CHAPTER III

METHODOLOGY

3.1 Experimental framework

Figure 3.1 presents experimental framework. This study aimed to investigate effect of AgNPs on nitrification process. Also, the use of cell entrapment technique to lessen the effect was conducted. The work started with nitrifying activated sludge enrichment. The sludge was then entrapped using PVA and CA at small and large sizes. The nitrification activity test of the free and entrapped cells was performed. Lastly, the selected cells from the activity test were observed the physiological change using electron microscopic technique.

The tasks were divided into four parts followed experimental framework (Figure 3.1). It includes: 1) chemical and culture preparation, 2) nitrification experiment, 3) entrapped cell procedure, and 4) microscopic observation. Details of each part were described in the following sections.

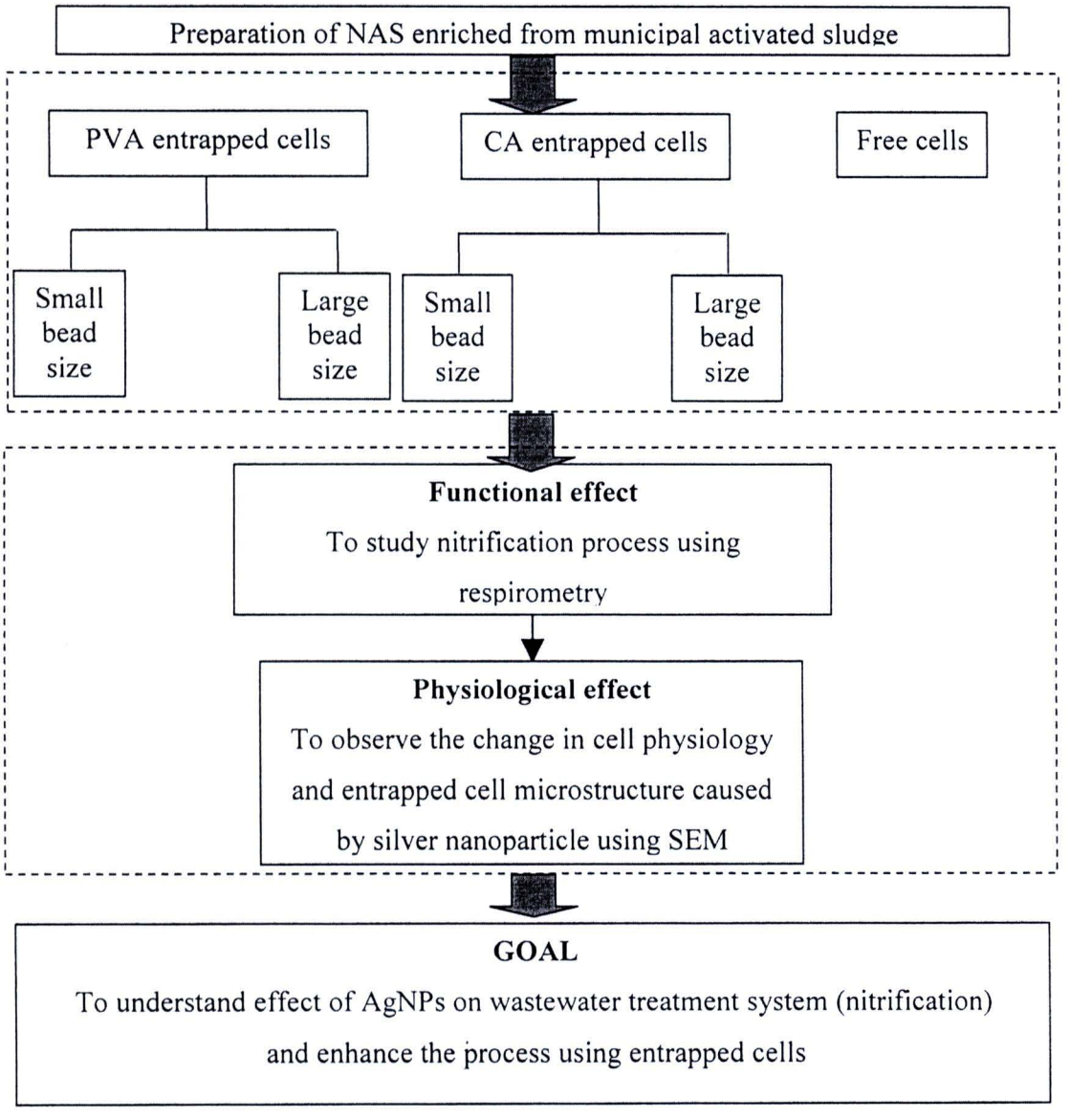

Figure 3.1 Experimental framework

3.2 Chemicals and culture preparation

3.2.1 Chemicals

Silver nanoparticles were obtained from Sensor Research Unit at Department of Chemistry, Chulalongkorn University, Thailand. The AgNPs were synthesized by reducing silver nitrate with sodium borohydride in the methyl cellulose solution followed previous reports (Hyning and Zukoski, 1998; Shirtcliffe *et al.*, 1999; Zhang *et al.*, 2000; Ngeontae *et al.*, 2009). The spherical AgNPs with an average size of 14 nm were applied in the study since it is a typical size of AgNPs widely utilized in industrial section.

Polyvinyl alcohol (99.0-99.8% fully hydrolyzed, molecular weight 77,000-79,000) from J.T. Baker (NJ, USA) and alginic acid sodium salt (for immobilization) from Sigma-Aldrich (Singapore) were purchased. Other chemicals including chemicals for cultural medium, cell entrapment, nitrification parameter analysis, and sample preparation for microscopic observation were laboratory grade obtaining from local distributers (Bangkok, Thailand).

3.2.2 Nitrifying activated sludge and cultural condition

Returned activated sludge from Siphraya municipal wastewater treatment plant, Bangkok, Thailand was taken and cultivated in two 12-L sequencing batch reactors (SBR) under aerobic condition for 1.5 months. The nitrifying activated sludge in these bioreactors was enriched at a hydraulic retention time (HRT) of 2 d and a solid retention time (SRT) of approximately 24 d. The compositions of growth medium were shown in Tables 3.1 and 3.2. The enriched cultures were verified nitrification ability before use. The nitrification ability verification procedure is shown in the following subsection.

Table 3.1 Composition of growth medium

Chemical	Concentration
$(NH_4)_2SO_4$	0.33 g/L
$NaHCO_3$	0.75 g/L
K_2HPO_4	2.1 g/L
$Na_2HPO_4 \cdot 2H_2O$	5.07 g/L
Inorganic salt	1 ml/L

Table 3.2 Composition of inorganic salt

Chemical	Concentration
$MgSO_4 \cdot 7H_2O$	40 g/L
$CaCl_2 \cdot 2H_2O$	40 g/L
KH_2PO_4	200 g/L
$FeSO_4 \cdot 7H_2O$	1.0 g/L
Na_2MoO_4	0.1 g/L
$MnCl_2 \cdot 4H_2O$	0.2 g/L
$CuSO_4 \cdot 5H_2O$	0.02 g/L
$ZnSO_4 \cdot 7H_2O$	0.1 g/L
$CoCl_2 \cdot 6H_2O$	0.002 g/L

3.2.3 Nitrification ability verification of enriched NAS

Nitrification ability verification was performed for two objectives. The first one was to confirm whether the enriched culture got acclimatized and well nitrified. The second objective was to confirm the stability of the NAS before application.

The verification of the enriched NAS was performed by measuring the reduction of ammonia and the production of nitrate in each SBR cycle. The ammonia and nitrate concentrations were plot and calculated for slope. The stable slope of all cycles showed the steady state of NAS.

3.2.3.1 Ammonia reduction test

The influent and effluent from the enriched reactor of 20 mL were taken and filtered. Determination of ammonia concentration was using potentiometric method followed standard method (Ammonia-Selective Electrode Method, Standard Method for the Examination of Water and Wastewater 20th edition). The ammonium probe (WTW GmbH, NH_4 500/2, Germany) were applied to measure ammonia concentration in 10 mL of the filtered sample (pH of 7-8). The concentration of ammonia from the influent and effluent were used for observing nitrification (ammonia oxidation) activity. The ammonia reduction rates were monitored. The enriched NAS was used after the rate being stable.

3.2.3.2 Nitrate production test

The filtered sample was not only diluted but also adjusted volume to 2 mL with DI. The diluted sample was measured the absorbance at 220 nm and 270 nm (to eliminate the dissolved organic matter) with UV visible spectrophotometers (Thermo Electron Corporation, Hexious α, Cambridge, UK) (Ultraviolet Spectrophotometric Screening Method, Standard Method for the Examination of Water and Wastewater, the 20th edition).

3.3 Nitrification experiment

3.3.1 Respirometer setup and operation

The duplicate experiments were conducted. A respirometer was built from a 250-mL flask with a screw cap coupled with the oxygen probe (WTW inoLab Oxi 730, WTW GmbH, Germany). The apparatus was set as shown in Figure 3.2. The enriched NAS was taken and centrifuged at 4,000 rpm for 5 min. Supernatant was discarded. The centrifuged NAS was resuspended in a washing solution (an inorganic salt solution) and vigorously mixed using a vertical shaker. The resuspended NAS was centrifuged. The washing solution was then discarded. The washed NAS was then rewashed with this manner for 4 times.

The respirometer setup was tested for reduction of dissolved oxygen concentration in solutions as follows: 1) synthetic wastewater only, 2) synthetic wastewater with entrapment matrices, 3) synthetic wastewater with AgNPs, and 4) synthetic wastewater with AgNPs and entrapment matrices. These tests were conducted to confirm whether dissolved oxygen concentration decreased because of the setup. In addition, the test of dissolved oxygen reduction by heterotroph was performed in synthetic wastewater without ammonia. The result is shown in the Table A.1. The result indicated that the factors which could affect oxygen concentration did not influence in the case. Therefore, the setup was appropriate for monitoring nitrification process.

For the free cells, the cleaned NAS was transferred into the respirometer. The synthetic wastewater containing with different ammonia and AgNP concentrations as shown in Table 3.3 was fully added into the respirometer to avoid oxygen diffusion into the reactor (285 mL). The concentrations of AgNPs at 0.05 from a realistic to high exposure scenario (Mueller et al., 2008), 0.5 from the maximum allowable from USEPA, 1, and 5 from the extreme concentration for the future mg/L were investigated. Note that the final mixture in the respirometer contained the cleaned NAS at volatile suspended solid (VSS) of 500 mg/L. The respirometer was operated and monitored every minute until dissolved oxygen (DO) concentrations reached stable.

For the entrapped cells, the cell entrapment procedures are shown in the following subsection 3.4. Entrapped cells of 3.5 g (1 g VSS/L) were used in a respirometer. The synthetic wastewater was prepared followed medium formulation (as shown in Table 3.3) except the components described in Tables 3.4 and 3.5. The beads were added into the respirometer. The respirometer was monitored in the same manner with the free cell systems.

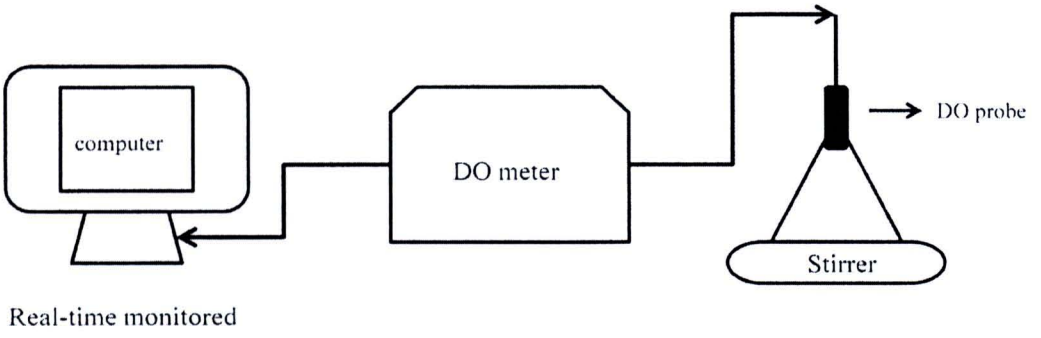

Figure 3.2 Respirometer setup

Table 3.3 Synthetic wastewater compositions for the free cells systems

No.	Test Name	AgNP concentration (mg/L)	NH_3 concentration (mg/L)
1	00528NP	0.05	28
2	0528NP	0.50	28
3	00570NP	0.05	70
4	0570NP	0.50	70
5	528NP	5.00	28
6	570NP	5.00	70

3.3.2 Oxygen Uptake Rate and Specific Oxygen Uptake Rate Calculation

Oxygen Uptake Rate was figured by DO value while SOUR was calculated by OUR and cell mass value in the respirometer. The rates were calculated as shown in equation 1 and 2. Note that the rates were calculated only during the log (rapid declining) period.

$$OUR = \frac{d[DO]}{dt} = Slope\ of\ DO\ value\ versus\ time\ graph \quad (1)$$

$$SOUR = \frac{OUR}{VSS} \quad (2)$$

3.4 Cell entrapment procedure

3.4.1 Calcium alginate entrapment

Sodium alginate at 2% (w/v) was dissolved in deionized water (DI). The enriched reactor mixture was centrifuged at 3,000-4,000 rpm for 3-5 min to acquire wet nitrifying bacteria cells. The wet cells were mixed with CA at ratio MLVSS of 2% (w/v). The CA-sludge mixtures were dropped into a calcium chloride solution of 3.5% (w/v) by a peristaltic pump (Masterflex L/S Tubing Pumps, Cole-Palmer Instrument Company, IL, USA) at a flow rate of 40 mL/min (bead sizes of 3 and 6 mm).

3.4.2 Polyvinyl alcohol entrapment

Polyvinyl alcohol at 10% (w/v) was well dissolved in DI (heated in water bath for 5 min). The enriched reactor mixture was centrifuged at 3,000-4,000 rpm for 3-5 min. The wet cells were mixed with PVA at ratio MLVSS of 2% (w/v). The PVA-sludge mixtures were dropped into a saturated boric acid solution by a peristaltic pump at a flow rate of 40 mL/min. The formed beads were transferred to 500 mL of 1 M sodium orthophosphate buffer (pH 7.0) for 1-2 hr. The beads were kept at 4 °C (bead sizes of 3 and 6 mm).

Tables 3.4 Composition of synthetic wastewater in respirometer for the CA-entrapped cell systems

No.	Name	Description	Components (mg/L)	
			AgNP	Others
1	NC	only AgNP test in respirometer	5	-
2	MNC1	only growth medium test	-	$Na_2B_4O_7 \cdot 10H_2O$ at 4.96 g/L instead of $Na_2HPO_4 \cdot {}_2H_2O$, H_3BO_3 at 0.8 g/L instead of K_2HPO_4, and KH_2PO_4 0.34 g/L
3	AGNC	only AgNP with the control (no cell) gel	5	Same as MNC1
4	AG00	growth medium with CA-entrapped cell	-	Same as MNC1
5	AG05	growth medium with CA-entrapped cell	0.5	Same as MNC1
6	AG10	growth medium with CA-entrapped cell	1	Same as MNC1
7	AG50	growth medium with CA-entrapped cell	5	Same as MNC1

Table 3.5 Composition of growth medium in respirometer for the PVA-entrapped cell systems

No.	Name	Description	AgNP (mg/L)
1	MNC2	only growth medium test	-
2	PVANC	only AgNP with the control (no cell) gel	5
3	PVA00	growth medium with PVA-entrapped cell	-
4	PVA05	growth medium with PVA-entrapped cell	0.5
5	PVA10	growth medium with PVA-entrapped cell	1
6	PVA50	growth medium with PVA-entrapped cell	5

3.5 Microscopic observation

3.5.1 Scanning electron microscopy (SEM) observation

3.5.1.1 Scanning electron microscopic sample preparation

The PVA-entrapped cells from respirometer were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for overnight at 4 °C. The beads were washed using phosphate buffer twice followed by DI for 10 min each. The fixed beads were dehydrated with ethanol 30%, 50%, 70%, and 90%, respectively for 10 min each. After that, the absolute ethanol was applied for 3 times for 10 min as the last step of dehydration. The dehydrated beads were critical point dried using a Critical Point Dryer (Balzers, CPD 020, Liechtenstein). Then, the beads were divided into two parts using razor blade in liquid nitrogen, attached to the stub by glue, coated with gold using an Ion Sputter (Balzers, SCD 040, Liechtenstein).

The CA-entrapped cells from respirometer were rinsed in 0.1 M $CaCl_2$ solution for 15 min twice. The rinsed beads were fixed with 2.5% glutaraldehyde in 0.1 M $CaCl_2$ for 1 hr. The fixed beads were washed in 0.1 M $CaCl_2$ solution for 15 min twice. Later, the washed beads were cut into two parts by ultramicrotome (Leica, CM 3000, Nussloch, Germany). After that, the beads were dehydrated with 30% ethanol and 0.07 M $CaCl_2$ solution, 50% ethanol and 0.05 M $CaCl_2$ solution, 70% ethanol and 0.03 M $CaCl_2$ solution, 90% ethanol and DI, and 100% ethanol, respectively. Then, the absolute ethanol was applied for 3 times for 10 min as the last step of dehydration. The dehydrated beads were critical point dried using a Critical Point Dryer (Balzers, CPD 020, Liechtenstein). Then, the beads were attached to the stub by glue, coated with gold using an Ion Sputter (Balzers, SCD 040, Liechtenstein).

3.5.1.2 Scanning electron microscopic observation procedure

The dried beads were observed using SEM with EDS Attachment (SEM-EDS) (JEOL, JSM-5410LV, Tokyo, Japan). The surface, surface edge and two levels of the internal parts of the beads were focused.

3.5.2 Transmission electron microscopy (TEM) observation

For the free cells, the NAS samples were transferred to a microcentrifuge tube of 2 mL and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for overnight at 4 °C. The fixed cell were washed using phosphate buffer twice followed by DI for 10 min each. The fixed cells were centrifuged at 2,000 rpm for 5-10 min. After that, the sediment was vigorously mixed and washed with 0.1 M phosphate buffer and leaved for 15-20 min. The washed cells were repeated in the same manner for 3 times. The buffer was discarded from the pellet by centrifugation at the same speed and vigorously mixed it with 1% osmium tetroxide in 0.1 M phosphate buffer. The mixed pellet was left in hood for 1-2 hr and centrifuged for disposing the supernatant. Then, the mixed pellet was washed with 0.1 M phosphate buffer and DI for 15-20 min each. The supernatant from the centrifugation of the washed cells was eliminated. The wet pellet was mixed with melt agar of 1.5% and formed the gel at 45-50°C. The hardened agar was cut as a cube of 0.5 mm^3. The cubic cells were dehydrated with 35%, 50%, 70%, and 95% of ethanol, respectively

for 15-20 min each. Later, the dehydrated cubic cells were consecutively dehydrated for 3 more times with absolute ethanol for 15-20 min each. The dehydrated cubic cells were saturated in propylene oxide for 15-20 min for 3 times. The saturated cubic cells were infiltrated overnight with a series of Spur resin and propylene oxide mixture at ratios of 1:3, 1:1, and 3:1 consecutively. After that the cells were infiltrated in Spur resin overnight for 3 times. Next, the infiltrated cells were baked at 70 °C for 8-10 hr. The baked cells were cut by Ultramicrotome with size between 60 and 90 nm. The cut cells were pasted into copper grid and stained with uranyl acetate and lead citrate for increasing the contrast.

For the entrapped beads, the bead samples from respirometer were fixed with the same manner as the preparation for SEM. The beads were washed by phosphate buffer twice and DI for 10 min each. Five washed beads were transferred to a microcentrifuge tube of 2 mL with DI water of 1.5 mL. Then cut the beads in small pieces and squeezed the microbial cells out of the matrices. After discarding the matrices, centrifuged supernatant at 4,000 rpm for 5 min and drained out the supernatant. The pellet was washed with DI for 4 times. After that, the washed cells were prepared in the same manner as the free cells. The stained cells were observed using TEM (JEOL, JEM-2100, Tokyo, Japan).