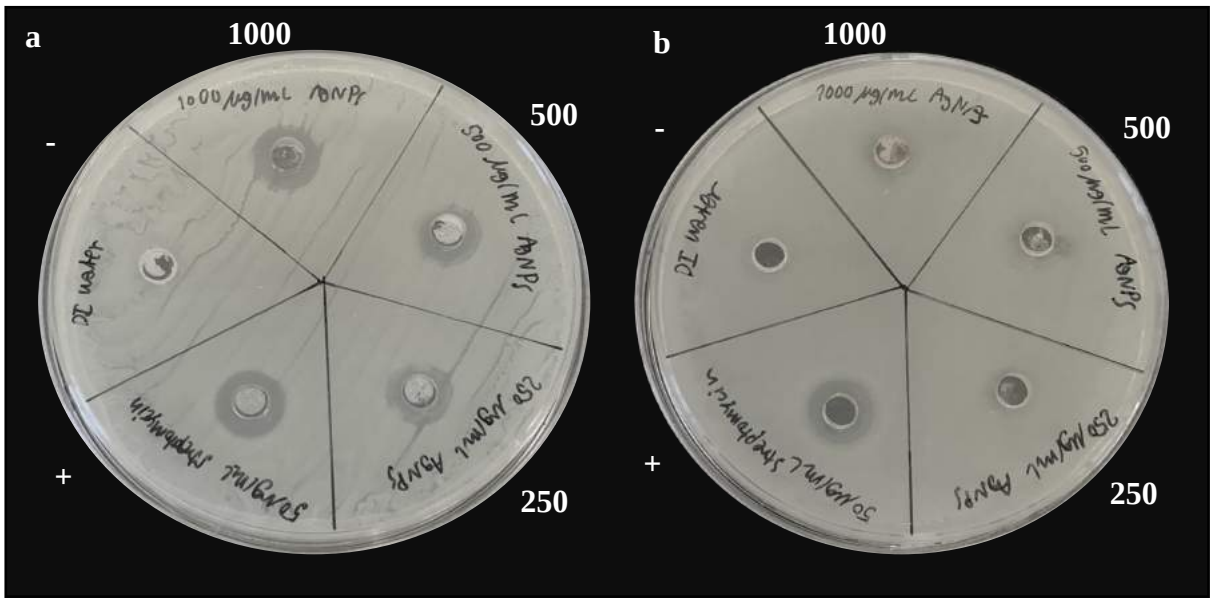


Figure 4.15 Antimicrobial activity of the 6 mM $AgNO_3$-synthesized AgNPs (concentrations of 1000, 500 and 250 µg/mL) by agar-well diffusion method: (a) *S. aureus* plate, (b) *E. coli* plate (The AgNPs dispersions used in this study were synthesized from the filtrate of *A. niger* grown on CDB medium.) Streptomycin and DI water was used as a positive and negative control respectively.

Chapter 5
Conclusion and Discussion

The biological method of AgNP production is a simple, fast, clean and eco-friendly alternative method. AgNPs can be produced using fungal cell free filtrates as reducing agents (Rheder et al. 2018). Several groups of fungi were utilized for biogenic synthesis of AgNPs (Zhao et al. 2018). *A. niger* was report as a filamentous fungus that showed an ability to reduce aqueous silver ions resulting in the production of AgNPs (Gade et al. 2008). Some studies suggested that the process of silver ions reduction occurred through reductase enzymes and electron shuttle quinones. (Durán et al. 2005).

In our study, we aimed to improve the AgNP production in the biological synthesis using *A. niger* MSCU 0361. We were interested in the factor of $AgNO_3$ concentration that might affect the production yields as well as the AgNP characteristics. To investigate this, we performed the biosynthesis of AgNPs in different concentrations of $AgNO_3$ by dilute 10 mM $AgNO_3$ solution with fungal filtrate to obtain the final concentration of $AgNO_3$ ranging from 1 mM to 8 mM (the fungal filtrate : $AgNO_3$ ratio was shown in Table 3.1). The formation of AgNPs was primarily observed by the change of fungal filtrate's color from pale brown to dark brown. This phenomenon was a result of the reduction of silver ions to form nanoparticles giving a unique optical property of AgNPs. The color intensity was consistently sustained even 4 days after incubation, which indicated that the AgNPs were well dispersed in the solution, and there was no obvious aggregation.

To observe more specific optical property of AgNPs, UV-visible absorption spectra of the AgNPs dispersions were performed in the range of 200-800 nm using a double-beam UV-visible spectrophotometer. The UV-Vis spectra of the AgNPs dispersions synthesized from the filtrate of *A. niger* which was cultured in PDB medium gave higher absorbance values compared to the one grown on CDB medium. This difference might be due to effects of media that contributed to the production of fungal metabolites and proteins involving in the reduction of silver ions. The PDB is a nutritionally rich medium that might induce a higher production of reductase enzymes compared to CDB medium. Our study also demonstrated that the optimum $AgNO_3$ concentration of the biosynthesis was 2 mM which gave the highest values of AgNPs specific wavelength at 420 nm.

The biosynthesized AgNPs were further characterized by TEM to observe their shapes and core sizes. Through the TEM analysis, the particles were spherical and monodispersed without obvious agglomeration. The particle size histograms showed that AgNP sizes ranged from 5 to 55 nm, and the majority of the particles were less than 25 nm.

Since TEM micrographs exhibited only the core size of AgNPs, in order to study the total size that include the silver core and coating molecules, DLS technique was performed. By this technique, the Zetasizer Nano range was used to measure the hydrodynamic size of AgNPs in water dispersions. The particle size measured by DLS technique was bigger than that the size obtained from TEM micrographs because the hydrodynamic diameter was affected by other molecules around the silver core, whereas TEM technique measured only the inner core of AgNPs. PDIs of all the AgNPs dispersions were between 0.3 to 0.4 suggesting the mid-range of polydispersity.

After the synthesis and characterization of AgNPs, the particles were tested for antibacterial activity. In the tests, *S. aureus* and *E. coli* were used as representatives of Gram-positive bacteria and Gram-negative bacteria, respectively. Compared with the control, the AgNPs demonstrated the inhibition against *S. aureus* as previously described (Kim et al. 2007), but unclear inhibition zones were observed in the tests with *E. coli*. The better results of antibacterial activity found in *S. aureus* compared to *E. coli* might be because of their different cellular structures and mechanisms used for protecting themselves from damaging by AgNPs.

To sum up, in this study, AgNPs were synthesized extracellularly by *A. niger* filtrate. Without using any toxic chemicals as capping agents as done in chemical methods, these biosynthesized AgNPs were still stable at least throughout half a year of our study. The spherical AgNPs ranged in size from 5 to 55 nm and showed promising antibacterial activity against *S. aureus*. The optimization study of $AgNO_3$ concentration suggested that 2 mM of $AgNO_3$ together with the condition parameters of 30°C incubation temperature, pH of 7, and fungal biomass of 25 grams gave the highest yield and pleasant characteristics of AgNPs. However, many further studies are still needed in order to understand molecular mechanisms involved in the biosynthesis of AgNPs.

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Appendix

Appendix A

Media recipes and preparation

1. Czapex Dox broth medium

Ingradients	**Grams/liter**
Sucrose	30.00
Sodium nitrate	3.00
Dipotassium hydrogen phosphate	1.00
Magnesium sulphate	0.50
Potassium chloride	0.50
Ferrous sulphate	0.01

Final pH (25°C) 7.3 ± 0.2

Suspend 35.01 grams in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Dispense in tubes or flasks as desired. Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes.

2. Potato dextrose broth medium

Ingradients	**Gms/liter**
Potatoes infusion from	200.00
Dextrose	20.00

Final pH (25°C) 5.1 ± 0.2

Suspend 24.0 grams in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Dispense in tubes or flasks as desired. Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes.

* Potato dextrose agar medium was prepared by adding 1.3% agar.

3. Nutrient broth medium

Ingradients	**Gms/liter**
Peptone	5.00
Sodium chloride	5.00
Meat extract	1.50
Yeast extract	1.50

Final pH (25°C) 7.4 ± 0.2

Suspend 13.0 grams in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Dispense in tubes or flasks as desired. Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes.

* Nutrient agar medium was prepared by adding 1.3% agar.

4. Mueller Hinton broth medium

Ingradients	**Gms/liter**
HM infusion B from [a]	300.00
Acicase™ [2]	17.50
Starch	1.50

Final pH (25°C) 7.3 ± 0.1

[1] Equivalent to Beef infusion from

[2] Equivalent to Casein acid hydrolysate

Suspend 13.0 grams in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Dispense in tubes or flasks as desired. Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes.

* Meuller Hinton agar medium was prepared by adding 1.3% agar.

Appendix B

Chemical agents

1. Silver nitrate ($AgNO_3$) **10 mM $AgNO_3$; 500 mL**

Suspend 0.85 grams in 500 mL sterile DI water and slowly mix to dissolve the solution completely. Store at 4°C.

* $AgNO_3$ molecular weight is 169.87 g/mol

2. Streptomycin sulphate **1.0 mg/mL**

Dissolve 1.0 mg in 1.0 mL of sterile DI water and slowly mix to dissolve the solution completely. Store at 4 °C.

* Streptomycin sulphate molecular weight is 728.69 g/mol

Appendix C

Microorganism strains

1. *Escherichia coli* MSCU 0349

Kingdom **Bacteria**
Subkingdom **Negibacteria**
Phylum **Proteobacteria**
Class **Gammaproteobacteria**
Order **Enterobacteriales**
Family **Enterobacteriaceae**
Genus ***Escherichia***
Species ***Escherichia coli*** Castellani and Chalmers, 1919

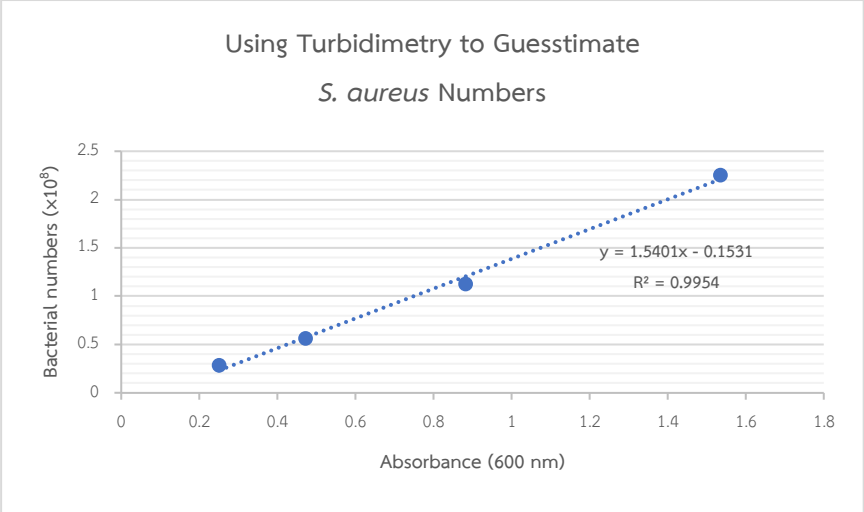
2. *Staphylococcus aureus* MSCU 0353

Kingdom **Bacteria**
Subkingdom **Posibacteria**
Phylum **Firmicutes**
Class **Bacilli**
Order **Bacillales**
Family **Staphylococcaceae**
Genus ***Staphylococcus***
Species ***Staphylococcus aureus*** Rosenbach, 1884

3. *Aspergillus niger* MSCU 0361

Kingdom **Fungi**
Division **Ascomycota**
Class **Eurotiomycetes**
Order **Eurotiales**
Family **Trichocomaceae**
Genus ***Aspergillus***
Species ***Aspergillus niger*** Tieghem, 1867

4. Calibration curve of *Staphylococcus aureus*



5. Calibration curve of *Escherichia coli*