CHAPTER III
RESERCH METHODOLOGY

3.1 Isolation and purification of *Streptomyces*

Soil samples were collected from the forest area in Sakaerat Environmental Research Station and Botanical Garden at the Suranaree University of Technology, Nakhon Ratchasima province, Thailand. Soil samples were taken from a depth of 10-15 cm from the upper surface of the soil. The soil samples were collected in sterile polyethylene bags, sealed tightly, immediately taken to the laboratory, and stored at 4°C in a refrigerator until used. For the *Streptomyces* isolation, soil samples were isolated and cultivated on the actinomycete isolation agar (AIA, Himedia) medium using a serial dilution procedure and incubated for 7 days or until the plates were examined for the presence of the colonies at 30-37°C. For purification, isolated colonies were picked up and streaked on International Streptomyces Project 2 (ISP2) agar medium. The purified isolates were cultured in ISP2 broth for 7 days before being added glycerol to make the final concentration 20% and preserved in a freezer at -80°C until required.

3.2 Identification of *Streptomyces* using 16S rRNA gene sequence analysis

A modified approach for fungal DNA extraction was used to extract genomic DNA. The *Streptomyces* strains were grown in 10 mL ISP2 medium for 3-4 days at 30-37°C. Bacterial cells were harvested by centrifugation at 8000 rpm for 10 minutes and the cell pellets were used for DNA extraction. The cell pellet was dispersed in 500 µL of lysis buffer solution and grinded until fine. The cell suspension was transferred to a microcentrifuge tube and mixed with 165 µL of 5M NaCl followed by centrifugation at 4°C, 10,000 rpm for 10 minutes. The supernatant was transferred to a new tube and mixed with 400 µL chloroform and 400 µL isoamyl alcohol by

inverting tube until the solution becomes milky. The sample was then centrifuged at 4°C, 10,000 rpm for 10 minutes. The upper layer was transferred into a new tube. After that, an equal volume of chloroform was added to supernatants. The sample was mixed by inverting the tube and centrifuged at 4°C, 10,000 rpm for 5 minutes. The DNA in an aqueous layer was transferred into a new tube and precipitated with two volumes of 95% (v/v) ethyl alcohol. The sample was mixed by inverting the tube and centrifuged at 4°C, 10,000 rpm for 5 minutes. The precipitated DNA was washed with 300 µL ice-cool 70% (v/v) ethyl alcohol and centrifuged at 4°C, 10,000 rpm for 1 minute. The DNA pellet was dried and resuspended in 50 µL of TE buffer. The quality of extracted DNA was checked using agarose gel electrophoresis.

The genomic DNA of soil isolated was used as a template for polymerase chain reaction (PCR) amplification of the 16S ribosomal RNA (rRNA) gene using universal 16S rRNA primers, 27F 5' AGAGTTTGATCCTGGCTCAG 3' and 1525R 5' AAGGAGGTGWTCCARCC 3' (Lane, 1991). The amplification was performed in a mixed reaction with a final volume of 50 µL containing 10 µL of genomic DNA template, 2 µL of 10 pM 27F primer, 2.0 µL of 10 pM 1525R primer, 25 µL of master mix (EconoTaq® PLUS 2x Master Mix, Lucigen), and 11 µL of nuclease-free water. The amplification was completed using a thermal cycler machine according to the following conditions: initial denaturation at 94°C, 1 cycle for 5 minutes followed by 20 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 second and a final extension of at 72°C for 7 minutes. Amplified products were electrophoresed at 100 V on a 0.7% (w/v) agarose gel in a TBE buffer. The target band in the gel has been trimmed and purified by using Gel/PCR Purification Mini Kit (Favorgen™). The purified PCR products were submitted for sequencing at Macrogen, Korea.

The sequence of the 16S rRNA gene was compared to the online 16S rRNA database by using the EzBioCloud website (http://www.ezbiocloud.net). After that, CLUSTAL W was used to align the sequences with closely related species. The phylogenetic trees were created by the phylogeny method using MEGA-X (Molecular Evolutionary Genetics Analysis software version X) with a 1000 bootstraps Neighbor-

Joining technique. Finally, Sequence analysis has been done by using the BLAST program (http://www.ncbi.nlm.nih.gov/).

3.3 Preparation of *Streptomyces* for Biosynthesis

Streptomyces sp. was grown in a 500 mL Erlenmeyer flask containing 100 mL of Starch Casein Broth (SCB) and incubated at 37°C under 200 rpm shaking conditions for 5 days. After incubation, the culture was centrifuged at 4°C, 8000 rpm for 5 minutes. The extracellular cell-free supernatant was collected and used in subsequent experiments. On the other hand, the cell pellet of *Streptomyces* sp. was washed using sterile deionized water three times with centrifuged at 4°C, 8000 rpm for 1 minute to remove residues of media. Then, the biomass of *Streptomyces* sp. was resuspended in 100 mL sterile deionized water in an Erlenmeyer flask and cultured for 24 hours in an incubator shaker at 37°C with 200 rpm shaking setting. After incubation, the culture biomass was centrifuged at 4°C, 8000 rpm for 5 minutes. The intracellular cell-free supernatant was collected and used in subsequent experiments.

3.4 Biosynthesis of gold nanoparticles

The extracellular or intracellular cell-free supernatant of *Streptomyces* sp. was added to 1 mM Hydrogen tetrachloroauric acid; $HAuCl_4 \bullet 3H_2O$ (Sigma-Aldrich, USA. ≥99.9%) by using various ratios with a pH of 7, After that, the mixture was incubated at 37°C, 200 rpm for 24-72 h. The biosynthesis of gold nanoparticles was visual identified by observing the color change of the gold aqueous solution. Then, the whole mixture was centrifuged at 4°C, 8000 rpm for 5 minutes. The AuNPs were washed with sterile DI water and collected for further studies. The two controls, a culture supernatant control without $HAuCl_4$ and 1mM $HAuCl_4$ were incubated under the same experimental conditions.

3.5 Characterization of gold nanoparticles

3.5.1 Analysis of gold nanoparticles

The biosynthesized AuNPs were characterized using a UV-visible spectrophotometer to measure the absorbance of colloidal AuNPs solutions (SkanIt Software 5.0 for Microplate Readers RE, ver. 5.0.0.42, Thermo Scientific Multiscan GO, Finland). The absorption spectra of the AuNPs were obtained in approximately wavelength range of 300-800 nm. Zeta potential measurement and particle size distribution from less than a nanometer to several microns of biosynthesis gold nanoparticles were recorded using Zeta-sizer (Malvern Instrument Ltd, USA). An X-ray diffractometer (D8 Advance, Bruker, Germany) was used to investigate the identity and crystallinity of AuNPs. The result of the sample was compared with the reference from Joint Committee on Powder Diffraction Standards (JCPDS). The elemental composition or chemical characterization of gold nanoparticles was notified through EDX spectroscopy analysis (X-ray Fluorescence Energy Dispersive Spectrometer, Model XGT-5200).

3.5.2 Morphological characterization of gold nanoparticles

TEM (FEI/TECNAI G^2 S-Twin, USA) was used to determine the size and morphology of the biosynthesized gold nanoparticles. The morphology and size of the biosynthesized AuNPs were determined by TEM. The morphology of the nanoparticles could be highly variable. Gold nanoparticle formation was confirmed using TEM images. The particle size distribution of biosynthesized AuNPs was measured by dynamic light scattering or zeta-sizer (Malvern Instrument Ltd, USA).

3.5.3 Fourier transform infrared (FTIR) spectroscopy analysis

FTIR spectroscopy can be used to identify potential biomolecules involved in the reduction of Au^{3+} to Au^{0}. Functional groups of organic molecules attached to the surface of AuNPs as well as other surface chemical residues were detected using an FTIR spectrophotometer (FT-IR microscope, Tensor 27, Bruker, Germany). The spectra were scanned in the range of 4000-400 cm^{-1}. The spectral data were compared with the online database to determine the functional group in the sample at https://www.sigmaaldrich.com/TH/en/technical-documents/technical-article/analytical-chemistry/photometry-and-reflectometry/ir-spectrum-table.

3.5.4 X-ray absorption spectroscopy (XAS) analysis

XAS analysis was performed to determine local geometry and structure of gold in the produced AuNPs. The samples were measured by XANES at Synchrotron Light Research Institute (SLRI), Thailand. As a standard, $HAuCl_4$ and gold foil were employed.

3.6 Antimicrobial activity of gold nanoparticles

3.6.1 Antimicrobial activity determination using Agar well diffusion method

The agar well diffusion method is commonly used to assess antimicrobial activity. This method was used to test the ability of the AuNPs to inhibition of the test pathogens including *Bacillus subtilis* TISTR008, *Staphylococcus aureus* TISTR1466*,* methicillin-resistant *Staphylococcus aureus* (MRSA) DMST20651, methicillin-resistant *Staphylococcus aureus* (MRSA) DMST20654, methicillin-resistant *Staphylococcus epidermidis* (MRSE), *Escherichia coli* TISTR8465, *Pseudomonas aeruginosa* N90Ps, *Pseudomonas aeruginosa* TISTR781, *Pseudomonas aeruginosa* TISTR1287, *Acinetobacter baumanii, Serratia marcescense, Klebsiella pneumonia* TISTR1617, *Proteus mirabilis* TISTR100*, Salmonella typhi* TISTR292 and *Enterobacter aerogenes* TISTR1540. The culture supernatant control without $HAuCl_4$ and $HAuCl_4$ was used as the negative control. The Mueller–Hinton agar (Himedia™, India) plate surface was inoculated by spreading on the overnight culture of mid-log phase $5x10^5$ CFU/mL test pathogens over the agar surface. Then, a 6 mm diameter hole was punched aseptically with a sterile cork borer. One hundred microliters of the sample as AuNPs solution or the control were transferred into each well and left at room temperature for 1 h to allow the sample to diffuse into the agar. All plates were incubated at 37°C for 24 h. After the incubation, the plates were observed for inhibition zone formation around the wells. Diameters of the inhibition zone around the well and the diameters of the wells were measured (in mm).

3.6.2 Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was used to evaluate the antimicrobial activity of the AuNPs against pathogenic organisms. MIC determination of AuNPs against tested pathogens was achieved by using 96-well sterile microplates. Two-fold serial dilutions of AuNPs were inoculated with 50 µL of $5.0x10^5$ CFU/ml of tested pathogen and incubated at 37°C, 100 rpm for 24 h. After incubation, the plates were examined visually for the growth of test organisms. The lowest concentrations of AuNPs that inhibit the growth of tested pathogens were recorded as the MIC value.