

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Nitrifying activated sludge acclimatization

Nitrifying activated sludge acclimatization was investigated to confirm the complete nitrification in the enriched NAS on the steady state before application. The enriched NAS has a hydraulic retention time at 2 days and sludge retention time at 18 days. Ammonia reduction and nitrate production were calculated as the efficiency of nitrification process. Base on the result in Figure 4.1, the efficiency of nitrification (based on ammonia removal) was approximately 90%. The ammonia reduction and nitrate production were similar. This obviously indicated that the NAS was acclimatized. The NAS performed full nitrification because most of ammonia transformed to nitrate at the end of the nitrification process.

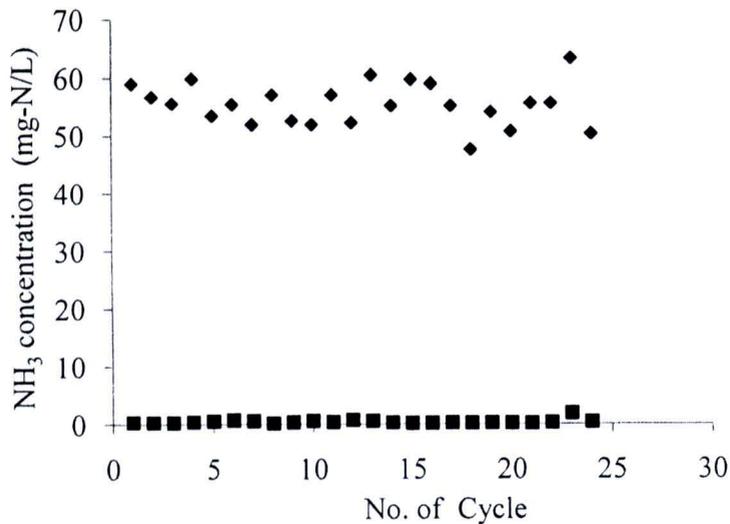


Figure 4.1 Ammonia concentrations during NAS acclimatization in: a) influent (◆) and b) effluent (■)

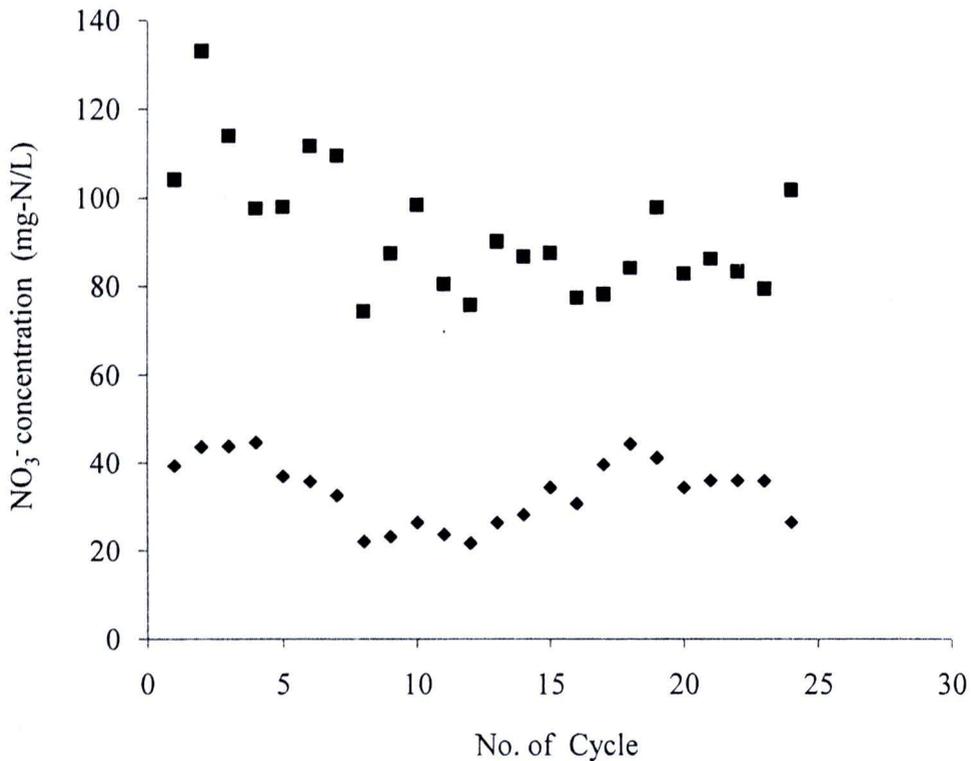


Figure 4.2 Nitrate concentrations during NAS acclimatization in: a) influent (◆) and b) effluent (■)

4.2 Nitrification activities by the free cells

4.2.1 Effect of initial ammonia concentration

Nitrification of the AgNP-contaminated wastewater at the ammonia concentrations of 28 and 70 mg-N/L was performed. The concentrations (28 and 70 mg-N/L) were selected to represent ammonia concentrations in municipal wastewater treatment and sludge digestion systems, respectively. The observed DO values from the tests with each AgNP concentration and ammonia concentrations of 28 and 70 mg-N/L were similar as shown in Figure 4.3-4.6. From the tests with AgNP concentrations of 0, 0.05 and 0.5 mg/L and ammonia concentrations of 28 and 70 mg-N/L (Test No. 1 to 3 and 5 to 7), DO quickly decreased during the first 40-100 min and reached steady state at approximately DO of 0 mg-O₂/L thereafter. On the contrary, DO of the tests with AgNP concentration of 5 mg/L and ammonia concentrations of 28 and 70 mg-N/L (Test No. 4 and 9) gradually. After testing for 200 min, DO of 3 and 7 mg-O₂/L still observed in the systems with the ammonia concentrations of 28 and 70 mg-N/L, respectively.

Based on the results in Figure 4.3-4.6, OURs and SOURs were calculated as shown in Table 4.1. The OUR and SOUR values indicated that the different ammonia concentrations (28 and 70 mg-N/L) did not play any role in nitrification process. Normally, the initial ammonia concentration influences nitrification (OUR and SOUR) rate. Higher ammonia concentration gives higher nitrification rate while too high ammonia concentration inhibits the rate. Phenomenon observed in this study could be because the tested ammonia concentrations did not much different resulting in similar nitrification rates. The result indicated that the ammonia concentration of lower than 70 mg-N/L did not inhibit nitrification in the AgNP-contaminated wastewater. Similarly, Kim et al. (2008) reported ammonia concentration of lower than 350 mg-N/L did not affect nitrification process.

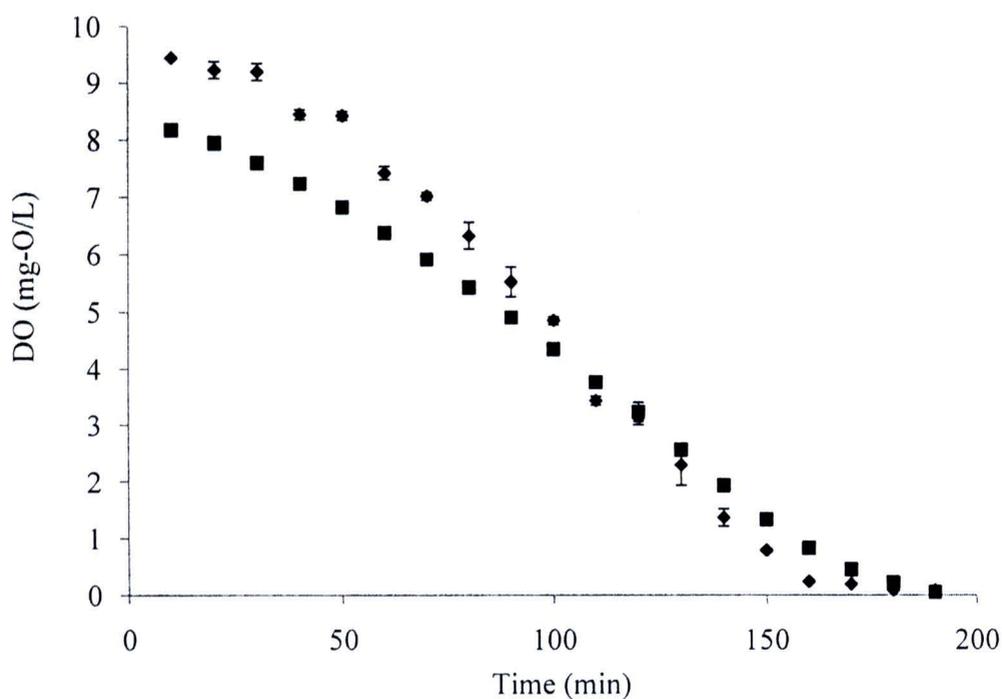


Figure 4.3 Dissolved oxygen concentrations from the tests at 28 (◆) and 70 (■) mg-N/L with AgNP concentrations of 0 mg/L

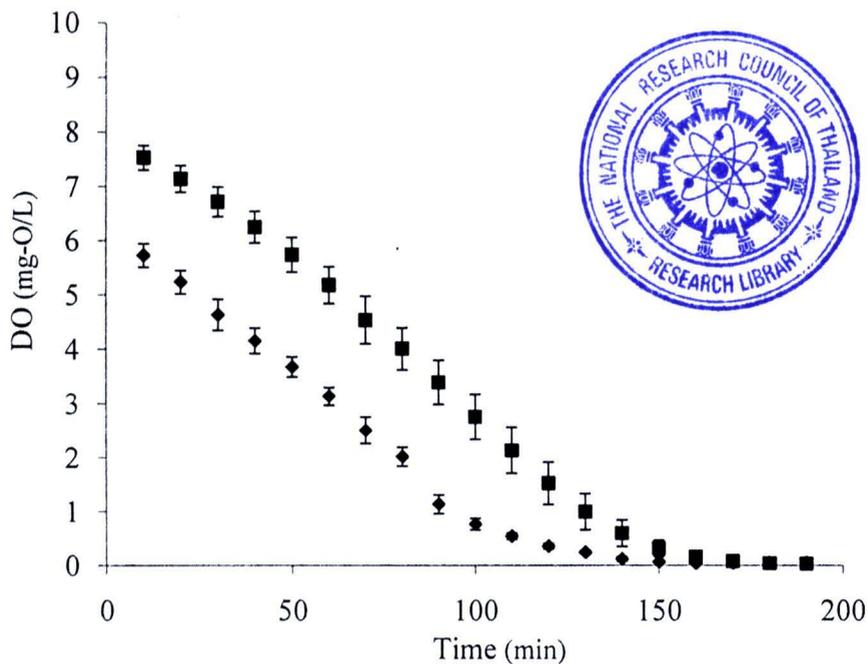


Figure 4.4 Dissolved oxygen concentrations from the tests at 28 (◆) and 70 (■) mg-N/L with AgNP concentrations of 0.05 mg/L

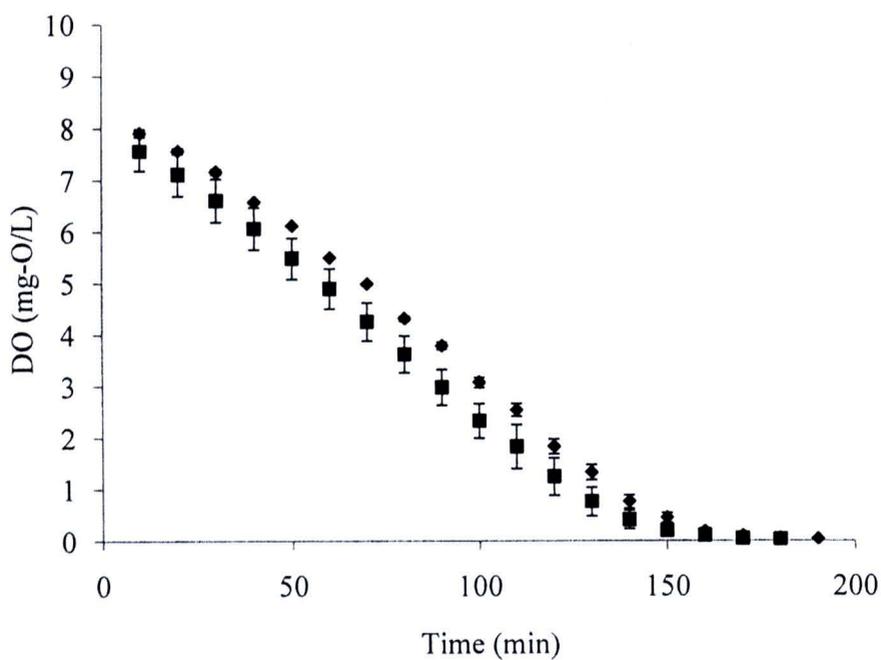


Figure 4.5 Dissolved oxygen concentrations from the tests at 28 (◆) and 70 (■) mg-N/L with AgNP concentrations of 0.5 mg/L

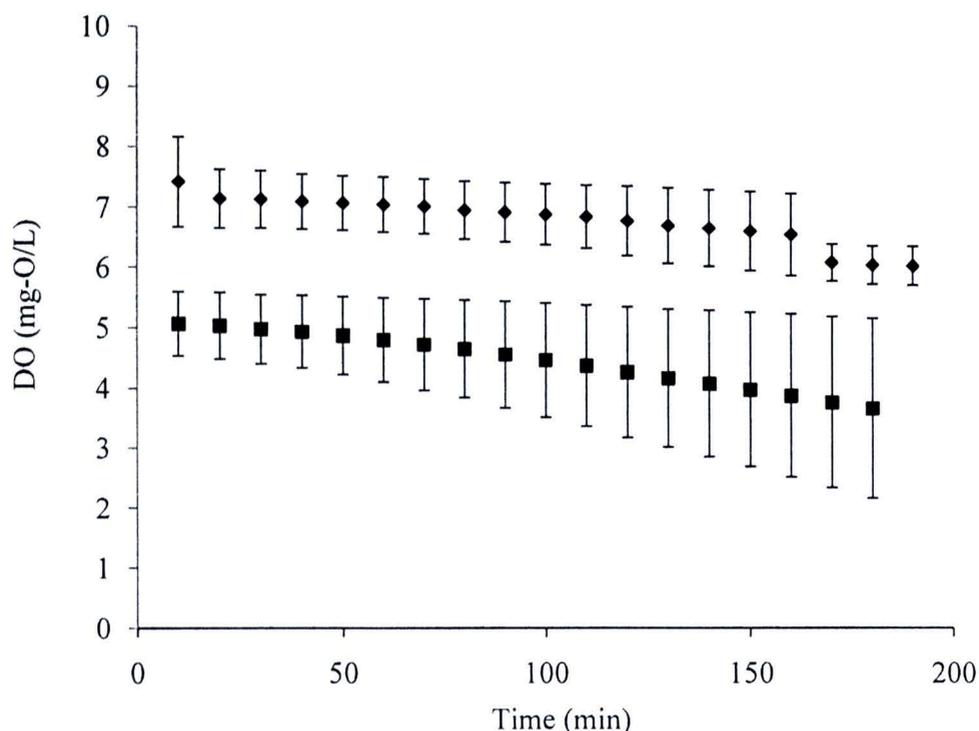


Figure 4.6 Dissolved oxygen concentrations from the tests at 28 (◆) and 70 (■) mg-N/L with AgNP concentrations of 5 mg/L

4.2.2 Effect of initial silver nanoparticles concentration

Nitrification of the AgNP-contaminated wastewater at different AgNP concentrations were performed (Figures 4.7 and 4.8). Figures 4.7 and 4.8 present similar results. The DO concentrations of the tests at the AgNP concentrations of 0, 0.05, and 0.5 mg/L were similar. The DO values dramatically decreased during the first hour to accomplished DO of 0 mg-O₂/L. On the other hand, DO at the AgNP concentration of 5 mg/L was slightly reduced and did not reach steady state. It is also noticed that the tests at the AgNP concentration of 5 mg/L were much more time-consuming. Based on the OUR and SOUR results in Table 4.1, it is obvious that different AgNP concentrations played an important role in nitrification process. The rates of the tests at AgNP concentrations of 0.05, 0.5, and 1 mg/L were nearly the same (OUR of 0.011 to 0.016 mg-O₂/L/min and SOUR of 0.21 to 0.44 mg-O₂/g VSS/min) while the rates of the tests at AgNP concentration of 5 mg/L were 50-times less than those from other tests. This indicated that AgNPs apparently inhibited nitrification process. However, the concentrations of AgNPs also influenced nitrification differently. It could imply that threshold concentration of AgNPs to NAS

and nitrification process in this study may be between 1 and 5 mg/L. Similarly, Çeçen et al. (2010) concluded that the increasing of heavy metal concentration inhibited the OUR activity in nitrifying bacteria. According to Choi et al. (2008), the concentration of AgNPs of 1 mg/L had the inhibition percentages of 86% on respiration. The study also reported that the inhibition might be from the toxicity to the cell metabolism but the cells did not get killed. In later study by Choi and Hu (2008), they confirmed that AgNPs inhibited microbial cells since the intracellular reactive oxygen species (representing damage in the cells) were observed. However, the toxicity mechanism is still inconclusive.

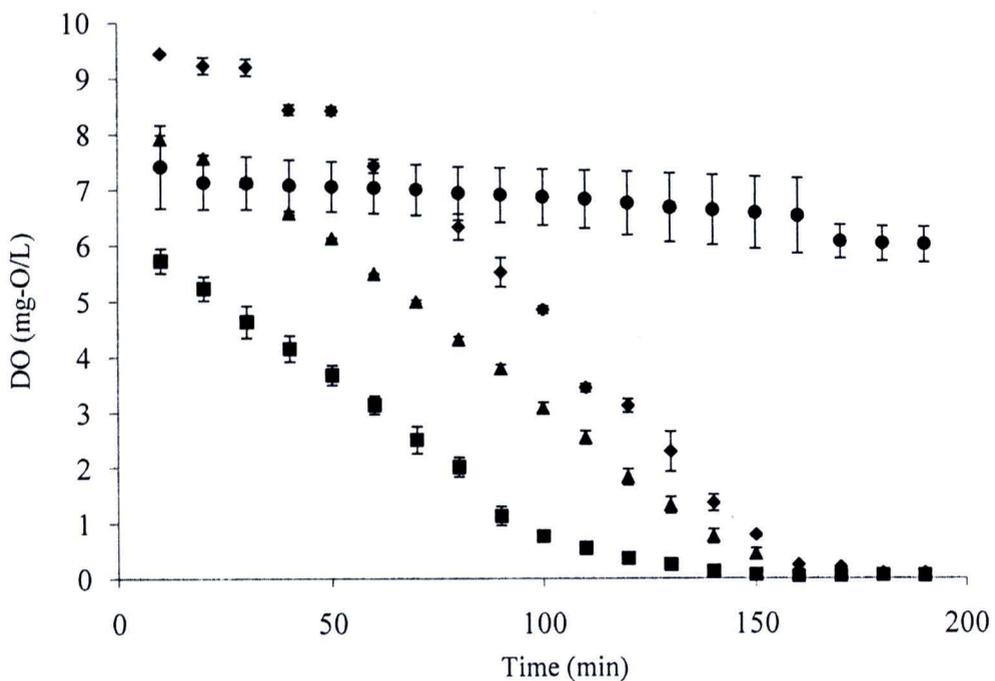


Figure 4.7 Dissolved oxygen concentrations from the tests at 28 mg-N/L with AgNP concentrations of 0 mg/L (◆), 0.05 mg/L (■), 0.5 mg/L (▲), and 5 mg/L (●)

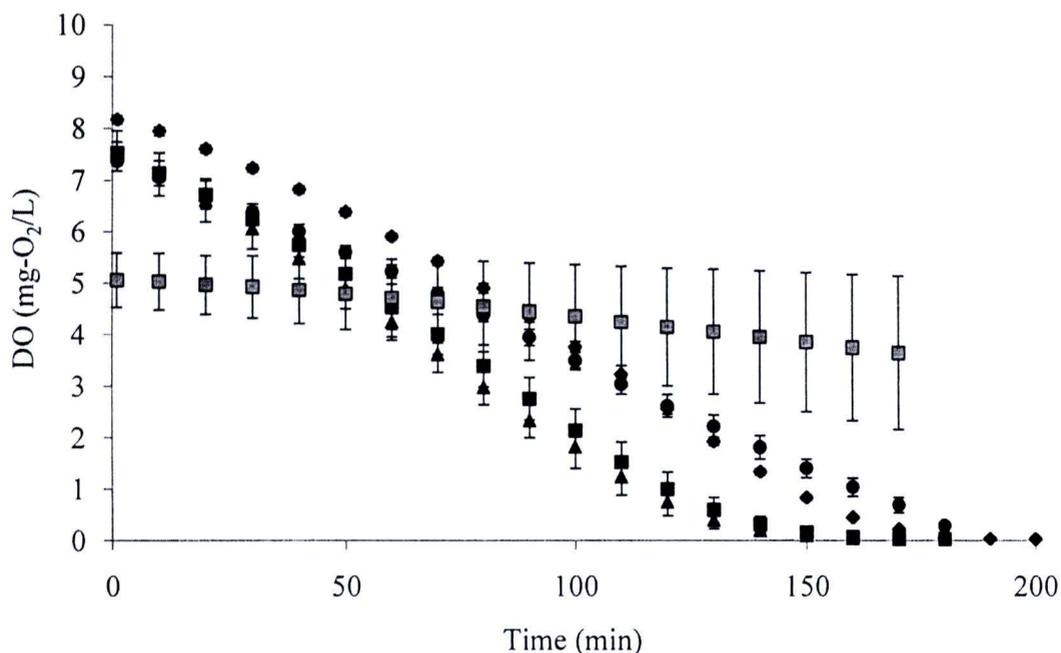


Figure 4.8 Dissolved oxygen concentrations from the tests at 70 mg-N/L with AgNP concentrations of 0 mg/L (◆), 0.05 mg/L (■), 0.5 mg/L (▲), 1 mg/L (●), and 5 mg/L (□)

Table 4.1 Oxygen uptake rates and specific oxygen uptake rates

No.	Test name	NH ₃ concentration (mg-N/L)	AgNP concentration (mg/L)	OUR (mg O ₂ /L/min)	SOUR (mg O ₂ /g VSS/min)	Activity (% compared to control)
1	028NP	28	0.00	0.1675	0.4437	100.0000
2	00528NP	28	0.05	0.1649	0.4368	98.4449
3	0528NP	28	0.50	0.1197	0.3171	71.4672
4	528NP	28	5.00	0.0038	0.0101	2.2763
5	070NP	70	0.00	0.1455	0.3854	100.0000
6	00570NP	70	0.05	0.1359	0.3600	93.4094
7	0570NP	70	0.50	0.1196	0.3168	82.2003
8	170NP	70	1.00	0.0822	0.2178	56.5127
9	570NP	70	5.00	0.0058	0.0152	3.9440

Note: The average initial VSS was 0.3775±0.1 mg-VSS/L.

4.3 Nitrification activities by the entrapped cells

Nitrification by two types (PVA and CA) and two sizes (3 and 6 mm in diameters) of entrapped cells were investigated. Nitrification activity tests of the AgNP-contaminated wastewater at different AgNP concentrations (0, 0.5, 1, and 5 mg/L) were performed. Note that based on the tests by the free cells, only the tests with ammonia concentration of 70 mg-N/L were selected.

4.3.1 Polyvinyl alcohol-entrapped cells

4.3.1.1 Small PVA-entrapped cells

Nitrifications of small (3 mm) PVA-entrapped cells in wastewater at different AgNP concentrations were performed (Figure 4.9). The DO concentration trends of the tests at the AgNP concentrations of 0, 0.5, and 1 mg/L were different. In the control test (AgNPs of 0 mg/L), the DO values continuously declined and accomplished stable within 25 hr. On contrarily, DO from the tests with the AgNP concentration of 0.5 mg/L was gradually decreased while DO from the tests with the AgNP concentration of 1 mg/L remained steady for entire of the experiment. In overall, it is noticed that the tests by the small PVA-entrapped cells took longer time for nitrification compared to the free cells. This could be from the limitation of substrate and oxygen diffusion into the entrapped cells (Siripattanakul et al., 2008).

The OUR values and activity were calculated as shown in Table 4.2. The OUR of the tests at AgNP concentrations of 0.5 and 1 mg/L were 0.0003 to 0.0006 mg-O₂/L/min, respectively. It indicated that the AgNP concentrations influenced on nitrification activities by the small PVA-entrapped cells. Interestingly, the results by the small entrapped cells were poorer than the free cells. When applying AgNPs into wastewater, nitrification activities by the entrapped cells and free cells were approximately 4-10 and 2-98% of the control, respectively. It is inconclusive but this could be from two reasons. The first reason could be from the entrapment environment having limitation of diffusion as stated earlier. This situation led to poorer nitrification performance. Another reason could be because entrapment environment initiate or promote toxicity of AgNPs.

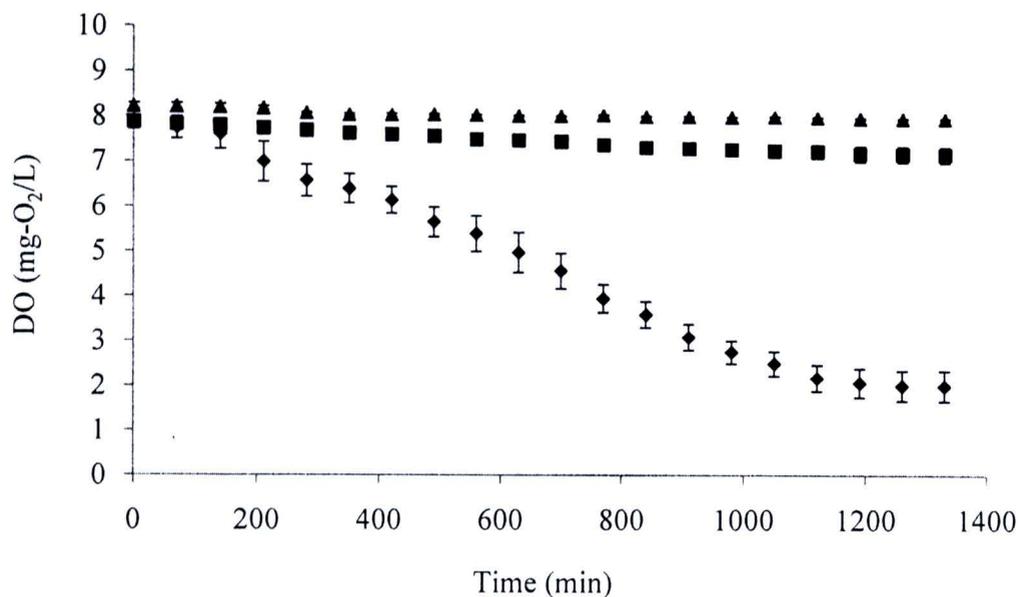


Figure 4.9 Dissolved oxygen concentrations from small PVA-entrapped cell tests with AgNP concentrations of 0 mg/L (◆), 0.5 mg/L (■), and 1 mg/L (●)

4.3.1.2 Large PVA-entrapped cells

The tests of nitrifications on the large (6 mm) PVA-entrapped cells at the different concentration of AgNPs (Figure 4.10). The DO concentration trends of the AgNP concentrations of 0, 0.5, 1, and 5 mg/L were similar. The DO values gently decreased and reached stable in 8 hr. Table 4.2 presents the OUR and activity of the large PVA-entrapped cells which the rates were in range of 0.0060-0.014 mg-O₂/L/min. Higher AgNP concentrations provided lower nitrification performance. As expected, the large PVA-entrapped cells can reduce the toxic effect of AgNPs on the nitrification process (Dwyer et al., 1986; Keweloh et al., 1989; Keweloh et al., 1990). This could be because the large entrapped cells have wider layer of matrix leading to protecting the microorganisms away from AgNPs.

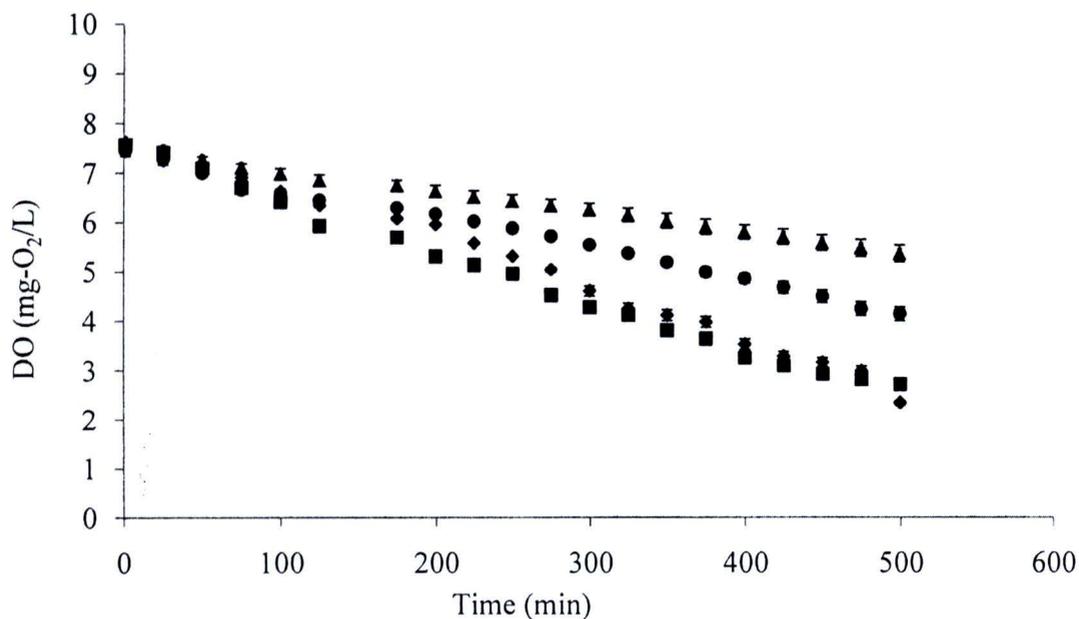


Figure 4.10 Dissolved oxygen concentrations from large PVA-entrapped cell tests with AgNP concentrations of 0 mg/L (◆), 0.5 mg/L (■), 1 mg/L (▲), and 5 mg/L (●)

Table 4.2 Oxygen uptake rate from PVA-entrapped cells

No.	Test name	Bead size (mm)	AgNP concentration (mg/L)	OUR (mg O ₂ /L/min)	Activity (% compared to control)
1	SPVA0	3	0	0.0056	100.0000
2	SPVA05	3	0.5	0.0006	10.8108
3	SPVA1	3	1	0.0003	4.5045
4	LPVA0	6	0	0.0144	100.0000
5	LPVA05	6	0.5	0.0126	87.5000
6	LPVA1	6	1	0.0118	81.9444
7	LPVA5	6	5	0.0064	44.0972

4.3.2 Calcium alginate-entrapped cells

4.3.2.1 Small CA-entrapped cells

Nitrifications of the AgNP-contaminated wastewater at different AgNP concentrations in small CA-entrapped cells were presented in Figure 4.11. The DO concentrations of the tests at the AgNP concentrations of 0, 0.5, 1, and 5 mg/L were similar. The DO values gradually reduced during the first 13 hr to accomplish the stable; however, only DO of the tests at the AgNP concentrations of 0 and 0.5 mg/L reached to 0 mg-O₂/L. Similar to the PVA-entrapped cells, it is noticed that the tests by the CA-entrapped cells took longer time compared to the free cells. This was because of the oxygen diffusion limitation from outside of the bead into the cells. Based on the OUR and activity results in Table 4.3, the rates of the tests at AgNP concentrations of 0, 0.5, 1, and 5 mg/L were 0.0090 to 0.0139 mg-O₂/L/min. The nitrification activities in the tests with AgNPs were 64-109% of control. Silver nanoparticles affected nitrification performance. Higher AgNP concentrations resulted in lower nitrification activities.

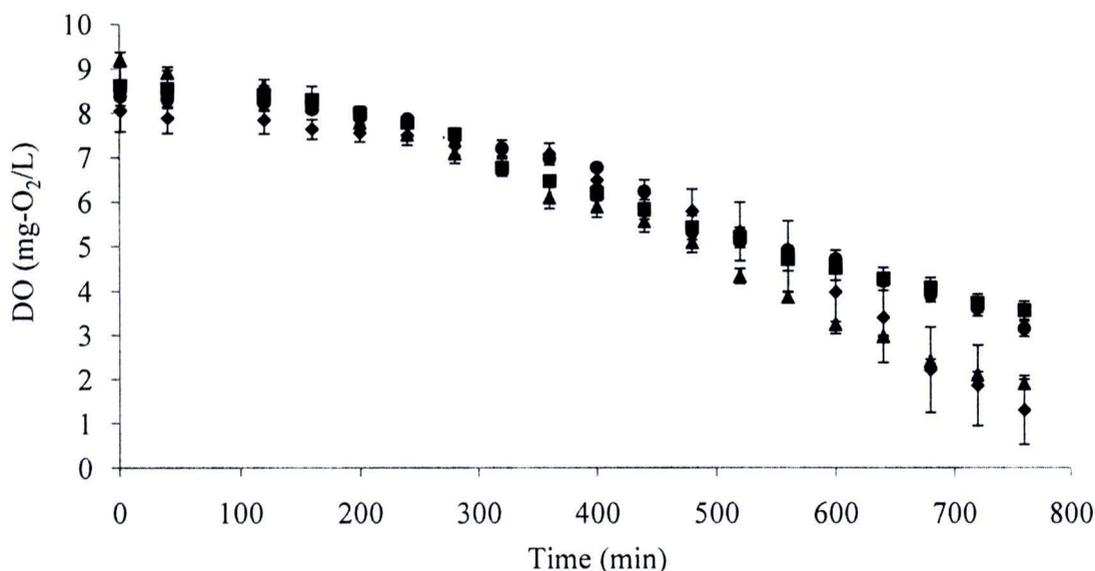


Figure 4.11 Dissolved oxygen concentrations from small CA-entrapped cell tests with AgNP concentrations of 0 mg/L (♦), 0.5 mg/L (■), 1 mg/L (▲), and 5 mg/L (●)

4.3.2.2 Large CA-entrapped cells

The tests of nitrifications on large CA-entrapped cells at the different concentration of AgNPs were determined (Figures 4.12). The DO concentration trends of the AgNP concentrations of 0, 0.5, 1 and 5 mg/L were similar. The DO values continuously decreased and remained stable within 7 hr. Table 4.3 presents OUR and nitrification activity of large CA entrapped cells, the rates of the entrapped cells at each AgNP concentrations were in range of 0.0194-0.0240 mg-O₂/L/min. The nitrification activities in the tests with AgNPs were 80-93% of control. Silver nanoparticles affected nitrification performance. Higher AgNP concentrations resulted in lower nitrification activities.

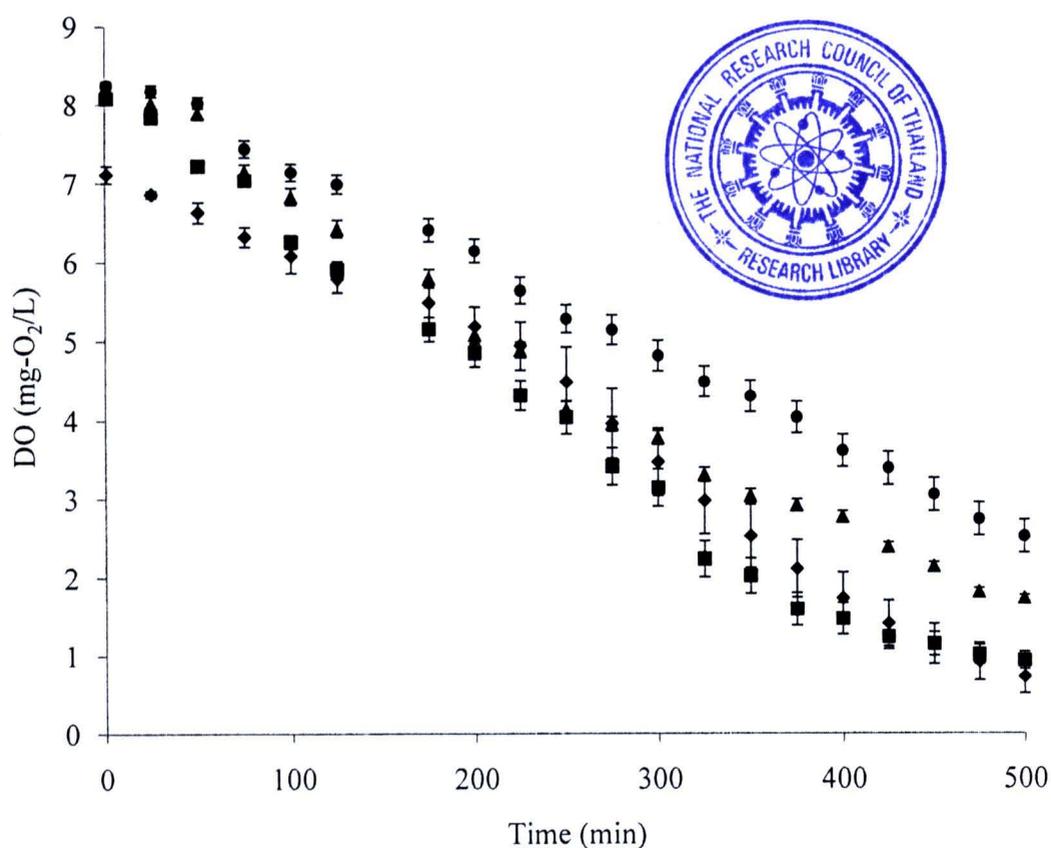


Figure 4.12 Dissolved oxygen concentrations from large CA-entrapped cell tests with AgNP concentrations of 0 mg/L (◆), 0.5 mg/L (■), 1 mg/L (▲), and 5 mg/L (●)

Table 4.3 Oxygen uptake rate from CA-entrapped cells

No.	Test name	Bead size (mm)	AgNP concentration (mg/L)	OUR (mg-O ₂ /L/min)	Activity (% compared to control)
1	SCA0	3	0	0.0139	100.0000
2	SCA05	3	0.5	0.0125	89.5683
3	SCA1	3	1	0.0153	109.7122
4	SCA5	3	5	0.0090	64.3885
5	LCA0	6	0	0.0240	100.0000
6	LCA05	6	0.5	0.0225	93.7500
7	LCA1	6	1	0.0201	83.7500
8	LCA5	6	5	0.0194	80.6250

4.3.3 Comparison of nitrification activities of PVA- and CA-entrapped cell

As a result from Table 4.4, the CA-entrapped cells showed better performances on the nitrification activity than those of the PVA-entrapped cells. The activities of the large CA-entrapped cells in the tests with AgNP concentration at 5 mg/L were twice higher than those of the PVA-entrapped cells. This indicated that CA entrapment provided better environment for microorganisms resulting in higher nitrification performances. Likewise, Yan and Hu (2009) compared ammonia and nitrite removal by CA and PVA entrapped cells. They found that CA gave better ammonia and nitrite removal. This is because CA had better adsorption capacities than PVA. In addition, CA entrapment might provide better environment for microorganisms. The insight information on better protection from toxic substance in CA matrices needs to be continued.

Moreover, Yao et al. (2010) reported that bifunctionalized AgNPs could react with monovalent and divalent ions, such as Co^{2+} , Fe^{2+} , Mn^{2+} , Hg^{2+} , Ni^{2+} , Cd^{2+} , Cu^{2+} , Zn^{2+} , Pb^{2+} , Li^+ , Na^+ , K^+ , and Cs^+ leading to the change in color. In this study, the CA-entrapped cells may release Ca^{2+} during the application. The reaction may change the AgNP physical property (color) which also may relate AgNP toxicity.

4.3.4 Comparison of nitrification activities of small and large entrapped cells

There was an assumption which can conclude from small and large entrapped cells. Better performance of the nitrification on the large entrapped cells was accomplished (Table 4.4). This could be from the large entrapped cells had thicker matrices resulting in lower contact of AgNPs to the microbial cell leading to lower cell damage. Consequently, the large entrapped cells performed better nitrification than the small entrapped cells. It was found in several previous studies that small beads attributed better mass and gas transfer (Aksu and Bülbül, 1999; Dursun and Tepe, 2005; Partil and Karegoudar, 2005). This also included to toxic substance as AgNPs; hence, the particle inhibited to the small entrapped cells more than the large one.

Table 4.4 Comparison of oxygen uptake rate between PVA- and CA-entrapped cells

AgNP concentration (mg/L)	Nitrification activity (% of control)				
	PVA		CA		Free cells
	Small	Large	Small	Large	
0	100.0000	100.0000	100.0000	100.0000	100.0000
0.5	10.8108	87.5000	89.5683	93.7500	82.2003
1	4.5045	81.9444	109.7122	83.7500	56.5127
5	-	44.0972	64.3885	80.6250	3.9440

4.4 Comparison of nitrification process by free and entrapped cells

From Table 4.4, it was obvious on better efficiency of nitrification activity by entrapped cells except the tests by the small PVA-entrapped cells. The nitrification activity percentage of the small PVA-entrapped cells was lower than free cells may be because of less oxygen diffusion in the entrapped cells as discussed earlier. The other cases (large PVA, small CA, and large CA), the activities from the entrapped cells were higher than free cells at the high concentration of AgNPs, even though the cells had limitation of oxygen diffusion. This obviously illustrated that the treatment efficiency from the entrapped cells performed better than free cells (Scott, 1987; Cassidy et al., 1996).

This study aimed to investigate the potential of the entrapped cells in nanoparticles contaminated wastewater treatment. It was found that in most of the cases, the entrapped cells successfully performed. However, it was observed about entrapped bead damage after application (data not shown), the further studies on matrix modification were required for real practice. The modification may focus on bead durability. Similar scheme, some previous studies modified the entrapped cells to promote biological oxidation capacity of Fe^{2+} on *Acidithiobactillus ferrooxidans* entrapped cells by mixing PVA and CA matrices (Yujian et al., 2007) and increase the physical strength of sodium alginate gel by barium alginate (Paul and Vignais, 1980).

4.5 Scanning electron microscopic observation

The morphologies of the PVA- and CA-entrapped cells were observed. The observation locations of entrapped cells were shown in Figure 4.13. There were four locations to observe the bead structure in each entrapped cells which were presented in Figure 4.13.

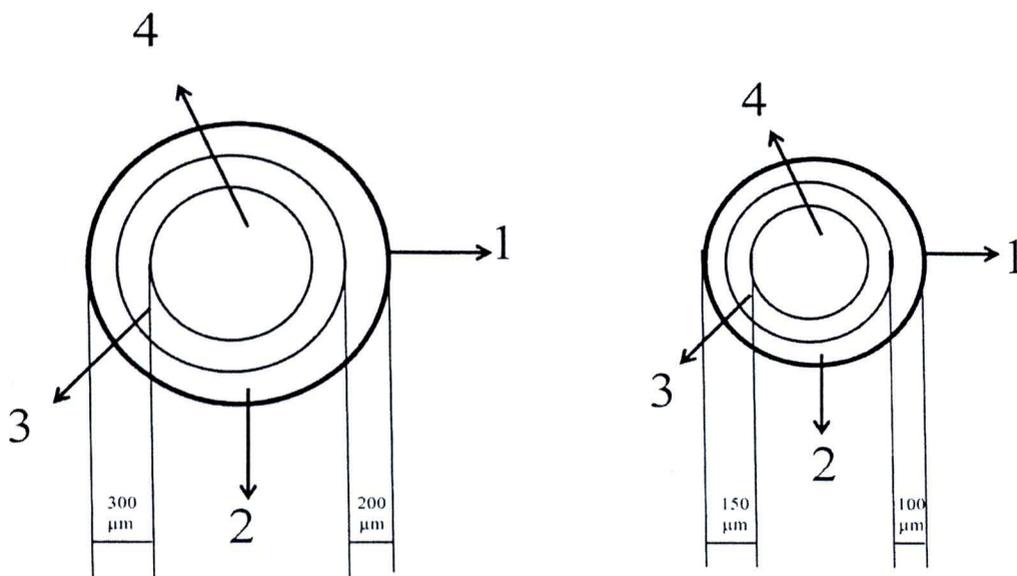


Figure 4.13 Observed location of the entrapped cells (1-surface of the bead, 2-second location (cross-sectional outer layer), 3-third location (cross-sectional inner layer), and 4-fourth location (cross-sectional core layer))

4.5.1 Polyvinyl alcohol-entrapped cells

The overview structure of the PVA-entrapped cells was observed (Figures 4.14 to 4.15). From Figure 4.14a, there were plenty of pores and smooth surface. The pores were quite uniform. The pore sizes ranged between 10 to 20 μm . The microorganisms occupied the pores as shown in Figures 4.14b. Moreover, the surface of the beads looked rough because of the microorganisms. Interestingly, from Figure 4.15, there was an agglomeration of AgNPs with PVA (Figure 4.15b and 4.15c). Similarly, Kim et al., (2006) synthesized AgNPs using the technique containing PVA; they also have the agglomeration between AgNPs and PVA. This observation supported the nitrification activity test that better nitrification performance by the PVA entrapped cells (compared to the free cells) was from the matrices physically

protected the microbial cells from AgNPs. The matrices worked as a wall to reduce opportunity of AgNP-cell contact. The matrices also combined with AgNPs and adhered on its net structure (Figure 4.15c). After testing, it was found a large amount of microorganisms, such as filamentous and rod colonies. However, the SEM observation could not really say whether the cells were alive. The continued work on the cell viability was recommended.

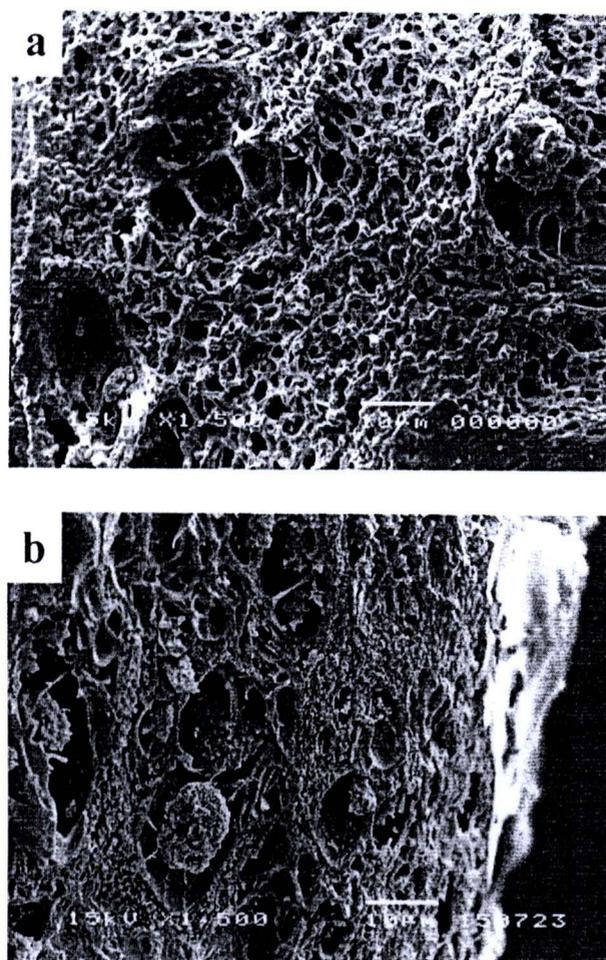


Figure 4.14 SEM images of the PVA-entrapped cells; a) only matrix (no microbial cell) at the second location (1,500 \times) and b) bead at the second location (1,500 \times)

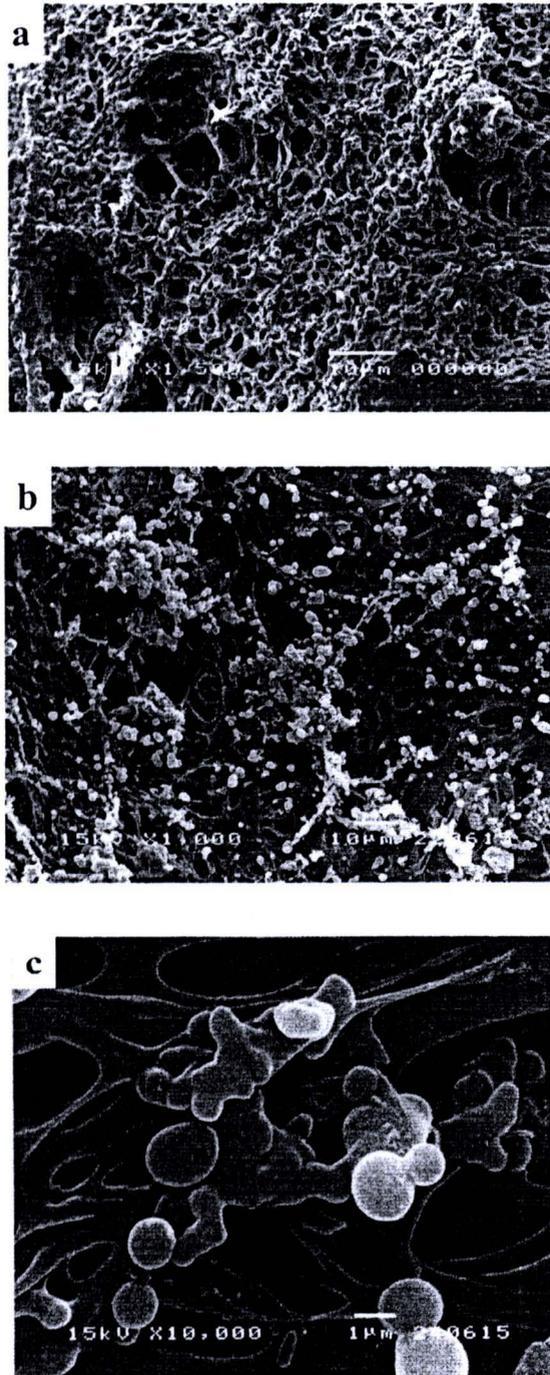


Figure 4.15 SEM images of the large PVA-entrapped cells a) only matrix (no microbial cell) at the second location (1,500 \times), b) bead from the test with 1 mg/L of AgNPs at the second location (1,000 \times), and c) bead from the test with 1 mg/L of AgNPs at the fourth location (10,000 \times)

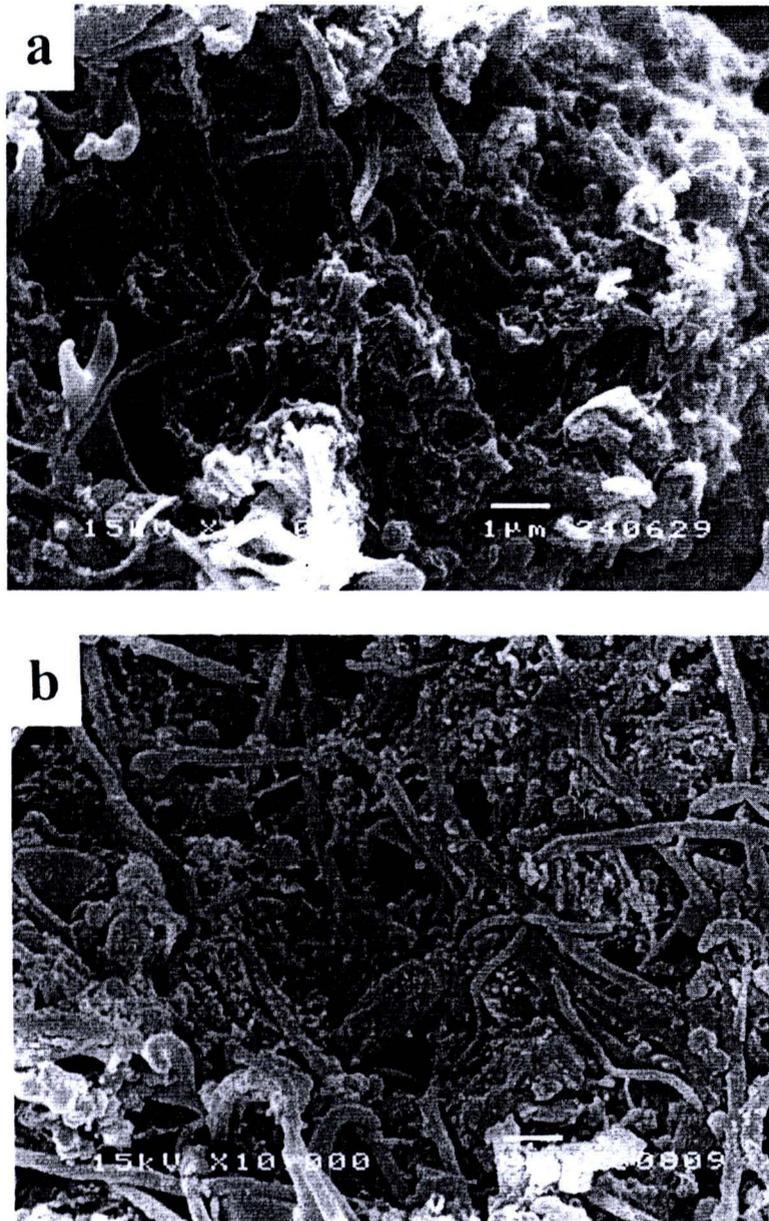


Figure 4.16 SEM images of the small PVA-entrapped cells; a) bead from the test with 0 mg/L of AgNPs and b) bead from the test with 1 mg/L of AgNPs from the forth location (10,000×)



4.3.2 Calcium alginate-entrapped cells

The structure diagrams of CA-entrapped cells were monitored (Figures 4.17 to 4.18). From Figure 4.17a, the surface layer was smooth as a sheet of paper, which was differed from Figure 4.17b. The surface of layer was rough and full of microorganisms. The pore sizes of CA matrices were less than 1 μm , therefore; the sheet structure was formed. Even though the CA matrices did not bind with AgNPs as occurring in the PVA matrices, the nitrification performance was high (even higher than the PVA entrapped cells). This might be from the CA structure (much smaller pores). Therefore, it could say that the protection of microbial cells in the entrapment matrices was mainly by physical protection from the matrix microstructure. The microorganisms in CA-entrapped cells were monitored. (Figure 4.18). Similarly to PVA-entrapped cells, it was found numerous microorganisms, such as filamentous and rod colonies.

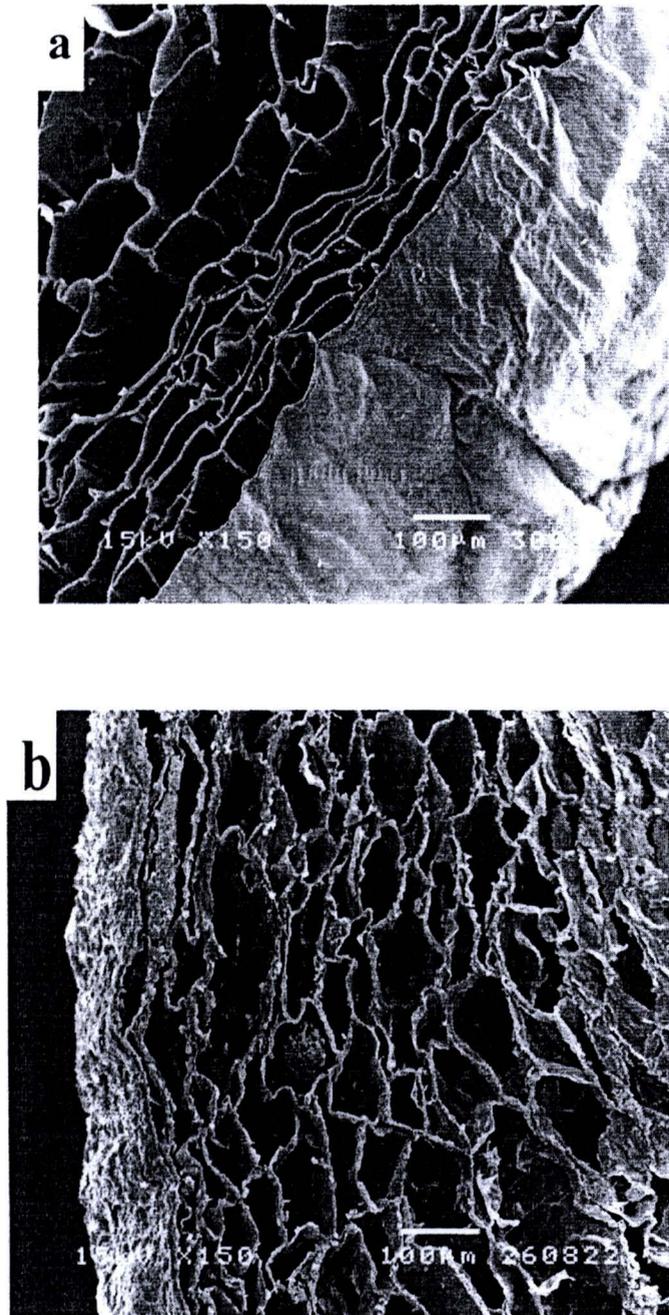


Figure 4.17 SEM images of the CA-entrapped cells; a) only matrix (no microbial cell) and b) bead before apply into the respirometer at the second location (150×)



Figure 4.18 SEM images of the small CA-entrapped cells; a) bead from the test with 0 mg/L of AgNPs at the second location, b) bead from the test with 0.5 mg/L of AgNPs at the third location, c) bead from the test with 1 mg/L of AgNPs at the fourth location, and d) bead from the test with 5 mg/L of AgNPs at the third location (10,000 \times)

4.6 Transmission electron microscopic observation

The spherical synthetic AgNPs at 14 nm were investigated (Figure 4.19). The free cells and cells separated from PVA entrapment after treating with AgNPs were observed using TEM (Figures 4.20 and 4.21). The free cell images are in Figures 4.20a and 4.20b while the images of cells separated from the PVA entrapment are in Figure 4.21a and 4.21b. It is obviously that AgNPs could penetrate into the microbial cells (Figure 4.20-4.21). The result was similar to previous studies that nanoparticles

including AgNPs went through cell membrane and wall causing cell damage (Andrä et al., 2008; Wu et al., 2010).

The results from the TEM images indicated that cell membrane and wall damaged after treating in wastewater contaminated with AgNPs. The free cell that cell membrane and wall were noticeably damaged whereas membrane and wall of cells separated from PVA entrapment were slightly injured (Figure 4.20-4.21). In addition, it was found that the microbial cells were ruined and broken inside (Figure 4.20b). This is similar to previous studies that reported about cell breakage after treating in toxic substances (Andrä et al., 2008; Wu et al., 2010). The toxic substance attached on cell membrane and wall attributing to their abrasion (soft edge). After that, outer layer was detached. The cytoplasm then moved out as white area in the microbial cells as shown in Figure 4.21a.

According to Choi and Hu (2008), they found that the cell presented the damage inside. The intracellular oxygen reactive species was investigated. The result from this study also well correlated. In previous study, the damage inside the cells was proved by molecular biological approach while in this study the result was discovered by physiological approach. However, in previous study, cell membrane damage which was determined using fluorescent staining technique did not observed. In the present study, it is apparent that the cell membrane and wall got damage. This could be from the interference in analytical technique in the previous study, difference in AgNP characteristics, or difference in tested condition and environment.

Based on the TEM images, it is clear that cell entrapment can lessen the opportunity of the cell damage by toxic substance. The result was well supported the nitrification activities discussed earlier. Silver nanoparticles cause cell damage and affect cell functionality. However, the result can only preliminarily indicated that AgNPs affected on nitrification and microorganisms, the result could not clearly conclude about effect on cell viability and community change. The continued works on effect of AgNPs on cell viability and community change were needed for better understanding.

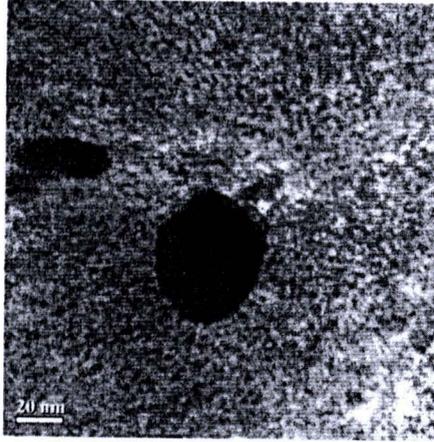


Figure 4.19 TEM images of the spherical synthetic AgNPs

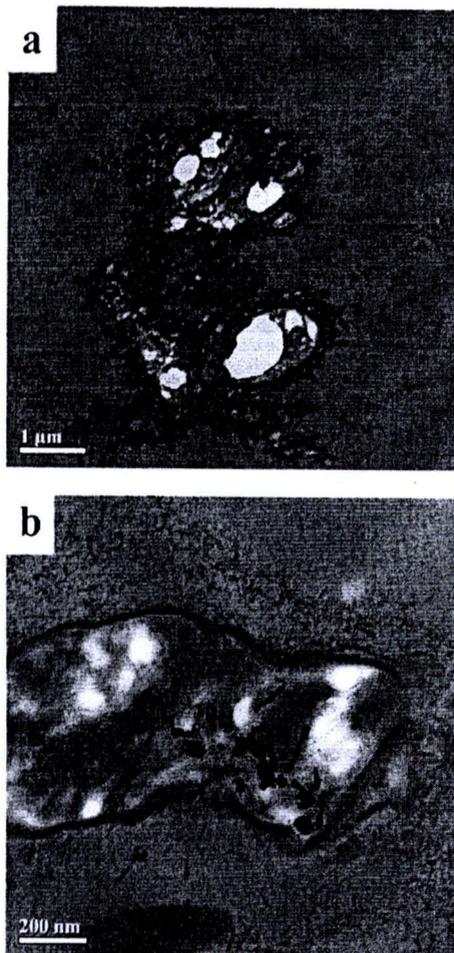


Figure 4.20 TEM images of free cells treated from the test with 5 mg/L of AgNPs

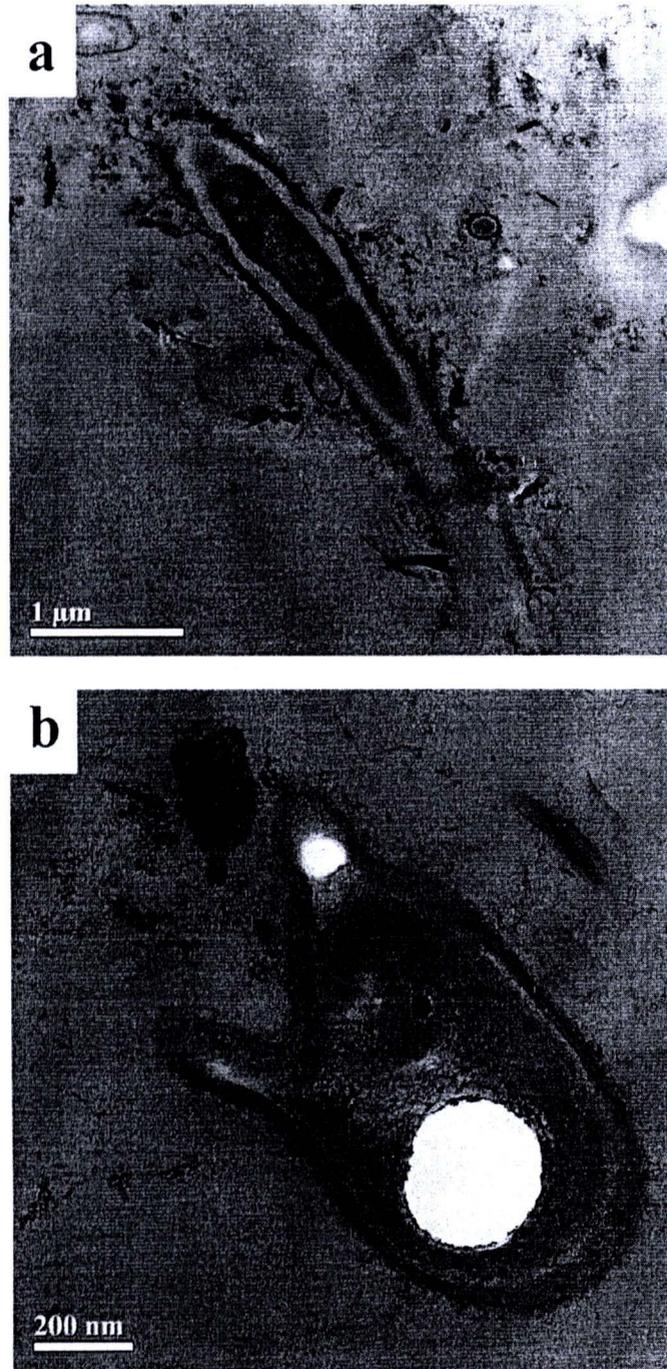


Figure 4.21 TEM images of separated cells from PVA entrapped cells treated from the test with 5 mg/L of AgNPs