ผลกระทบของซิลเวอร์อนุภาคนาโนต่อกระบวนการไนตริฟิเคชั่นในเซลล์ดักติด

นางสาวชุติมา พลอยจันทร์กุล

EFFECT OF SILVER NANOPARTICLES ON NITRIFICATION
BY ENTRAPPED CELLS

Miss Chutima Ploychankul

A Thesis Submitted in Partial Fulfillment of the Requirements

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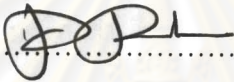
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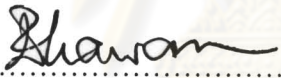
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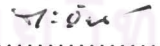
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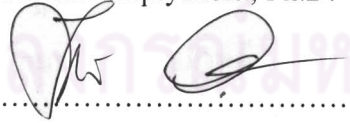
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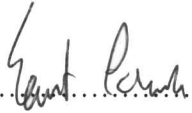
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ชุติมา พลอยจันทร์กุล : ผลกระทบของซิลเวอร์ขนาดนาโนต่อกระบวนการไนตริ
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การศึกษาเป็นการศึกษาผลกระทบของซิลเวอร์อนุภาคนาโน (AgNP) ต่อกระบวนการ
ไนตริฟิเคชันโดยใช้เซลล์ดักติด   การศึกษาประกอบด้วยการศึกษาผลของ AgNP ต่อ
สมรรถนะการไนตริฟิเคชันและลักษณะทางกายภาพของจุลินทรีย์ โดยศึกษาครอบคลุม
อิทธิพลของความเข้มข้นแอมโมเนียและ AgNP เริ่มต้นต่อสมรรถนะการไนตริฟิเคชันของ
เซลล์ดักติดซึ่งใช้วิธีการเรสไพโรเมตริก การศึกษาเซลล์ดักติดจำแนกออกได้เป็นเซลล์ดักติด
สองชนิด (แคลเซียมแอลจีเนต (CA) และพอลีไวนิลแอลกอฮอลล์ (PVA)) และสองขนาด (เล็ก
และใหญ่) การทดลองโดยใช้เซลล์อิสระได้กระทำควบคู่ไปกับเซลล์ดักติด เพื่อใช้เปรียบเทียบ
ผลการศึกษา ส่วนการศึกษาผลต่อลักษณะทางกายภาพของจุลินทรีย์และวัสดุดักติดศึกษาด้วย
กล้องจุลทรรศน์อิเล็กตรอนแบบสแกนและแบบทรานซ์มิสชั่น   ผลการศึกษาพบว่าความ
เข้มข้นของแอมโมเนียเริ่มต้น (28 and 70 mg-N/L) ไม่ส่งผลกระทบต่อกิจกรรมการไนตริฟิเค
ชัน ในขณะที่ความเข้มข้นของ AgNP (0.05 to 5 mg/L) ส่งผลกระทบต่อการไนตริฟิเคชันอย่าง
ชัดเจน (กิจกรรมไนตริฟิเคชันเท่ากับร้อยละ 2 ถึง 98 เมื่อเปรียบเทียบกับชุดควบคุมที่ไม่มี
AgNP) สำหรับเซลล์ดักติดทั้งสอง (CA และ PVA) สามารถช่วยลดปัญหาดังกล่าวได้   แต่
พบว่าเซลล์ดักติดด้วย CA ซึ่งมีกิจกรรมไนตริฟิเคชันเท่ากับร้อยละ 64 ถึง 93 มีสมรรถนะการ
ไนตริฟิเคชันดีกว่าเซลล์ดักติดด้วย PVA ซึ่งมีกิจกรรมไนตริฟิเคชันเท่ากับร้อยละ 4 ถึง 87
นอกจากนี้ยังพบว่าทั้งเซลล์ดักติดด้วย CA และ PVA ขนาดใหญ่ซึ่งมีกิจกรรมไนตริฟิเคชัน
เท่ากับร้อยละ 44 ถึง 93 มีสมรรถนะดีกว่าขนาดเล็กซึ่งมีกิจกรรมไนตริฟิเคชันเท่ากับร้อยละ 4
ถึง 89 ผลการศึกษาลักษณะทางกายภาพด้วยกล้องจุลทรรศน์อิเล็กตรอนสอดคล้องกับผล
ข้างต้น กล่าวคือ AgNP แทรกซึมเข้าไปภายในเซลล์และทำลายเยื่อหุ้มเซลล์และไซโตพลา
สซึมผลส่งให้กิจกรรมไนตริฟิเคชันลดลง รวมทั้งยังพบอีกว่าวัสดุดักติดสามารถลดทอนปัญหา
นี้ได้

สาขาวิชา การจัดการสิ่งแวดล้อม   ลายมือชื่อนิสิต...

ปีการศึกษา 2553   ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก...............

                                      ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.................

5187523820 : MAJOR   ENVIRONMENTAL MANAGEMENT

KEYWORDS : CELL ENTRAPMENT NITRIFICATION NITRIFYING BACTERIA RESPIROMETER SILVER NANOPARTICLES

CHUTIMA PLOYCHANKUL : EFFECT OF SILVER NANOPARTICLES ON NITRIFICATION BY ENTRAPPED CELLS THESIS ADVISOR : SUMANA SIRIPATTANAKUL, Ph.D. THESIS CO-ADVISOR : TAWAN LIMPIYAKORN, Ph.D., 95 pp.

Effect of silver nanoparticles (AgNP) on nitrification by entrapped cells was investigated. The study consisted of the AgNP effect on nitrification performance and microorganism physiology. The influences of initial ammonia and AgNP concentrations on the nitrification performance by the entrapped cells were conducted using respirometric method. Two types (calcium alginate (CA) and polyvinyl alcohol (PVA)) and two sizes (small and large) of the entrapped cells were chosen. The test using the free cells was also performed for comparative purpose. For physiological effect, scanning electron microscopic (SEM) and transmission electron microscopic (TEM) observations of the microbial cells and matrices were performed. The result showed that the initial ammonia concentrations (28 and 70 mg-N/L) did not play the important role on the nitrification activity while the AgNP concentrations (0.05 to 5 mg/L) obviously affected on the nitrification (activities of 2 to 98 % compared to the control (no AgNPs). Both PVA- and CA-entrapped cells could lessen the problem but nitrification performance by the CA-entrapped cells (activities of 64-93%) was better than that of the PVA-entrapped cells (activities of 4 to 87 %). For both PVA- and CA- entrapped cells, large entrapped cells (activities of 44 to 93 %) performed better than small entrapped cells (activities of 4 to 89 %). The microscopic observation supported the respirometric result. Silver nanoparticles penetrated and damaged microbial cell membrane and cytoplasm resulting in decreased nitrification activity. The entrapment matrices could minimize the problem.

Field of Study : Environmental Management   Student's Signature _Chutima Ploychankul_

Academic Year : 2010   Advisor's Signature _____

Co-Advisor's Signature _____

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CHAPTER I

INTRODUCTION

1.1 General introduction

Nanotechnology relates to a wide range of technologies that incorporate materials feature with a range of dimensions between approximately 1 and 100 nm. Nanoparticles, particles in nano scale, are a part of nanotechnologies claimed to substance, such as silver, iron, gold, and nanotubes (nano-scale carbon) (Kaiser et al., 2008; Savage and Diallo, 2005). The nanoparticles have been incorporated in numerous consumer products. Silver nanoparticles (AgNPs) are one of the most well-liked nanoparticles utilized in the world. It is known as the antimicrobial agent. In industrial sector, AgNPs were broadly utilized in products, such as bicycles frames, plastic containers, drug delivery, and textiles (Nanohorizon, 2007; Yang et al., 2005; Hong et al., 2006; Benn and Westerhoff, 2008). Although there has not been the published document of AgNPs contamination levels in the environment, the trend of AgNPs utilization significantly increases. Nearly one-third of AgNPs products on the market in 2007 had the potential to disperse into the environment. A few information of the adverse effect of AgNPs were presented. Consequently, in the near future, the contamination of nanomaterials especially AgNPs would be a problematic issue in surface water, soil, groundwater, or wastewater treatment systems.

It has been known that silver nanopaticles affected on microorganisms. Antimicrobial achievement of silver nanoparticles has been assumed to its surface oxide layer release of silver ion species which is highly toxic to organisms (McGeer, 2000; Fan and Bard, 2002). It is also known that silver is toxic to cell because it can destroy or attach to cell membrane resulting in inhibition in growth or effect on viability (Lok et al., 2006; Choi et al., 2008). Nonetheless, most of previous studies were focus on effect of silver particles in micro scale. There were only a few reports on effect of AgNPs (Lok et al., 2006; Choi et al., 2008). Silver nanoparticles could penetrate though cell membrane and cause more 100-time severe effect to the cells (Lok et al., 2006).

As mentioned earlier, biological wastewater treatment system would be critically affected by AgNP contamination. Nitrification is known as the most sensitive process among wastewater treatment processes. The nitrification process is an important process for removing nitrogen from wastewater treatment plants (WWTPs). It is a biological transformation of reduced forms of nitrogen to nitrate by nitrifying activated sludge (NAS). In prior study, it was found failure in nitrification process (Choi et al., 2008). The nitrification decreased up to $86\pm3\%$. Although there are a few studies on fate and toxicity mechanism of AgNPs in wastewater treatment systems, there was no such the problem abatement of AgNPs in the systems.

Cell entrapment technique, microorganism immobilization in a porous polymeric matrix, is a potential method to alleviate the problem. The technique was used in pollutant removal such as, nitrogen, carbon, herbicide and other hazardous substance in wastewater and contaminated sites (Chen et al., 1998; Siripattanakul et al., 2008). The polymeric materials widely used as cell entrapment matrices were calcium alginate (CA), polyvinyl alcohol (PVA), carrageenan (CN), and cellulose triacetate (CTA) (Siripattanakul and Khan, 2010). The entrapment matrices can increase biological activities and protect the cells from toxic substances (Chen et al., 1998; Siripattanakul et al., 2008). Based on the advantages, the technique is promising to lessen the problem.

This study aims to investigate effect of AgNPs on the nitrification process and to protect toxic substance from microorganisms using cell entrapment technique. Effects of initial AgNP concentrations, entrapment material types (CA and PVA), and bead sizes (small and large beads) of the entrapped NAS on the nitrification kinetics were conducted. The free NAS was tested for comparative purpose. The nitrification kinetics in the present study was used respirometric approach followed the previous studies (Surmacz-Gorska et al., 1996; Ginestet et al., 1998; Schramm et al., 1998; Chandran and Smets, 2001; Moussa et al, 2003; Ciudad et al., 2006; Chandran and Love, 2008).

1.2 Objectives

Main objective of this study is to investigate effect of AgNPs on the nitrification process using entrapped cells compared to free cells. The specific objectives are:

1. to examine effects of AgNP and initial ammonia at different concentrations on nitrification performance,

2. to examine effect of cell entrapment matrices (different sizes and types) on nitrification performance of the entrapped NAS that was exposed by AgNPs, and

3. to observe interaction of AgNPs on the free and entrapped NAS microstructure.

1.3 Scopes

This study has been held at the environmental laboratory, National Center of Excellence for Environmental and Hazardous Waste Management (NCE-EHWM), Chulalongkorn University since September 2008. It is expected to complete all tasks in September 2010. The experiment was in a bench-scale experiment. The following details are the specific informations on the scope of study.

1. Returned activated sludge used in the study was taken from returned activated sludge at Siphraya municipal wastewater treatment plant, Bangkok, Thailand.

2. The NAS was enriched from the returned activated sludge at 28 and 70 mg/L of ammonia concentration.

3. Respirometer was used for studying nitrification.

4. Silver nanoparticles were varied at 0.5, 1, and 5 mg/L.

5. The NAS was entrapped using CA and PVA entrapment techniques.

6. The entrapped cell sizes of 3 and 6 mm were tested.

7. Microstructure of cells was observed using Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM).

1.4 Hypotheses

1. Entrapped cells perform better than free cells on nitrification with AgNP contamination.

2. Both CA- and PVA-entrapped cells were protected from AgNPs. The PVA-entrapped cells are more effective because the matrix structure is more suitable for the cell protection from AgNPs.

3. The large entrapped cells perform better than the small entrapped cells. Thicker matrix provides lower contact of AgNPs to the microbial cell leading to lower cell damage.

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 Silver nanoparticles

Nanotechnology relates to a wide range of technologies that incorporate materials feature with a range of dimensions between approximately 1 and 100 nm. presents scale of materials related to nanotechnology. Nanoparticles, particles in nano scale, are a part of nanotechnologies claimed to substances, such as silver, iron, gold, carbon nanotubes, and nano-scale organic compounds (Kaiser et al., 2008; Savage and Diallo, 2005).

2.1.1 Properties of silver nanoparticle

Silver nanoparticles (AgNPs) were found in ashes, soil particles or biomolecules. The sizes of the nanoparticles are varied from 1 to 100 nm causing the difference in physico-chemical properties compared to their bulk material. Table 2.1 is shown typical properties of silver nanoparticles.

2.1.2 Application of silver nanoparticles

Silver nanoparticles are widely used in many applications because of their properties. Normally, AgNPs are applied for antimicrobial purpose. Estrin et al. (2008) examined the property of AgNPs as antimicrobial and antifungal agents on *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger* and *Penicillium phoeniceum*. The result demonstrated that AgNPs produced from a novel electrochemical method had strong antibacterial and antifungal properties. In food industries, AgNPs were applied as an antimicrobial agent by coating on the food container (Del Nobile et al., 2004; Jain and Pradeep, 2005; An et al., 2008; Tankhiwale and Bajpai, 2009; Fernandez et al., 2009; Fernandez et al., 2010). It was found that number of spoilage-related microorganisms in melon kept in cellulose-AgNPs-hybrid materials decreased compared to the control. Another application of AgNPs utilization as antimicrobial agent were textiles industry (Lee and Jeong,

2005; Dubas et al., 2006; Benn and Westerhoff, 2008; Yoksan and Chirachanchai; 2010). Lee and Jeong (2005) found that AgNPs at sizes of 2-3 nm were skin-innoxious which can be used as antibacterial agents on fabrics.

Table 2.1 Properties of silver nanoparticles

Properties	Expressions
Shape	Triangular, spherical, rod-shape[a]
Size	9-25 nm[b]
Types	Polymeric silver, colloidal silver, spun silver, nanosilver powder, ionic silver[c]
Specific surface area	9-11 m^2/g[d]
Color	Red, brown , and green[e]

[a] Sun et al. (2003), [b] Choi and Hu (2008), [c] Luoma (2008), [d] Navarro (2008), [e]Van Hyning (1998)

Other utilizations of AgNPs were for medical application. Silver nanoparticles were exploited for medical apparatus (Furno et al, 2004; Biju et al., 2008; Kassaee, 2008). For example, Biju et al. (2008) examined that AgNPs can be applied to in vitro and in vivo imaging of living cell, and in vivo imaging of cancers, tumor vasculature, and lymph nodes. In addition, AgNPs were synthesized and demonstrated to use as a potentiometric redox marker in a glucose biosensor (Ngeontae et al., 2009).

2.1.3 Fate and transport of silver nanoparticles to environment

Silver nanoparticles are one of the most well-liked nanoparticles utilized in the world. Although there has not been published document of AgNPs contamination levels in the environment, the trend of AgNPs utilization significantly increases. Silver nanoparticles could spread throughout the environment.

Fauss (2008) classified AgNPs into the five types which are polymeric silver, colloidal silver, spun silver, nanosilver powder, and ionic silver. First, polymeric silver is AgNPs from handrails, medical devices, food storage containers, dressing for wounds, and female-hygeine products. It forms a complex long chain molecule as gelatin. Second, colloidal silver is AgNPs at the size of 0.6-25 nm. Third, spun silver is AgNPs from fabric, impregnating sheets, clothing, and sportswear. Forth, nanosilver powder is AgNPs from the first wash from sock and shoes. Fifth, ionic silver is AgNPs from washing machines and dishwashers. The highest amount of AgNPs that was released into water is from spun silver as mention in Benn and Westerhoff (2008).

Luoma (2008) calculated the release of AgNPs from manufacturers' information and came up with fate and transport scenarios for three new AgNPs applications including silver sock, silver wash machines, and swimming pool or spa equipments. All applications were applied AgNPs as antibacterial agent. Silver nanoparticles can react and fate into many characteristics to the water as an individual particles, aggregation, dissolution, and dissolved organic matter. Silver nanoparticles could be discharged up to 150 tons per year. Silver nanoparticles could be degraded in acidic condition and room temperature (JR Nanotech, 2002). The abundant of chloride or dissolved organic matter could activate dissolution rate of AgNPs. On the other hand, natural water containing a large amount of dissolved organic matter can reduce the reactivity of AgNPs.

Besides estimated fate and transport in natural water, Mueller and Nowack (2009) predicted that AgNPs released into soil and water as the wet deposition. There are many processes to dispose AgNPs such as, waste incineration plants, landfills, and sewage treatment plants. They analyzed the percentage of releasing in AgNPs from products. The result showed that the highest percentages of AgNPs were released to dissolution form from textiles, cosmetics, spray agents, metal products, plastics, and paint. Blaser et al. (2008) reported that AgNPs could leach from landfill to soil. In addition, the digested sludge from

sewage treatment plants is sold as an agricultural fertilizer, this leads to contaminate of AgNPs into soil (FOE, 2007).

For fate of AgNPs in living organisms, AgNPs could transfer from soil to heterotrophic (ammonifying/nitrogen fixing) and chemolithotrophic bacteria (FOE, 2007). Moreover, AgNPs in the suspended particulates form in water transported to sediments. Then, animals consume AgNPs sediment as food (JR Nanotech, 2002). Silver nanoparticles could pass though membranes of the digestive tract though all over animal body.

2.1.4 Effect of silver nanoparticles in the environment

After AgNPs discharged to soil and water, it can damage to organism resulted from an antimicrobial function of AgNPs. The toxicity of AgNPs on human, fish, algae, crustaceans, some plants, fungi, and bacteria was reported (Eisler, 1996; Yeo and Kang, 2008).

2.1.4.1 Human

Silver nanoparticles can cause argyria as reported in White et al. (2003). This study reported the case of a 58-year-old man who drank colloid silver protein solution for at least 1 year. He believed the prevention of silver from various diseases. Consequently, he has a deep blue/grey discoloration of the skin on face, neck, bald scalp, hands, and forearms. The ingestion of AgNPs has also related to neurological problems, kidney damage, stomach upset, headaches, fatigue, and skin irritation (White et al., 2003; Hori et al., 2002)

2.1.4.2 Animal

There are some studies demonstrated the effect of AgNPs to the mammalian cells. For example, Hussain et al. (2005) evaluated the acute toxic effects of metal nanopartciels using the *in vitro* rat liver derived cell line. Silver nanoparticles meaningfully decreased the function of mitochondria. Lactate dehydrogenase leakage significantly extended in the treated cells by AgNPs. Likewise, Braydich-Stolle and colleagues (2005) examined the cytotoxicity on male germline *in vitro* of mouse. Silver nanoparticles were the most toxic to a mouse spermatogonial stem cell line. Moreover, AgNPs could induce the neurotransmitter dopamine depletion which was essential for the normal functioning of the central nervous system (Hussain et al., 2006).

2.1.4.3 Microorganisms

It is well known that AgNPs is a toxic substance for microorganisms (Sondi and Salopek-Sondi, 2004; Pal et al., 2007; Kim, 2007; Estrin, 2008; Fabrega et al., 2009). Sondi and Salopek-Sondi investigated the antimicrobial activity of AgNPs on *Escherichia coli* as a model of Gram-negative bacteria. The result showed that the cell wall of *E. coli* was damaged by the formation of pits. Similarly, Pal and colleagues (2007) revealed the images of treated *E. coli* with AgNPs by energy-filtering transmission electron microscopy. There was a change in the cell membrane on the treated cell, resulting in cell death. Besides, Kim and co-worker (2007) researched the antimicrobial activity of AgNPs against yeast, *E. coli*, and *S. aureus.* Silver nanoparticles at the low concentration obviously inhibited the growth of yeast and *E. coli* while the growth-inhibitory impact on *S. aureus* was light. Moreover, they studied generation of free radicals from AgNPs which attack to membrane lipids and this cause a breakdown of membrane function.

According to Choi and Hu (2008), the relationship between the inhibition and reactive oxygen species (ROS) on nitrifying bacteria was determined. The reactive oxygen species including singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radical, may be free radical species as mentioned in Kim et al. (2007). On the other hand, the toxicity of AgNPs also reacts to HIV-I (Elechiguerra, 2005) by preferential binding to the gp I20 glycoprotein knobs. This interaction inhibits the virus from binding to host cells.

2.1.4.4 Wastewater treatment plant

As mentioned in fate and transport topic, there are many industries that directly release AgNPs to water. The wastewater is contaminated with AgNPs effect on microorganisms in wastewater treatment plants because of the strong antimicrobial properties of AgNPs. The toxicity damages useful bacteria. Choi and Hu (2008) investigated the size-dependent inhibition by AgNPs on nitrifying bacteria. The result showed that the less AgNPs size than 5 nm and the concentration of AgNPs at 1 mg/L could be more toxic to bacteria than their bulk materials. Moreover, the relationships of ROS were different for the various form of silver.

2.2 Nitrification

Nitrification, a two-step nitrogen conversion process, is one of the most important part of nitrogen cycle, since it provide nitrate (NO_3^-) in soil water from ammonia in soil particles for plants and wastewater treatment plants. The nitrification process relates to two groups of lithoautotrophic bacteria which are ammonia oxidizers and nitrite oxidizers (known as nitrifier). The process generates the energy by the oxidation of ammonia to nitrite (ammonia oxidizers) or nitrite to nitrate (nitrite oxidizers) as shown on Equation (2.1) and (2.2).

$$NH_4^+ + 1.5O_2 \quad \rightarrow \quad 2H^+ + 2H_2O + NO_2^- + energy \qquad (2.1)$$

$$NO_2^- + 0.5O_2 \quad \rightarrow \quad NO_3^- + energy \qquad (2.2)$$
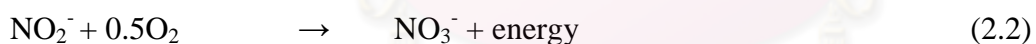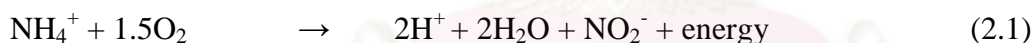
Nitrifying bacteria are useful for agriculture because it reduces the acidification on unbuffered soils, which are phytotoxic by transforming nitrate in soil particles to mobile phase. In addition, nitrifying bacteria remove nitrogen from wastewater leading to eutrophication and reducing the toxic effect of ammonia on aquatic organisms.

2.2.1 Microbial Nitrification

As mentioned earlier, nitrification processes relate with two groups of bacteria, ammonia oxidizer (AOB) and nitrite oxidizer (NOB). The most frequently determined genus with the step from Equation (2.1) is *Nitrosomonas*. Furthermore, there are the other genera associate with this step which is *Nitrosococcus* and *Nitrosospira* (Watson et al., 1981). *Nitrobacter* is the most frequently determined genus with the step from Equation (2.1), including with *Nitrococcus* and *Nitrospira* (Watson et al., 1981).

The lithoautotrophic ammonia oxidizing bacteria has the ability to use ammonia as the major source of energy and carbon dioxide as the main source of carbon. The characteristics of the AOB genera are shown in Table 2.2 (Koops and Pommerening-Röser, 2006).

Table 2.2 Characteristics of the genera of AOB

Characteristic	*Nitrosomonas*	*Nitrosospira*	*Nitrosococcus*
Cell shape	Spherical to rod shaped	Tightly coiled spirals	Spherical to ellipsoidal
Intracytoplasmic membranes	Peripherally located flattened vesicles	Occasional tubular invaginations	Centrally located stack of membranes
Flagella	Polar flagella	Peritrichous flagella	Tuft of flagella

The major groups of AOB, located within the *Betaproteobacteria*, enclose two clusters (Figure 2.1) by the use of 16S rDNA. The phylogenetic analyses of AOB were demonstrated into two classes which are *Betaproteobacteria* and *Gammaproteobacteria* (Woeses et al., 1984; Woeses et al., 1985). The genera *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus* belong to the class *Betaproteobacteria*, while the genus *Nitrosococcus* is branched with the class *Gammaproteobacteria*.
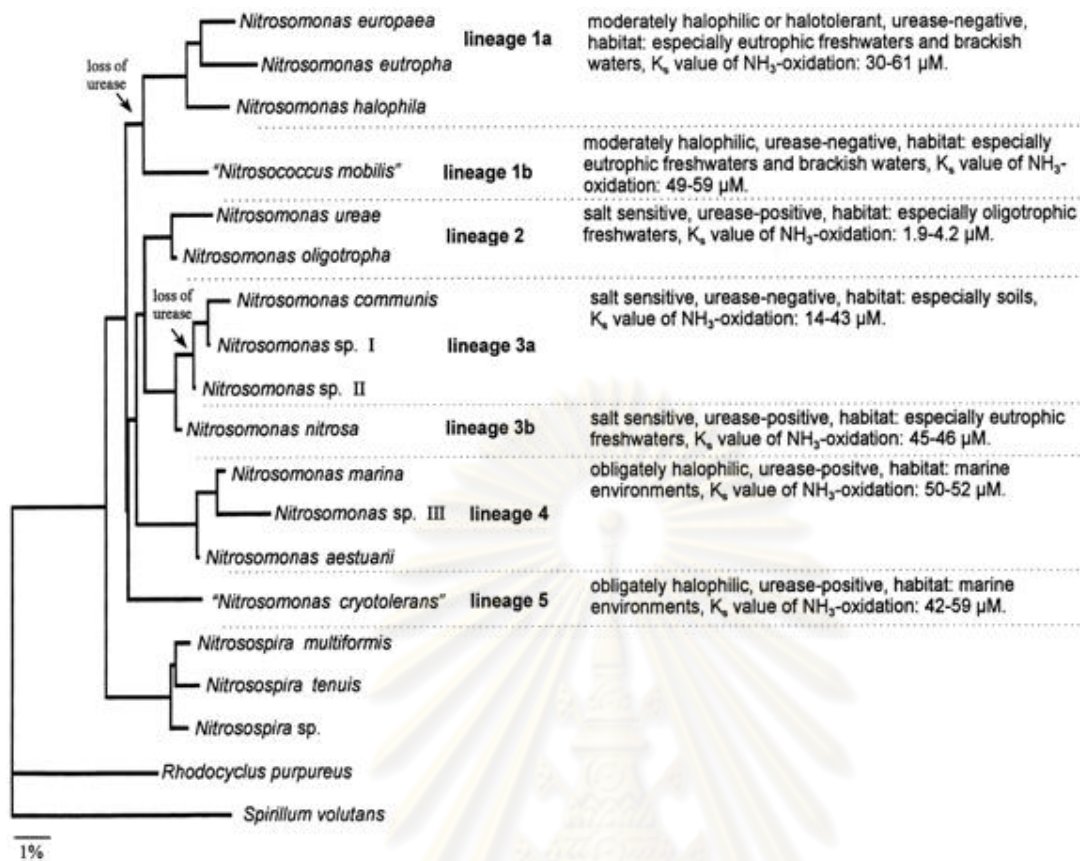
Figure 2.1 16S rDNA-based phylogenetic tree nearly complete sequences, in the class *Betaproteobacteria* and the most important ecophysiological characteristics of the five genera (Koops and Pommerening-Röser, 2005).

The lithoautotrophic nitrite oxidizing bacteria has the ability to use nitrite as the main source of energy and carbon dioxide as the sole source of carbon. The characteristics of NOB are rods, cocci, and spirilla (Spieck and Bock, 2005b). There are four genera classifications which are *Nitrobacter*, *Nitrococcus*, *Nitrospina*, and *Nitrospira* (Table 2.3). Unlike AOB, NOB is more scattered phylogenetically as demonstrated in Figure 2.2. The dominate genus of NOB in most natural environments is *Nitrobacter*. However, NOB is more likely to live in the extreme environments such as concrete and natural stones, desert soils, and sulfidic ore mines. Besides, they can survive long periods of starvation and dryness without endospores.

Table 2.3 Differentiation of the four genera of NOB (Spieck and Bock, 2005)

Characteristics	*Nitrobacter*	*Nitrococcus*	*Nitrospina*	*Nitrospira*
Phylogenetic position	*Alphaproteo-bacteria*	*Gammaproteo-bacteria*	*Deltaproteo-bacteria*	Phylum *Nitrospirae*
Morphology	Pleomorphic short rods	Coccoid cells	Straight rods	Curved rods to spirals
Intracytoplasmic membranes	Polar cap	Tubular	Lacking	Lacking
Size (µm)	0.5-0.9x1.0-2.0	1.5-1.8	0.3-0.5x1.7-6.6	0.2-0.4x0.9-2.2
Reproduction	Budding or binary fission	Binary fission	Binary fission	Binary fission
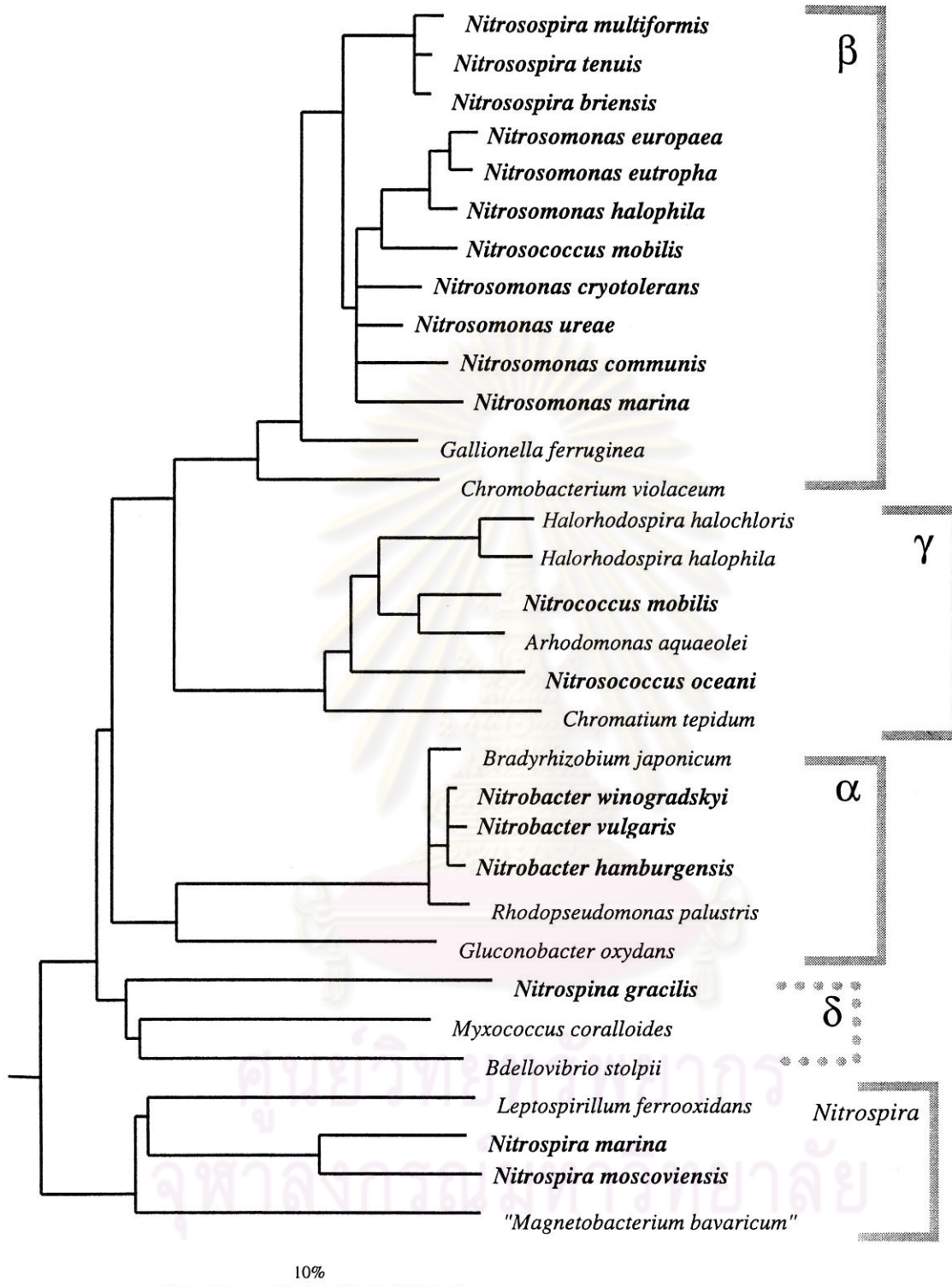
Figure 2.2 16S rRNA-based tree reflecting the phylogenetic relationship of NOB (Spieck and Bock, 2005a).

2.2.2 Influential factors for nitrification

Nitrification process is the first important pathway to remove the reduced nitrogen in term of ammonia and organic nitrogen by nitrification and denitrification processes in wastewater. Since, nitrifying bacteria have a slower growth rate (Prosser, 1989) and lower competitor of oxygen than aerobic heterotrophs, it is difficult to manipulate nitrification in municipal and industrial wastewater treatment plants. Several influential factors affecting nitrification are as follows.

2.2.2.1 Ammonia

From Equation (2.3), ammonia is the substrate in the nitrification process, nitrifiers use ammonia as the sole source of energy. Kemp and Dodds (2002) determined the influence of ammonium on nitrification rates with prairie stream substrata. The conclusion of the study was nitrification response to increasing NH_4^+ concentrations but depending on the substrata type. Furthermore, the 98% of ammonia conversion to nitrite can be produced in the case of saturated oxygen concentration (Ruiz et al., 2003).

2.2.2.2 Biochemical Oxygen Demand

Biochemical oxygen demand (BOD_5) represents the level of organic pollutant in wastewater samples by measuring the oxidized organic matter from bacteria. Wastewater treatment plants are plenty of microorganisms like autotrophs and heterotrophs. The increasing of BOD leads to the competition in both types of microorganism which affect on nitrification process. Therefore, the high amount of BOD_5 could reduce the performance of nitrification process. Parallels to Coskuner and Jassim (2008) indicated that the nitrification performance was decreased by the higher BOD concentrations results from the competition for DO.

2.2.2.3 Sludge Retention Time (SRT)

For biological treatment process in wastewater treatment plants, SRT is one of the most important for operational factor. Longer SRT leaded to accumulation of biomass (sludge), which interrupted with the transfer of oxygen in bioreactor (Huang et al., 2001). Likewise, Hallin et al (2005) also studied the effects of different SRTs on the nitrification activity. Prolonged SRT reduce the nitrification activity causing by the physiological changes

in the AOB community rather than a change in community composition. On the other hand, too short SRT caused reduction of nitrification activity by decreasing of the sludge concentration (Huang et al., 2001).

2.2.2.4 Salinity

High saline concentrations were occurred in the high nitrogen concentrated waste streams. Due to the fact that they contain plenty of ions such as chloride from fish canning industry and wet lime-gypsum desulphurization process, sulphate from tannery wastes (Campos et al., 2002). Organic matter, nitrogen, and phosphorus removal are affected from this situation (Panswad and Anan, 1999; Intrasungkha et al., 1999). Panswad and Anan (1999) investigated that the more increasing of chloride concentration was added, the lower of ammonia and nitrate uptake rate were made. Likewise, Campos et al. (2002) discovered the inhibition of nitrification activity became completed at the higher of salt concentration than 525 mM.

2.2.2.5 Temperature

Temperature is one of the factors that influence for nitrification as Antoniou and colleagues (1990) determined the maximum growth rate of nitrifying bacteria on temperature. They found the maximum growth rate is in the range of 15-25 °c. Besides, Mulder et al. (2001) constructed the SHARON process operating without sludge retention. The optimal operating temperature of 30-40 °c supported N-removal over nitrite. The growth rates of nitrifying bacteria increase with extend the temperature up to 30 °c (Coskuner and Jassim, 2008).

2.2.2.6 pH and alkalinity

The optimum pH for the growth of nitrifying bacteria is in the range of 7.5-8.0 (Prosser, 1989; EPA, 2002). EPA (2002) informed the reduction of alkalinity leads to the change of buffering capacity as bicarbonate which is consumed in the conversion of ammonia to nitrite. Moreover, an increasing in pH to higher than 9 can reduce the nitrification activity.

2.2.2.7 Dissolved oxygen concentration

Since oxygen is the important substrate to nitrification process as can be seen from Equation (2.3). The optimal oxygen concentration in the operation of nitrification process should not less than 2.0 mg/L (Coskuner and Jassim, 2008). Prosser (1989) stated that the low concentrations of oxygen decrease rates of nitrification result from the nitrifiers become a poor competitors at the low concentration of oxygen compare to heterotrophic nitrifiers. The K_s value of hetrerotrophs is lower for oxygen leading them to be a competitive advantage in this situation.

2.2.2.8 Light

There are some studies which investigated in the effect of light through the nitrification process because of the light penetration in the different light sources and intensities (Yoshioka and Saijo, 1984; Diab and Shilo; 1988; Guerrero and Jones, 1996). Guerrero and Jones (1996) demonstrated the effect of light on nitrifying bacteria depends on both types of nitrifiers and the conditions of their environment. Yoshioka and Saijo (1984) concluded that light can be also lethal and bacteriostatic to nitrifying bacteria.

2.2.3 Nitrification process monitoring

Due to the various influential factors of nitrification, many of the monitoring ways were produced to support the researchers. Nitrifying bacteria are sensitive microorganism to the stress condition so the process for monitoring should produce the less effect to the nitrification process.

2.2.3.1 Decrease in dissolved oxygen

This method is applied for determination the nitrification activity which impact from toxic substance, called respirometric method (Surmacz-Gorska et al., 1996; Hu et al., 2002; Moussa et al., 2003; Ciudad et al., 2006; Cecen et al., 2010). The method is convenient, accurate, precise, and less-time consuming. The saturated oxygen concentration which is required for nitrification is available; therefore, it is easy to control the concentration of oxygen.

2.2.3.2 Decrease in ammonia concentration

As ammonia is the main source of energy to nitrifying bacteria, the method to monitor the ammonia concentration is applied. Panswad and Anan (1999) investigated in the specific oxygen, ammonia, and nitrate uptake rates of a biological nutrient removal process treating uplifted salinity wastewater. Moreover, You et al. (2009) examined the effect of heavy metal in nitrification by measuring the specific ammonia uptake rate.

2.2.3.3 Real-time Polymerase Chain Reaction (Real-time PCR)

Nowadays, PCR is widely used for quantification of nitrifying bacteria. by amplification of ammonia monooxygenase (amoA) and *Nitrospira* spp. 16S rRNA genes (Dionisi et al., 2002; Harms et al., 2003). Harms et al. (2003) developed the real-time PCR for the quantification of total bacteria (NOB and AOB) in mixed liquor suspended solids (MLSS) from wastewater treatment plants.

2.3 Cell entrapment

Immobilized cell is widely utilized in the field of sciences and technologies to develop the production. Mostly, it is used in bioreactor, and production of the useful compounds such as amino acids, organic acids, antibiotics, steroids, and enzymes (Cassidy et al., 1996). According to Tampions (1987) introduced the advantages of immobilized cell which are long-term stability of biocatalyst, low leakage of cells, high resistance to abrasion, resistance to microbial degradation, low diffusional limitation, high surface area, cheap support materials, and non-toxic materials. There are many different forms to produce the entrapped cells which can be shown in Table 2.4. There still are two more techniques to form the immobilized cell which are cross-linking of cells and encapsulation in polymer-gel (Cassidy et al., 1996). The application of immobilized cell is used in agriculture, bio-control, pesticide application, and pollutant biodegradation in contaminated soil or groundwater (Connick and William, 1982; Bettmen and Rehm, 1984; Bashan, 1986; Axtell et al, 1987; Hu et al., 1994).

2.3.1 Principle of cell entrapment

Cell entrapment is one of the immobilized cell techniques which contain two main steps (Dulieu, 1999; Siripattankul, 2010). First, the mixture of cells and matrix are well blended by magnetic stirrer to become a homogenous phase. Second, the droplets of cell mixture is formed the gelation by peristaltic pump. The gelation is produced in the formation of cross-linking between a matrix and a cation in gel formation solution such as calcium alginate beads. The spherical shape has been applied for the formation of the beads because there is much surface area than the other shape.

2.3.2 Types of cell entrapment materials

There are two types of matrix materials using for cell entrapment which are natural and artificial types. Natural matrices are polysaccharides made from algae or seaweed, such as calcium alginate, carrageenan, agarose, and gelatin. On the other hand, the artificial types are polymer, such as polyvinyl alcohol, cellulose triacetate, and polyacrylamide.

Table 2.4 Classification of the immobilized cell techniques (Tampion, 1987)

Technique		Advantages	Disadvantages
Adsorption			
	Neutral supports	Cheap	Cell leakage
		Mild	Sensitive to pH changes
		Reusable	
		Simple	
	Charged supports	Mild	
		Reusable	
		Simple	
Flocculation		Simple	Cell leakage
		Mild	Diffusional limitations
Entrapment			
	Natural polymers	Mild	Diffusional limitations
		Simple	
	Synthetic polymers	May be simle	Toxicity
			Expensive
			Diffusional limitations
Covalent coupling		Permanent	Toxicity
			Expensive
Containment		Mild	Diffusional limitations
		Simple	Expensive
		Reusable	

2.3.2.1 Calcium alginate

Calcium alginate is a cross-linking of alginate with divalent cation as Ca^{2+}. Alginate is a non-toxic natural polysaccharide from brown algae, such as *Macrocystis pyrifera*, *Laminaria digitata*, *L. hyperborea*, and *Eklonia cava* and some bacteria, principally *Azotobacter vinelandii* (Fett et al., 1986, Fett et al., 1995). Alginate is provided as a sodium salt of alginate. While the sodium alginate matrix contact with the Ca^+ solution, a gelation is

formed suddenly in its outer layer through the entire alginate bead (Siripattankul, 2010). The powder of sodium alginate is dissolved in DI water at 2% (w/v) by slowly add into stirred DI for overnight. The concentrated microbial cells were centrifuged at 7,00 rpm for 10 min. The mixture of sodium alginate solution and wet cells is dropped into calcium chloride solution of 3.5% (w/v) using peristaltic pump. The droplets are left in the solution for 2.5-3.0 hr. for the hardening of the beads.

2.3.2.2 Carrageenan

Carrageenan is produced from red algae, mainly *Chondrus crispus*, *Eucheuna cottonii*, *Gigartina stellata* and *G. radula* (Guisely, 1989). The gealation of carragenans depend on temperature and the further strengthening of the polymer network with K^+, or Al^{+3} ions. The 2-5% (w/v) of carragenan in physiological saline is warmed to 70-80 °c and maintain at 42 °c, including the cell suspension. The warm cells at 40-50 °c are added to the carragenan solution. The droplets are expelled into cold KCl solution to make a gelation.

2.3.2.3 Polyvinyl alcohol

Polyvinyl alcohol is a non-toxic synthetic polymer. The characteristics of raw PVA are white and free-flowing granule. There are several gelation techniques for producing PVA gels for immobilized cell, for instance boric acid-PVA (BPVA), freezing and thawing of PVA (FPVA), and phosphorylated-PVA (PPVA) methods. The BPVA technique is a one-step droplet gelation method (Hashimoto and Furukawa, 1987) and a cross-linking of boron. The FPVA technique is a physical cross-linking during temperature-induced condition (Lozinsky and Plieva, 1998). PPVA method is less time consuming and cell damage than BPVA. The PPVA technique is a two-step droplet gelation method that is spherical bead formation and hardening. The spherical bead formation is the first step by cross-linking of the PVA-boron as in BPVA. After that, the spherical beads are transfer to a sodium phosphate solution for bead hardening process. A sodium phosphate increases the surface gel strength through PVA phosphorylation.

2.3.2.4 Cellulose triacetate

The other natural polymer used for matrix of entrapped cell is cellulose which containing of a chain of glucose

molecules. The modification of cellulose with esterification and etherification are applied for the entrapped cell. The entrapped cells of CTA are prepared by the plated gelation (Khan et al., 1994; Yang et al., 1997; Jittawattanarat et al., 2007; Siripattankul, 2010). The 10% (w/v) powder of CTA is dissolved in methylene chloride. Concentrated suspension cells are mixed with CTA solution. After that the mixture is plated into toluene for hardening. The hardened CTA is cut to small cube and washed with water.

2.3.3 Applications of entrapped cells

As a result of the entrapped cells properties, they are broadly used in many industries for increased metabolic activity and metabolite production, protection from toxic substances, and increased plasmid stability (Dwyer et al., 1986; Keweloh et al., 1989; Keweloh et al., 1990; Gadkari, 1990).

2.3.3.1 Biomedical

The cell entrapment was used in the biomedical area for the production of pharmaceuticals and reagents. Koshcheyenko et al. (1983) studied on the transformation of steroid by living entrapped cells in polyacrylamide gel. Likewise, O'Shea (1984) concluded that the entrapped cells have great potential in the treatment of diabetes and liver diseases.

2.3.3.2 Food and beverage

In food industries, pack-bead systems are being applied to give flavors for addition to cheese (Cavin et al., 1985). The entrapped cell also is utilized for cream fermentations of milk in cheese manufacturing (Linko, 1984). In addition, the entrapped cell is used in the alcoholic beverage industry for wine treatment and beer brewing (Onaka, 1985).

2.3.3.3 Wastewater treatment

Biodegradation properties of the entrapped cells are used in wastewater treatment by adding mixed culture the matrix (Chen, 1998; Siripattankul, 2010). Siripattanakul et al. (2008) investigated the effect of cell to matrix ratio in PVA on atrazine degradation. Moreover, Chen (1998) examined the carbon and nitrogen removal in wastewater using phosphorylated PVA-entrapped microorganisms. Due to the prevention of eutrophication effect on surface water, the removal of excess nitrogen has concerned. The entrapped cell is occupied for degradation of ammonia in the wastewater treatment plants (Tramper, 1985; Chen, 1994; Hill and Khan, 2008)

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.

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┌─────────────────────────────────────────────────────────────┐
│  Preparation of NAS enriched from municipal activated sludge  │
└─────────────────────────────────────────────────────────────┘
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PVA entrapped cells	CA entrapped cells	Free cells

Small bead size	Large bead size	Small bead size	Large bead size

Functional effect

To study nitrification process using respirometry

Physiological effect

To observe the change in cell physiology and entrapped cell microstructure caused by silver nanoparticle using SEM

GOAL

To understand effect of AgNPs on wastewater treatment system (nitrification) and enhance the process using entrapped cells

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.
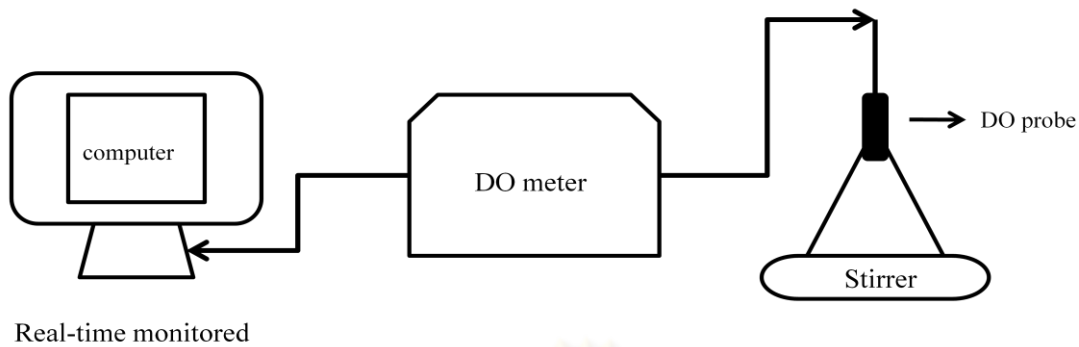
Figure 3.2 Respirometer setup

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.

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

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

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 $Na_2HPO_4 \cdot_2 H_2O$, 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).

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.
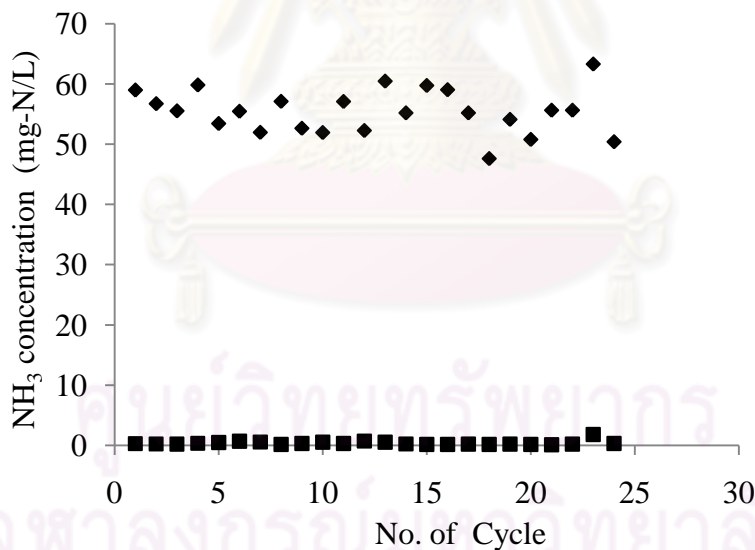
Figure 4.1 Ammonia concentrations during NAS acclimatization in: a) influent (♦) and b) effluent (■)
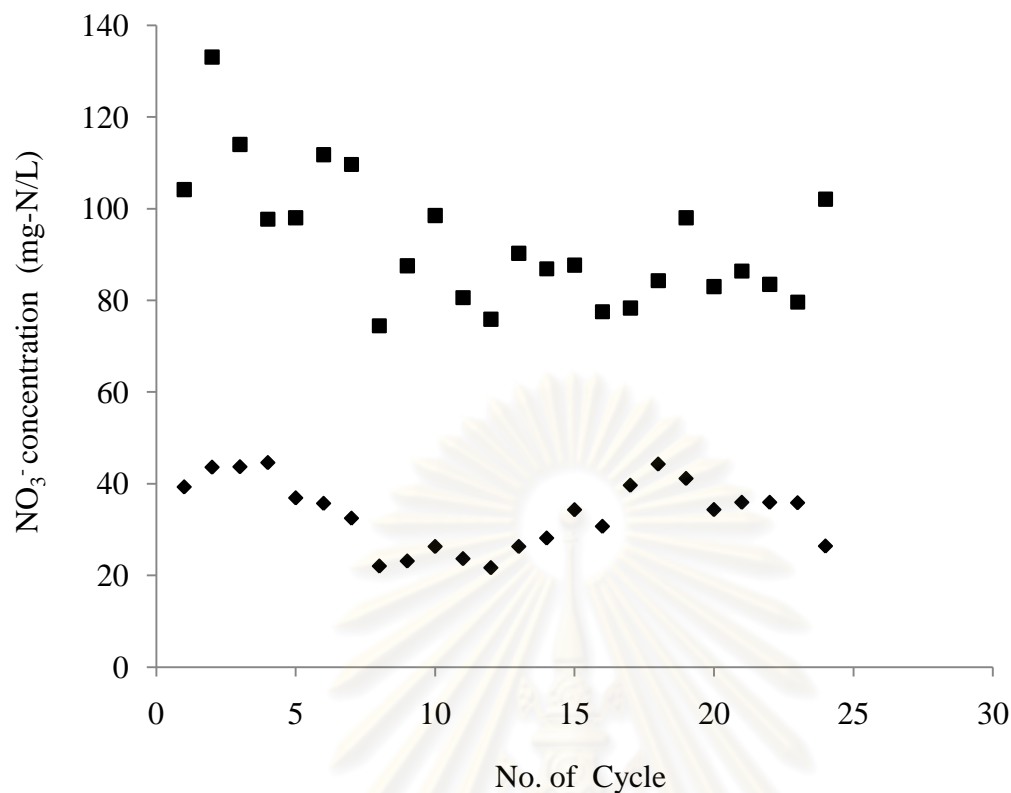
Figure 4.2 Nitrate concentrations during NAS acclimatization in: a) influent (♦) and b) effluent (■)

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-O_2/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-O_2/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.
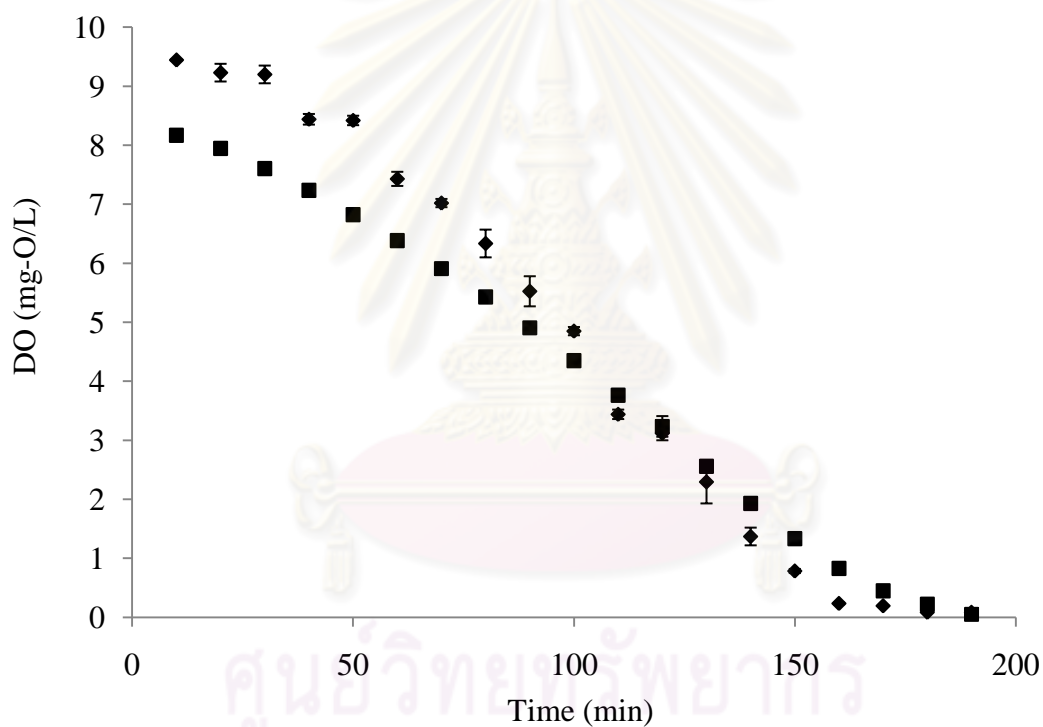
Figure 4.3 Dissolved oxygen concentrations from the tests at 28 (♦) and 70 (■) mg-N/L with AgNP concentrations of 0 mg/L
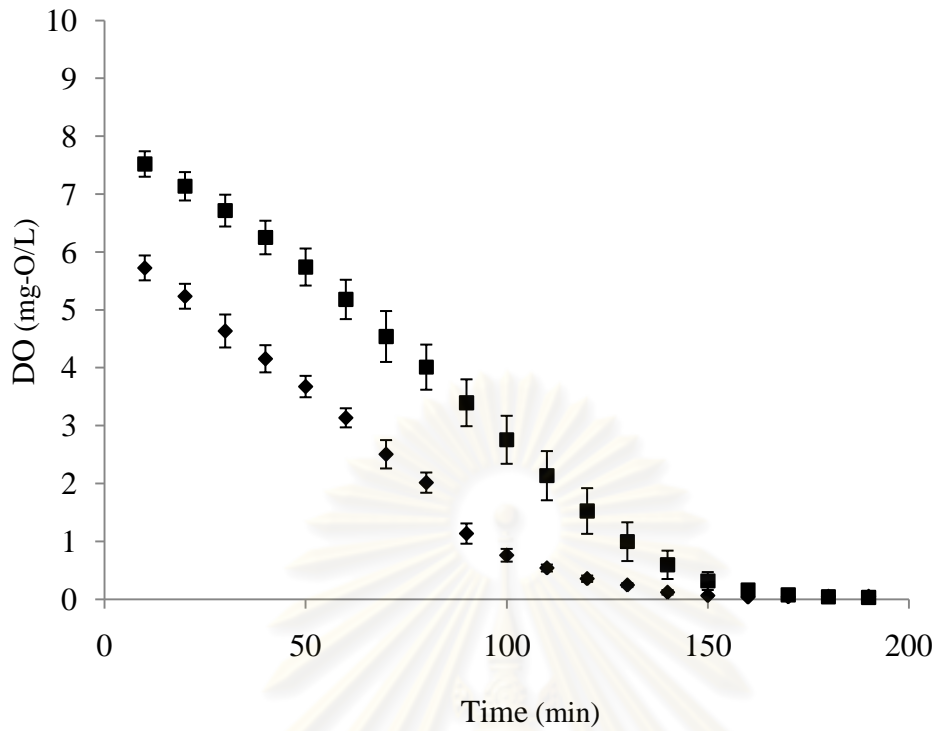
Figure 4.4 Dissolved oxygen concentrations from the tests at 28 (♦) and 70 (■) mg-N/L with AgNP concentrations of 0.05 mg/L
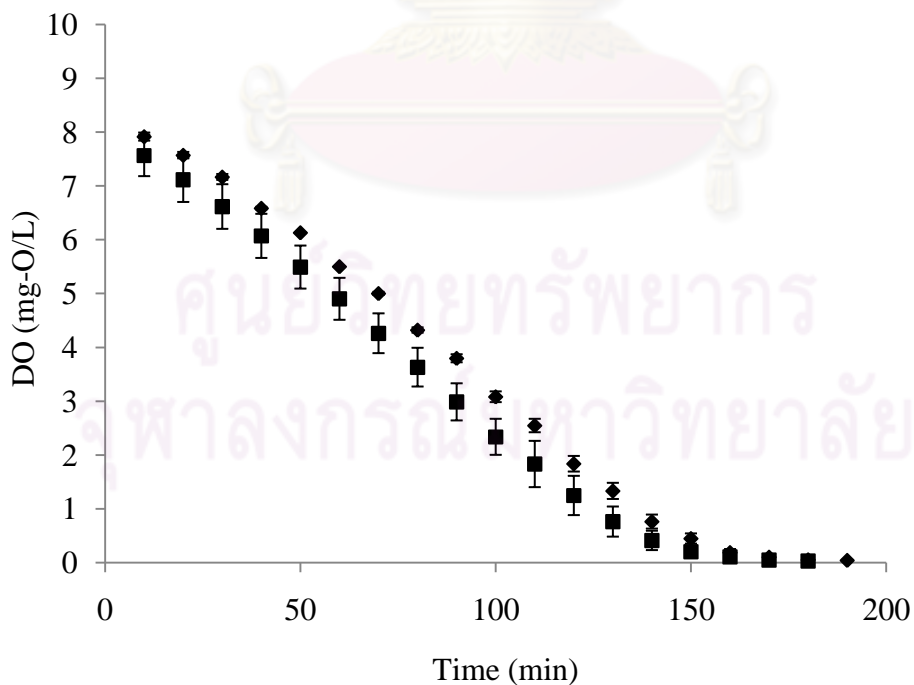
Figure 4.5 Dissolved oxygen concentrations from the tests at 28 (♦) and 70 (■) mg-N/L with AgNP concentrations of 0.5 mg/L
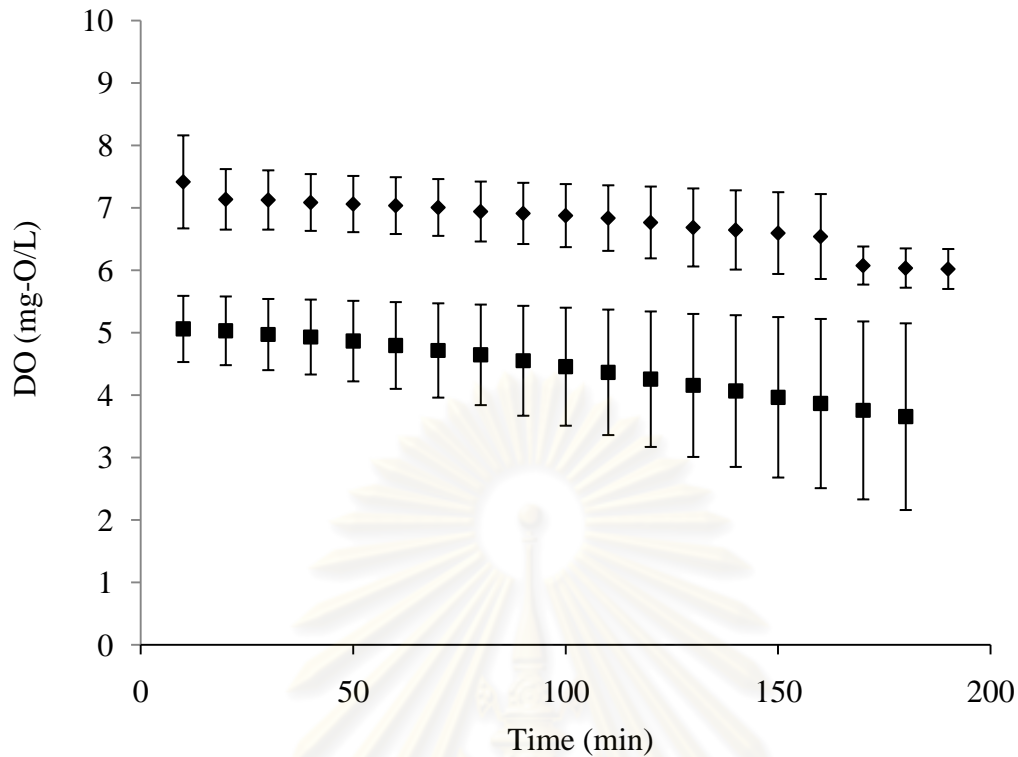
Figure 4.6 Dissolved oxygen concentrations from the tests at 28 (♦) and 70 (■) mg-N/L with AgNP concentrations of 5 mg/L

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-O_2/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-O_2/L/min and SOUR of 0.21 to 0.44 mg-O_2/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.
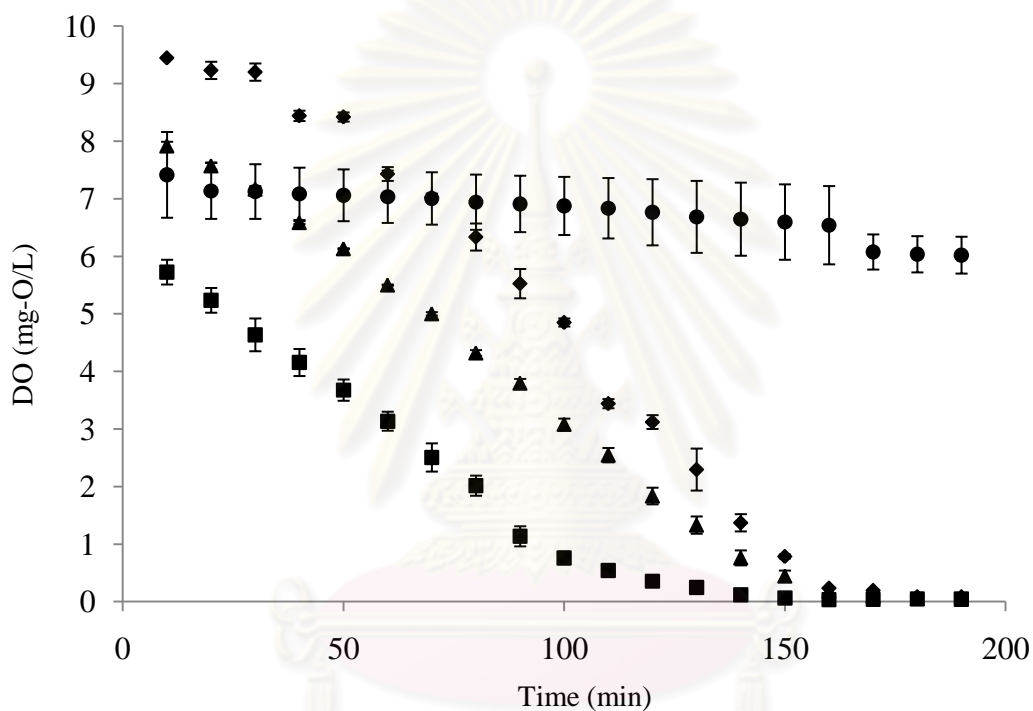
Figure 4.7 Dissolved oxygen concentrations from the tests at 28 mg-N/L with AgNP concentrations of 0 mg/L (♦), 0.05 mg/L (■), 0.5 mg/L (▲), and 5 mg/L (●)
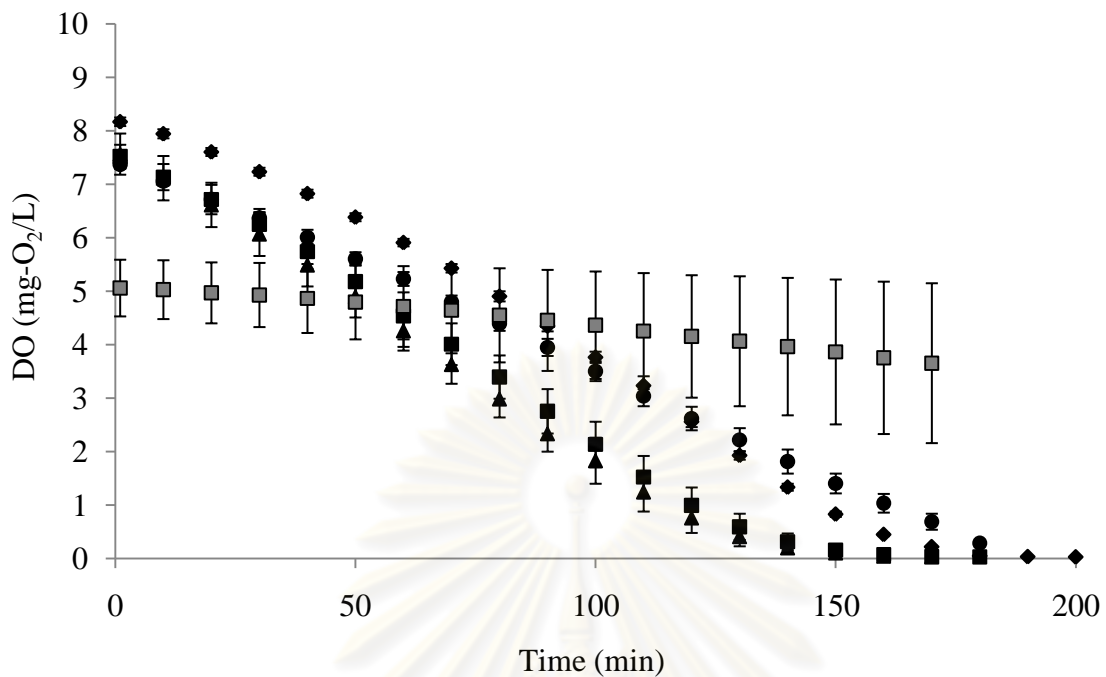
Figure 4.8 Dissolved oxygen concentrations from the tests at 70 mg-N/L with AgNP concentrations of 0 mg/L (♦), 0.05 mg/L (■), 0.5 mg/L (▲), 1 mg/L (●), and 5 mg/L ( ) ▇

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-O_2/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 leaded to poorer nitrification performance. Another reason could be because entrapment environment initiate or promote toxicity of AgNPs.
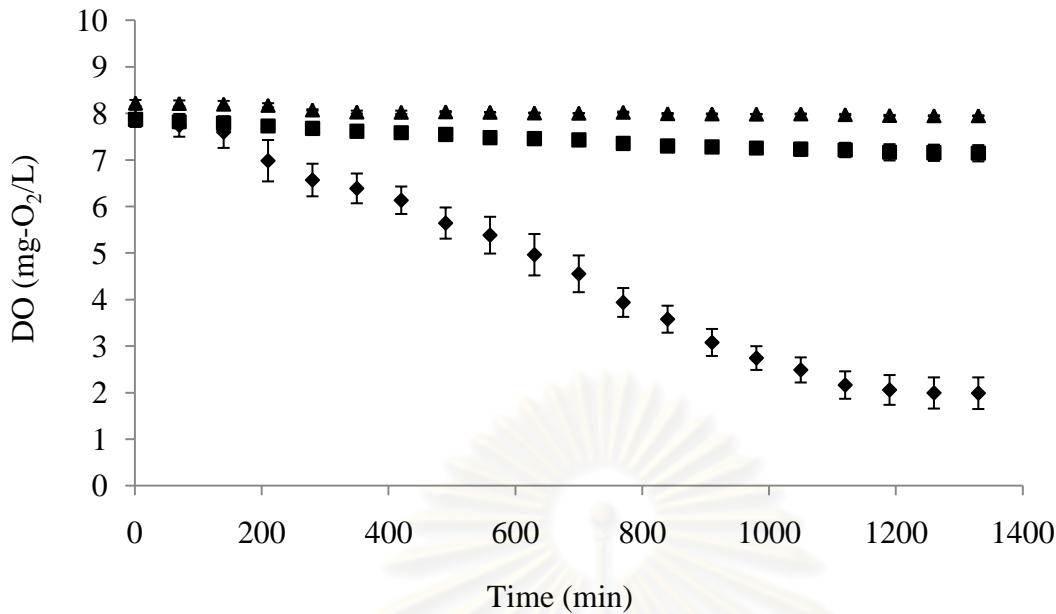
Figure 4.9 Dissolved oxygen concentrations from small PVA-entrapped cell tests with AgNP concentrations of 0 mg/L (♦), 0.5 mg/L (■), and 1 mg/L (●)

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-O_2/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.
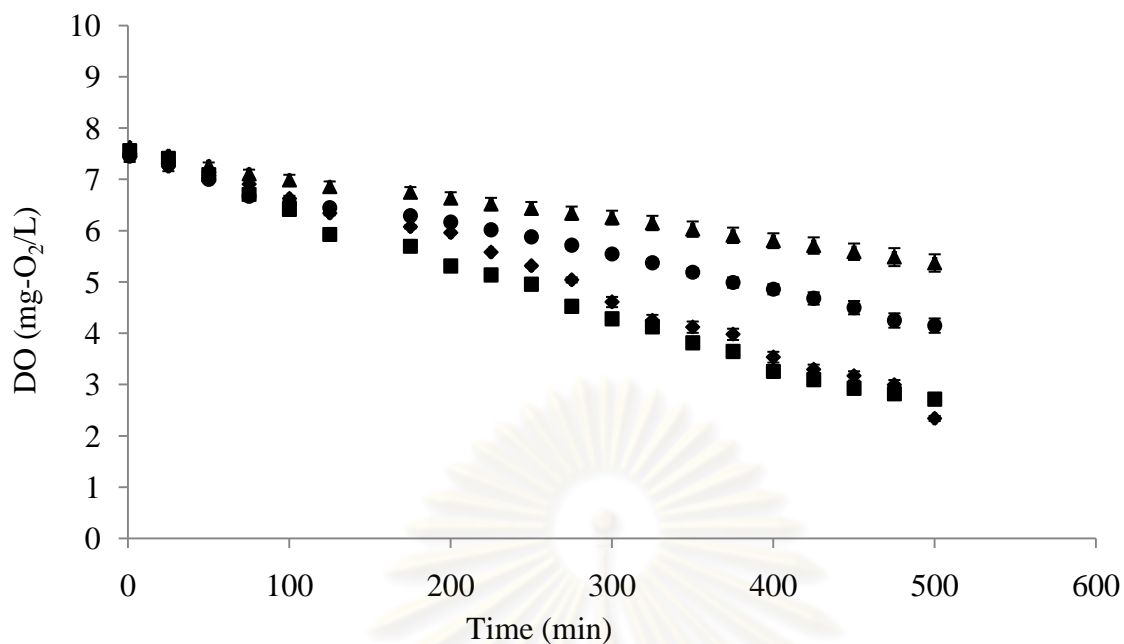
Figure 4.10 Dissolved oxygen concentrations from large PVA-entrapped cell tests with AgNP concentrations of 0 mg/L (♦), 0.5 mg/L (■), 1 mg/L (▲), and 5 mg/L (●)

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-O_2/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-O_2/L/min. The nitification activities in the tests with AgNPs were 64-109% of control. Silver nanoparticles affected nitrification performance. Higher AgNP concentrations resulted in lower nitrification activities.
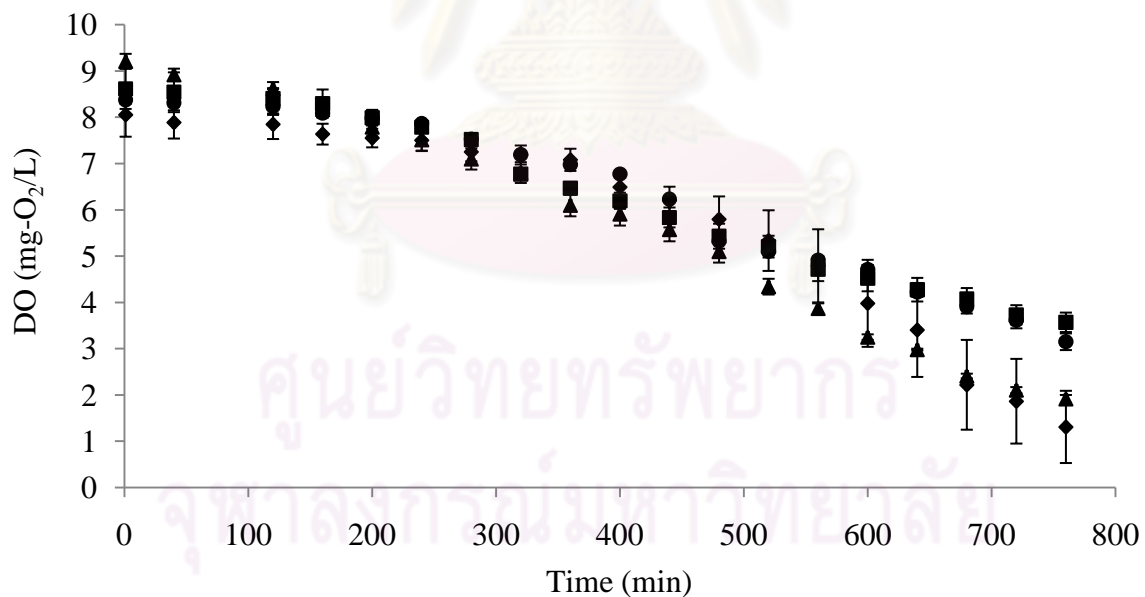
Figure 4.11 Dissolved oxygen concentrations from small CA-entrapped cell tests with AgNP concentrations of 0 mg/L (♦), 0.5 mg/L (■), 1 mg/L (▲), and 5 mg/L (●)

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-O_2/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.
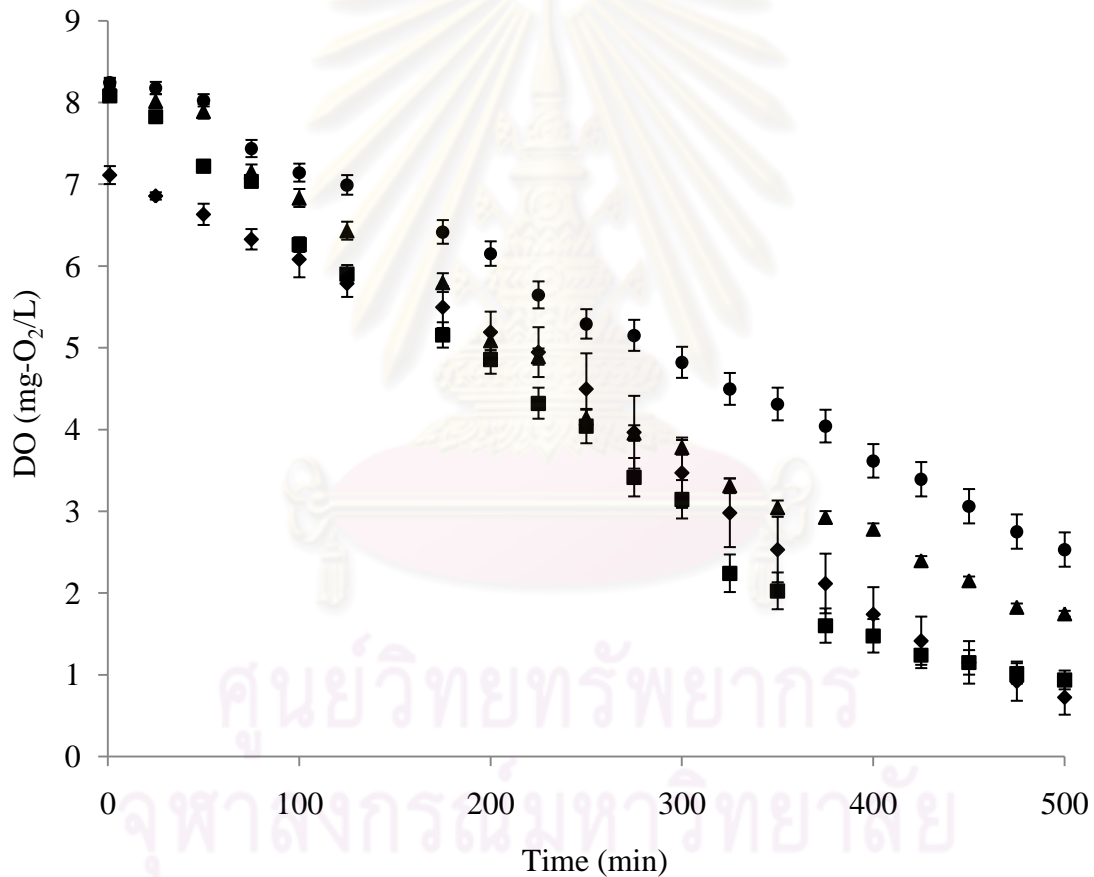
Figure 4.12 Dissolved oxygen concentrations from large CA-entrapped cell tests with AgNP concentrations of 0 mg/L (♦), 0.5 mg/L (■), 1 mg/L (▲), and 5 mg/L (●)

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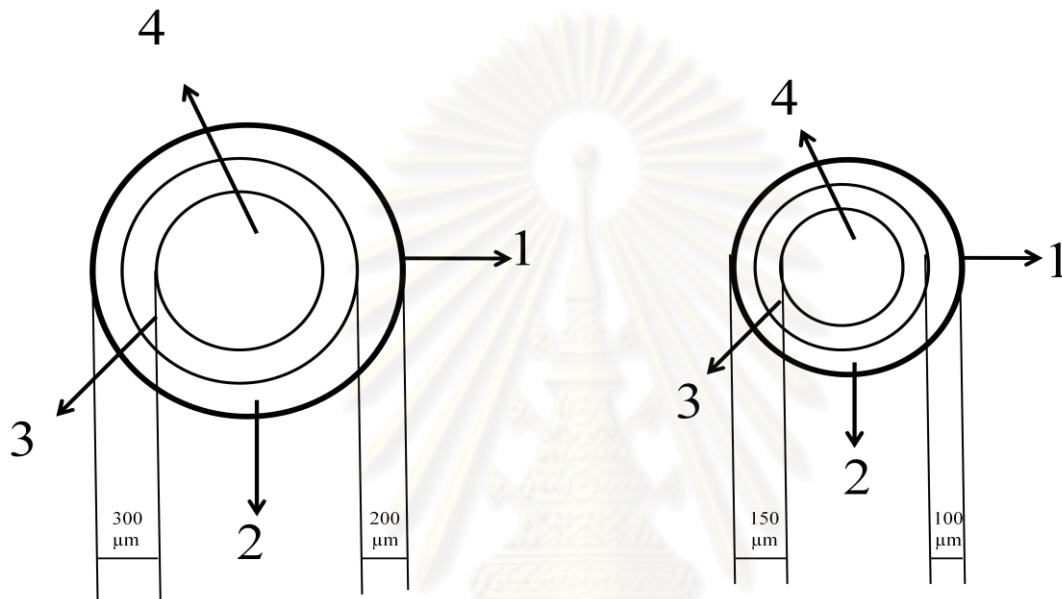
Figure 4.13 Observed location of the entrapped cells (1-surface of the bead, 2-second location (cross-sectional outer layer), 3-third location (cross-sectional inner layer), and 4-forth location (cross-sectional core layer)

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.
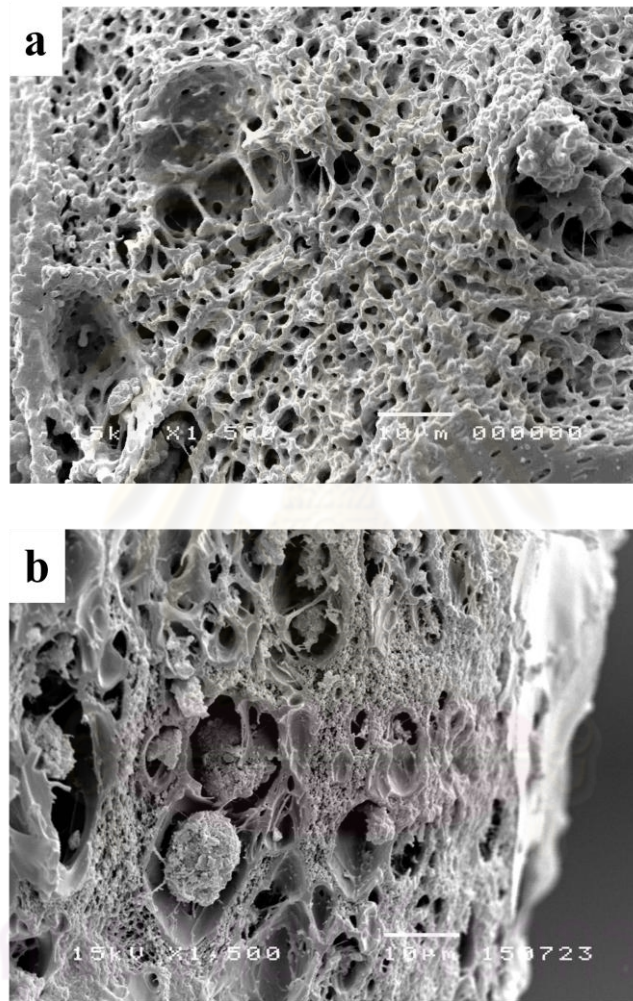
Figure 4.14 SEM images of the PVA-entrapped cells; a) only matrix (no microbial cell) at the second location (1,500×) and b) bead at the second location (1,500×)
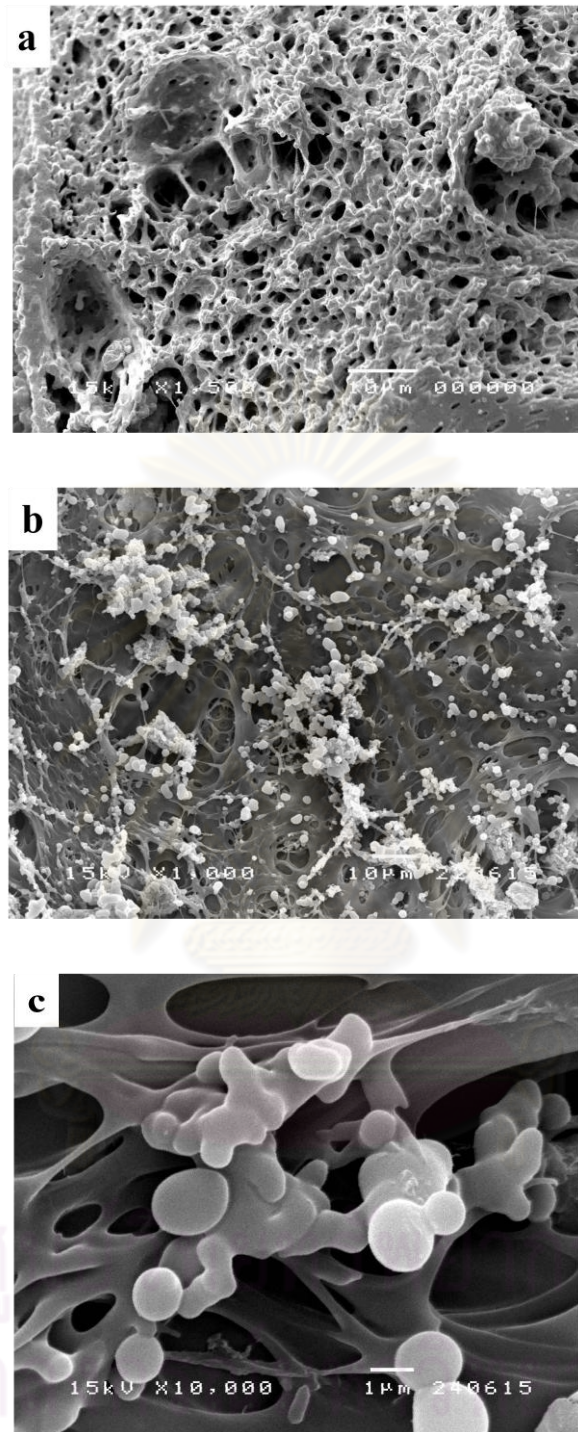
Figure 4.15 SEM images of the large PVA-entrapped cells a) only matrix (no microbial cell) at the second location (1,500×), b) bead from the test with 1 mg/L of AgNPs at the second location (1,000×), and c) bead from the test with 1 mg/L of AgNPs at the forth location (10,000×)
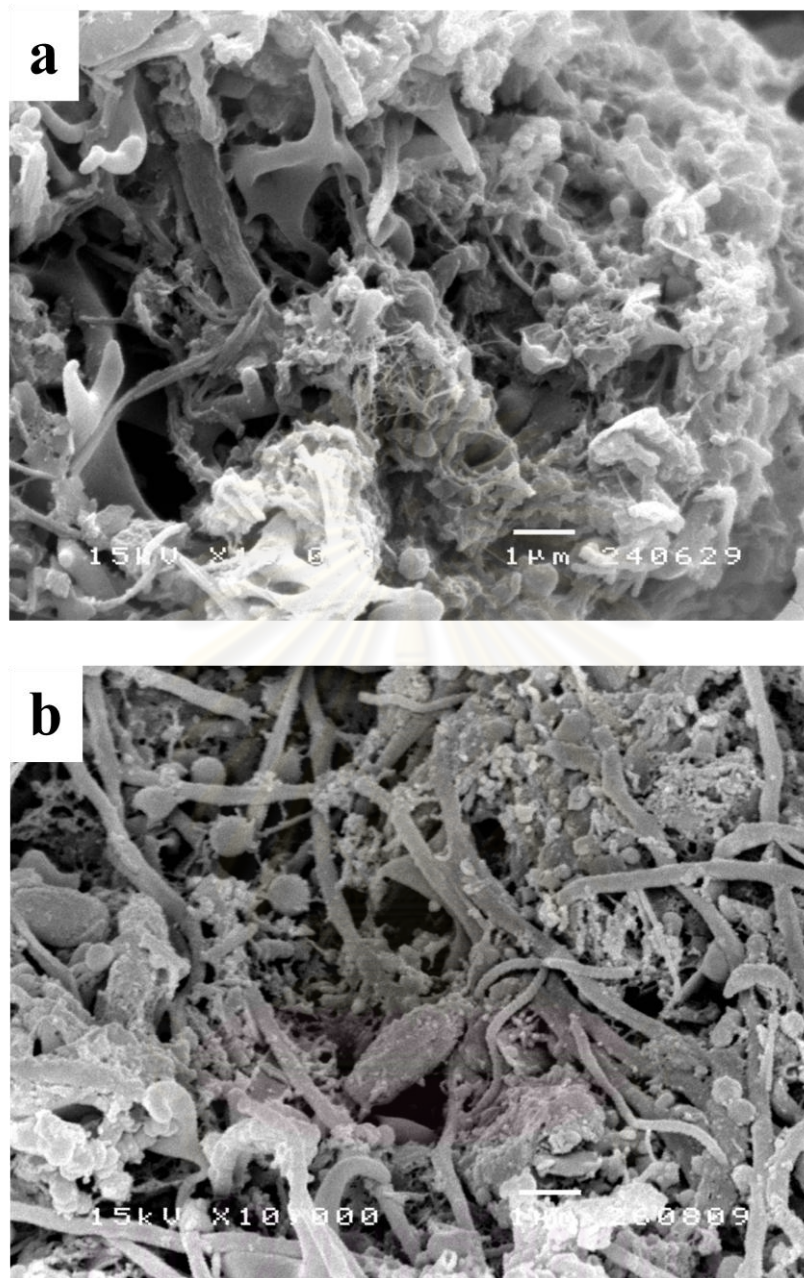
Figure 4.16 SEM images of the small PVA-entrapped cells; a) bead from the test with 0 mg/L of AgNPs and b) bead from the test with 1 mg/L of AgNPs from the forth location (10,000×)

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. (Firgure 4.18). Similarly to PVA-entrapped cells, it was found numerous microorganisms, such as filamentous and rod colonies.
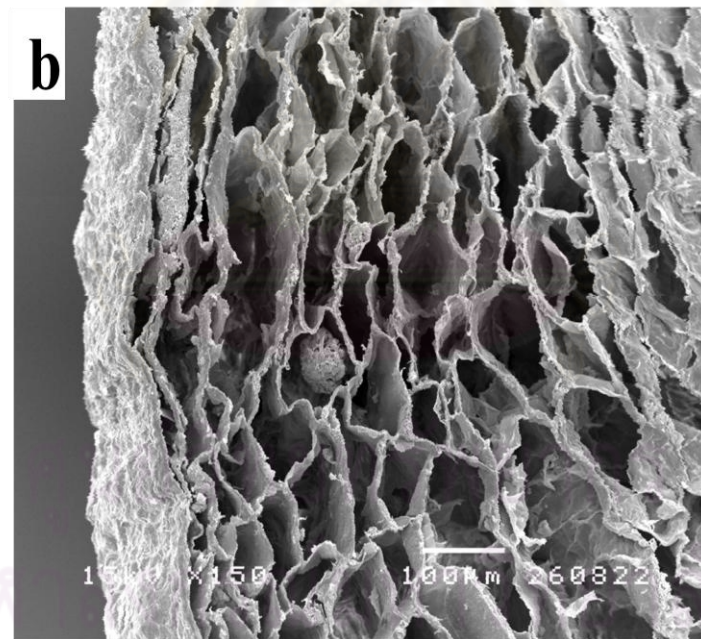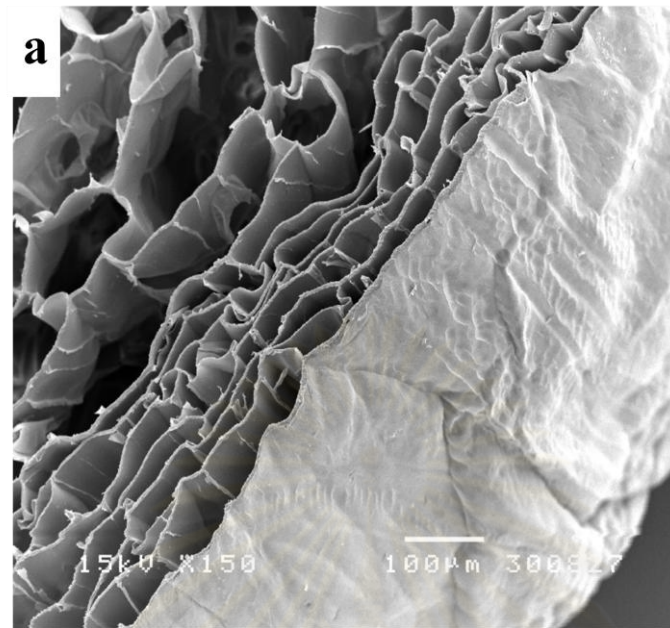
Figure 4.17 SEM images of the CA-entrapped cells; a) only matrix (no microbial cell) and b) bead before apply into the respirometer at the second location (150×)
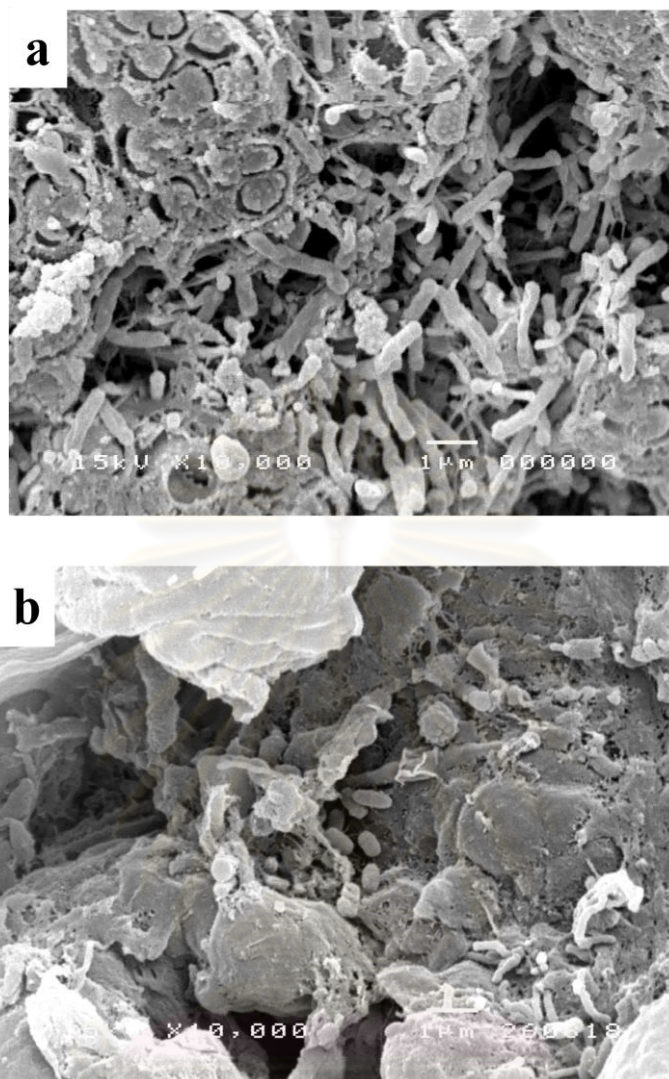
Figure 4.18 SEM images of the small CA-entrapped cells; a) bead from the test with 0 mg/L of AgNPs at the second location, b) bead from the test with 0.5 mg/L of AgNPs at the third location, c) bead from the test with 1 mg/L of AgNPs at the forth location, and d) bead from the test with 5 mg/L of AgNPs the third location (10,000×)

4.6 Transmission electron microscopic observation

The spherical synthetic AgNPs at 14 nm was 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 cell 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.
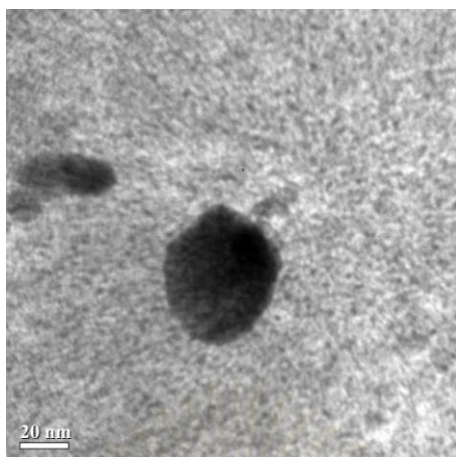
Figure 4.19 TEM images of the spherical synthetic AgNPs
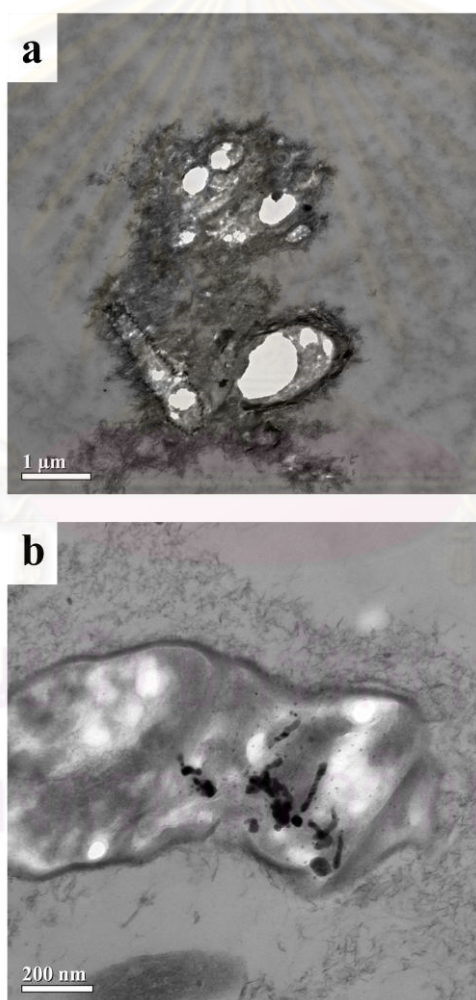
Figure 4.20 TEM images of free cells treated from the test with 5 mg/L of AgNPs
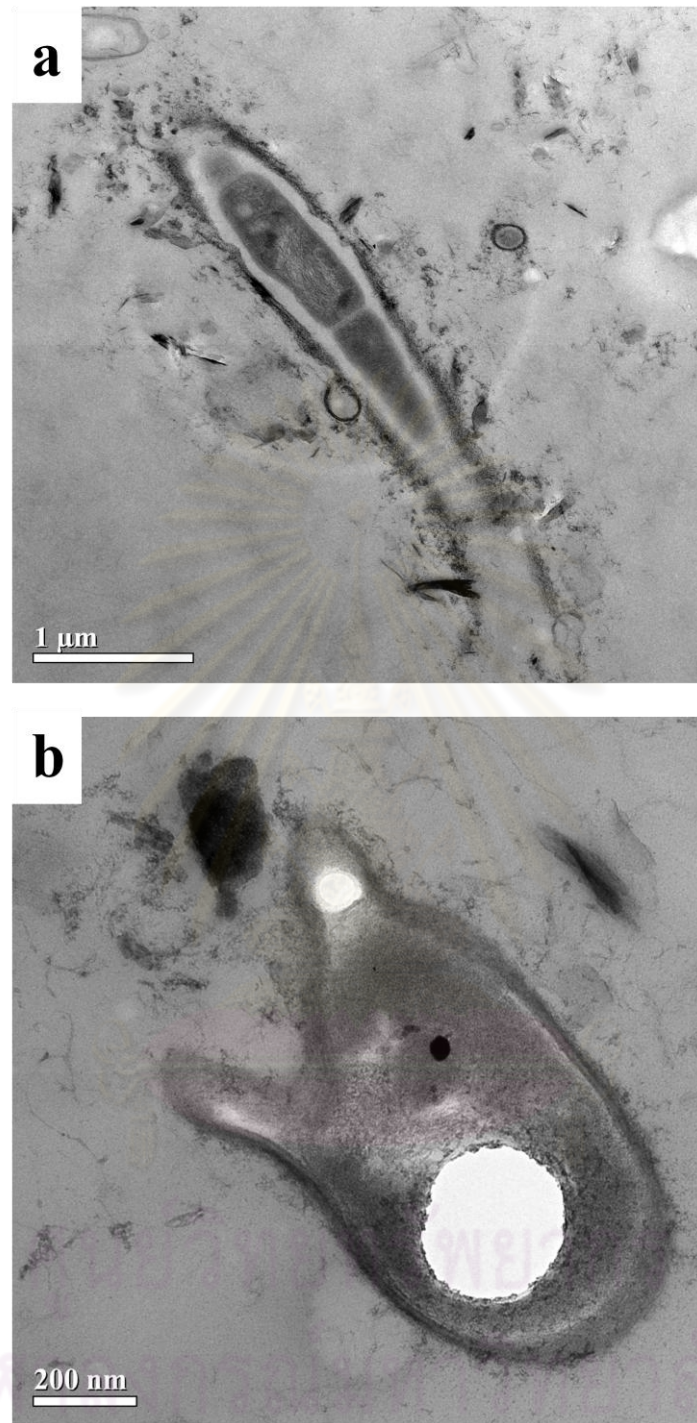
Figure 4.21 TEM images of separated cells from PVA entrapped cells treated from the test with 5 mg/L of AgNPs

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The effect of silver nanoparticles on the nitrification process was investigated. The nitrification activities performances were tested with free cells and the entrapped cells. The PVA and CA-entrapped cells were applied. The CA-entrapped cells represented of the natural matrices material while PVA-entrapped cells expressed in the synthetic matrices material. The results were concluded as followed.

1. The effects of initial ammonia (28 mg/L and 70 mg/L) and AgNP (0, 0.05, 0.5, and 5 mg/L) concentrations on nitrification activity were examined. The result presented that the initial ammonia did not play an important role on the nitrification performance. On the other hand, the effect of initial AgNP concentration apparently affected the nitrification process. Higher AgNP concentration inhibited higher nitrification process. The nitrification performance of the wastewater contaminated with AgNPs indicated by OUR, SOUR and activity values ranged from 0.004-0.168 mg-O_2/L/min, 0.010-0.444 mg-O_2/g-VSS/min, and 2.3-98.5 % of control (no AgNPs), respectively.

2. The nitrification performance of the PVA- and CA-entrapped cells were studied. The nitrification performance by the PVA- and CA-entrapped cells indicated by OUR and activity values ranged from 0.0003-0.0144 and 0.0090-0.0240 mg-O_2/L/min and 4.5-87.5 and 64.4-93.8% of control (no AgNPs), respectively. The CA-entrapped cells showed better performances on the nitrification activity than the PVA-entrapped cells. The activities of the large CA-entrapped cell in the tests with AgNP concentration at 5 mg/L were twice higher than those of the PVA-entrapped cells. This could be that CA- entrapment provided better environment for microorganisms resulting in higher nitrification performances. Additionally, AgNPs may interact on Ca^{2+} ion in the CA-entrapped cells causing the fewer amounts of AgNPs in the tests with CA.

3. Large entrapped cells (both PVA- and CA-entrapment) performed better than small entrapped cells. It is because AgNPs penetrated thoroughly in smaller bead.

4. Based on microscopic observation, pelnty of microorganisms were observed. Higher AgNPs concentration resulted in more movement of microoraganisms.

5. Agglomeration of AgNPs with PVA was observed. This observation supported that better nitrification performance by the PVA entrapped cells compared to free cells was from the matrices physically and chemically protected the microbial cells from AgNPs. Physically, the matrices worked as a wall for entrapping AgNPs. Chemically, the matrices could bind with AgNPs attributing in less cell damage by AgNPs.

6. CA-entrapped cells were protected from AgNPs by physical property. The matrices acted as a wall for entrapping AgNPs.

7. Silver nanoparticles could penetrate into the microbial cells. The cell membrane damaged after treating in wastewater contaminated with AgNPs. The free cell membrane and cytoplasm were more brutal damage compared to cells separated from PVA entrapment.

5.2 Recommendations

The continued works should be performed as follows.

1. The viability of microorganism affected by AgNPs should be performed for both free and entrapped cells.

2. The change in microbial community and effect of AgNPs on individual microorganisms is also needed.

3. The durability of the entrapped cells for long term test should be performed.

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APPENDICES

APPENDIX A

No.	Test	Dissolved Oxygen concentration (mg-O_2/L)
1	growth medium	8.34±0.03
2	growth medium with plain beads	7.85±0.05
3	growth medium with AgNPs	8.06±0.04
4	growth medium with plain beads and AgNPs	8.30±0.05
5	AgNPs with DI	7.93±0.04
6	AgNPs with plain beads	8.46±0.05

Table A.1 Dissolved oxygen concentration from respirometer setup test

APPENDIX B

Table B.1 Ammonia reduction and nitrate production tests

Cycle	Sample	NH$_3$ concentration (mg-N/L)			NO$_3^-$ concentration (mg-N/L)		
		Influent	Effluent	Reduction	Influent	Effluent	Production
1	Sample 1	58.9956	0.2984	58.6972	39.2765	104.1667	64.8902
	Sample 2	51.7062	0.2543	51.4519	26.0336	80.1034	54.0698
2	Sample 1	56.7312	0.2407	56.4904	43.5724	133.0749	89.5026
	Sample 2	56.5104	0.2778	56.2326	36.7248	108.8501	72.1253
3	Sample 1	55.5358	0.2190	55.3168	43.6693	114.0181	70.3488
	Sample 2	50.8062	0.1979	50.6083	40.6331	90.2778	49.6447
4	Sample 1	59.8366	0.3594	59.4773	44.5736	97.7067	53.1331
	Sample 2	48.1006	0.1648	47.9358	31.5568	84.1408	52.5840
5	Sample 1	53.4399	0.4910	52.9489	36.8863	98.0297	61.1434
	Sample 2	45.1918	0.2703	44.9215	27.1318	94.9612	67.8295
6	Sample 1	55.4696	0.7040	54.7656	35.6912	111.7571	76.0659
	Sample 2	52.9168	0.2432	52.6736	31.7183	86.0788	54.3605
7	Sample 1	51.9737	0.5754	51.3983	32.4612	109.6576	77.1964
	Sample 2	54.6617	0.3502	54.3115	28.1977	90.9238	62.7261
8	Sample 1	57.1176	0.1614	56.9562	22.0284	74.4509	52.4225
	Sample 2	56.6762	0.1366	56.5396	18.9922	68.7984	49.8062

Cycle	Sample	NH$_3$ concentration (mg-N/L)			NO$_3^-$ concentration (mg-N/L)		
		Influent	Effluent	Reduction	Influent	Effluent	Production
9	Sample 1	52.6494	0.3328	52.3165	23.1266	87.5323	64.4057
	Sample 2	58.9179	0.2519	58.6659	24.6770	84.7868	60.1098
10	Sample 1	51.9414	0.5328	51.4086	26.2920	98.5142	72.2222
	Sample 2	60.6303	0.4349	60.1954	23.3204	90.4393	67.1189
11	Sample 1	57.0948	0.3275	56.7673	23.6434	80.5879	56.9444
	Sample 2	59.7811	0.3238	59.4573	22.7067	89.7933	67.0866
12	Sample 1	52.2788	0.7295		21.6731	75.9044	54.2313
	Sample 2	64.7899	0.3847	64.4052	22.3514	73.8049	51.4535
13	Sample 1	60.4722	0.5374	59.9348	26.2920	90.2778	63.9858
	Sample 2	65.2883	0.3089	64.9794	26.8088	96.5762	69.7674
14	Sample 1	55.2062	0.2426	54.9636	28.1331	86.8863	58.7532
	Sample 2	52.6469	0.1944	52.4525	33.8501	90.4393	56.5891
15	Sample 1	59.7512	0.1509	59.6003	34.3023	87.6938	53.3915
	Sample 2	60.2258	0.1299	60.0959	34.8837	102.0672	67.1835
16	Sample 1	59.0464	0.1583	58.8881	30.6848	77.5194	46.8346
	Sample 2	61.6723	0.1796	61.4927	31.8152	103.8437	72.0284
17	Sample 1	55.2062	0.2334	54.972829	39.6318	78.3269	38.6951

Cycle	Sample	NH$_3$ concentration (mg-N/L)			NO$_3^-$ concentration (mg-N/L)		
		Influent	Effluent	Reduction	Influent	Effluent	Production
	Sample 2	65.9622	0.1785	65.7838	32.6550	97.3837	64.7287
18	Sample 1	47.6230	0.1522	47.4708	44.2506	84.3023	40.0517
	Sample 2	49.3708	0.2528	49.1181	38.2106	97.0607	58.8501
19	Sample 1	54.1241	0.2081	53.9160	41.1176	98.0297	56.9121
	Sample 2	55.6468	0.1748	55.4720	36.8863	103.5207	66.6344
20	Sample 1	50.7983	0.1516	50.6468	34.3346	83.0103	48.6757
	Sample 2	49.2129	0.1445	49.0683	34.9483	90.4393	55.4910
21	Sample 1	55.6468	0.1319	55.5149	35.9173	86.4018	50.4845
	Sample 2	55.8678	0.1144	55.7534	37.5000	89.1473	51.6473
22	Sample 1	55.6468	0.2254	55.4214	35.9173	83.4948	47.5775
	Sample 2	55.8678	0.1795	55.6883	37.5000	92.3773	54.8773
23	Sample 1	63.3084	1.8127	63.0830	35.8204	79.6189	43.7984
	Sample 2	67.6781	0.5944	67.4986	31.5568	91.5698	60.0129
24	Sample 1	50.4155	0.3417	48.6028	26.3889	102.0672	75.6783
	Sample 2	64.8174	0.2125	64.2230	33.6240	94.9612	61.3372

Table B.2 Dissolved oxygen concentration from free cells with ammonia concentration at 28 mg-N/L

Time (min)	0 mg/L of AgNPs (mg-O_2/L)		Average (mg-O_2/L)	Difference	|Difference|	0.05 mg/L of AgNPs (mg-O_2/L)		Average (mg-O_2/L)	Difference	|Difference|
10	9.43	9.46	9.45	-0.02	0.02	5.51	5.94	5.73	-0.22	0.22
20	9.38	9.08	9.23	0.15	0.15	5.02	5.45	5.24	-0.22	0.22
30	9.35	9.05	9.20	0.15	0.15	4.35	4.92	4.64	-0.29	0.29
40	8.53	8.35	8.44	0.09	0.09	3.92	4.39	4.16	-0.24	0.24
50	8.50	8.34	8.42	0.08	0.08	3.49	3.86	3.68	-0.19	0.19
60	7.55	7.31	7.43	0.12	0.12	2.97	3.30	3.14	-0.17	0.17
70	7.09	6.95	7.02	0.07	0.07	2.26	2.75	2.51	-0.25	0.25
80	6.57	6.10	6.34	0.24	0.24	1.84	2.19	2.02	-0.18	0.18
90	5.27	5.78	5.53	-0.26	0.26	0.96	1.31	1.14	-0.18	0.18
100	4.92	4.78	4.85	0.07	0.07	0.65	0.87	0.76	-0.11	0.11
110	3.52	3.36	3.44	0.08	0.08	0.48	0.60	0.54	-0.06	0.06
120	3.24	3.00	3.12	0.12	0.12	0.30	0.41	0.36	-0.06	0.06
130	2.66	1.93	2.30	0.37	0.37	0.21	0.28	0.25	-0.04	0.04
140	1.22	1.52	1.37	-0.15	0.15	0.08	0.16	0.12	-0.04	0.04
150	0.82	0.75	0.79	0.03	0.03	0.05	0.08	0.06	-0.02	0.02
160	0.25	0.22	0.24	0.02	0.02	0.02	0.05	0.04	-0.02	0.02
170	0.20	0.19	0.20	0.01	0.01			0.04	0.00	0.00
180	0.09	0.08	0.09	0.00	0.00			0.05	0.00	0.00
190	0.09	0.08	0.09	0.00	0.00			0.05	0.00	0.00
200	0.08	0.07	0.08	0.01	0.01			0.04	0.00	0.00

Time (min)	0.05 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|		1 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|
10	7.84	7.99	7.92	-0.08	0.08		8.16	6.67	7.42	0.75	0.75
20	7.51	7.63	7.57	-0.06	0.06		7.62	6.65	7.14	0.48	0.48
30	7.11	7.22	7.17	-0.05	0.05		7.60	6.65	7.13	0.48	0.48
40	6.56	6.61	6.59	-0.03	0.03		7.54	6.63	7.09	0.46	0.46
50	6.12	6.14	6.13	-0.01	0.01		7.51	6.61	7.06	0.45	0.45
60	5.51	5.49	5.50	0.01	0.01		7.49	6.58	7.04	0.46	0.46
70	5.03	4.97	5.00	0.03	0.03		7.46	6.55	7.01	0.46	0.46
80	4.37	4.27	4.32	0.05	0.05		7.42	6.46	6.94	0.48	0.48
90	3.87	3.72	3.80	0.07	0.07		7.40	6.42	6.91	0.49	0.49
100	3.18	2.98	3.08	0.10	0.10		7.38	6.37	6.88	0.51	0.51
110	2.67	2.42	2.55	0.13	0.13		7.36	6.31	6.84	0.53	0.53
120	1.98	1.69	1.84	0.15	0.15		7.34	6.19	6.77	0.58	0.58
130	1.48	1.18	1.33	0.15	0.15		7.31	6.06	6.69	0.63	0.63
140	0.89	0.63	0.76	0.13	0.13		7.28	6.01	6.65	0.64	0.64
150	0.54	0.35	0.45	0.10	0.10		7.25	5.94	6.60	0.66	0.66
160	0.25	0.12	0.19	0.07	0.07		7.22	5.86	6.54	0.68	0.68
170	0.13	0.07	0.10	0.03	0.03		6.38	5.77	6.08	0.31	0.31
180	0.05	0.05	0.05	0.00	0.00		6.35	5.72	6.04	0.32	0.32
190	0.03	0.05	0.04	-0.01	0.01		6.34	5.70	6.02	0.32	0.32
200	0.02	0.05	0.04	-0.02	0.02		6.32	5.71	6.02	0.31	0.31

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Table B.3 Dissolved oxygen concentration from free cells with ammonia concentration at 70 mg-N/L

Time (min)	0 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|		0.05 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|		0.5 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|
1	8.09	8.25	8.17	-0.08	0.08		7.30	7.74	7.52	-0.22	0.22		7.18	7.95	7.57	-0.39	0.39
10	7.86	8.03	7.95	-0.08	0.08		6.89	7.38	7.14	-0.25	0.25		6.70	7.53	7.12	-0.42	0.42
20	7.53	7.68	7.61	-0.07	0.07		6.44	6.99	6.72	-0.28	0.28		6.20	7.03	6.62	-0.42	0.42
30	7.16	7.31	7.24	-0.08	0.08		5.96	6.54	6.25	-0.29	0.29		5.66	6.48	6.07	-0.41	0.41
40	6.75	6.90	6.83	-0.08	0.08		5.42	6.06	5.74	-0.32	0.32		5.09	5.89	5.49	-0.40	0.40
50	6.31	6.46	6.39	-0.08	0.08		4.84	5.52	5.18	-0.34	0.34		4.51	5.29	4.90	-0.39	0.39
60	5.84	5.98	5.91	-0.07	0.07		4.10	4.98	4.54	-0.44	0.44		3.89	4.63	4.26	-0.37	0.37
70	5.35	5.51	5.43	-0.08	0.08		3.62	4.40	4.01	-0.39	0.39		3.27	3.99	3.63	-0.36	0.36
80	4.81	5.00	4.91	-0.10	0.10		2.99	3.80	3.40	-0.41	0.41		2.64	3.33	2.99	-0.35	0.35
90	4.25	4.45	4.35	-0.10	0.10		2.34	3.17	2.76	-0.42	0.42		2.00	2.67	2.34	-0.34	0.34
100	3.66	3.87	3.77	-0.11	0.11		1.71	2.56	2.14	-0.43	0.43		1.40	2.26	1.83	-0.43	0.43
110	3.06	3.41	3.24	-0.18	0.18		1.13	1.92	1.53	-0.40	0.40		0.88	1.61	1.25	-0.37	0.37
120	2.46	2.66	2.56	-0.10	0.10		0.66	1.33	1.00	-0.34	0.34		0.48	1.04	0.76	-0.28	0.28
130	1.85	2.01	1.93	-0.08	0.08		0.35	0.84	0.60	-0.25	0.25		0.23	0.59	0.41	-0.18	0.18
140	1.27	1.40	1.34	-0.06	0.06		0.16	0.47	0.32	-0.16	0.16		0.10	0.30	0.20	-0.10	0.10
150	0.78	0.88	0.83	-0.05	0.05		0.07	0.25	0.16	-0.09	0.09		0.05	0.16	0.11	-0.06	0.06
160	0.42	0.48	0.45	-0.03	0.03		0.03	0.12	0.08	-0.05	0.05		0.02	0.07	0.05	-0.03	0.03
170	0.20	0.24	0.22	-0.02	0.02		0.02	0.06	0.04	-0.02	0.02		0.01	0.05	0.03	-0.02	0.02
180	0.04	0.06	0.05	-0.01	0.01		0.02	0.04	0.03	-0.01	0.01						
190	0.02	0.05	0.04	-0.02	0.02												
200	0.02	0.04	0.03	-0.01	0.01												
210	0.02	0.04	0.03	-0.01	0.01												

Time (min)	1 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|	5 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|
1	7.29	7.45	7.37	-0.08	0.08	5.59	4.53	5.06	-0.53	0.53
10	6.97	7.15	7.06	-0.09	0.09	5.58	4.48	5.03	-0.55	0.55
20	6.62	6.82	6.72	-0.10	0.10	5.54	4.40	4.97	-0.57	0.57
30	6.27	6.47	6.37	-0.10	0.10	5.53	4.33	4.93	-0.60	0.60
40	5.87	6.15	6.01	-0.14	0.14	5.51	4.22	4.87	-0.65	0.65
50	5.48	5.73	5.61	-0.13	0.13	5.49	4.10	4.80	-0.70	0.70
60	5.10	5.36	5.23	-0.13	0.13	5.47	3.96	4.72	-0.76	0.76
70	4.68	4.92	4.80	-0.12	0.12	5.45	3.84	4.65	-0.81	0.81
80	4.26	4.53	4.40	-0.14	0.14	5.43	3.67	4.55	-0.88	0.88
90	3.79	4.11	3.95	-0.16	0.16	5.40	3.51	4.46	-0.95	0.95
100	3.32	3.69	3.51	-0.19	0.19	5.37	3.36	4.37	-1.01	1.01
110	2.85	3.23	3.04	-0.19	0.19	5.34	3.17	4.26	-1.09	1.09
120	2.40	2.84	2.62	-0.22	0.22	5.30	3.01	4.16	-1.15	1.15
130	2.00	2.44	2.22	-0.22	0.22	5.28	2.85	4.07	-1.22	1.22
140	1.59	2.04	1.82	-0.23	0.23	5.25	2.68	3.97	-1.29	1.29
150	1.22	1.59	1.41	-0.19	0.19	5.22	2.51	3.87	-1.36	1.36
160	0.86	1.21	1.04	-0.18	0.18	5.18	2.33	3.76	-1.43	1.43
170	0.54	0.84	0.69	-0.15	0.15	5.15	2.16	3.66	-1.50	1.50
180		0.29	0.29	0.00	0.00					
190										
200										
210										

Table B.4 Dissolved oxygen concentration from the small PVA-entrapped cells

Time (min)	0 mg/L of AgNPs (mg-O_2/L)		Average (mg-O_2/L)	Difference	|Difference|		0.05 mg/L of AgNPs (mg-O_2/L)		Average (mg-O_2/L)	Difference	|Difference|		1 mg/L of AgNPs (mg-O_2/L)		Average (mg-O_2/L)	Difference	|Difference|
1	7.71	8.08	7.90	-0.19	0.19		7.90	7.84	7.87	0.03	0.03		8.16	8.29	8.23	-0.06	0.06
70	7.50	7.99	7.75	-0.25	0.25		7.79	7.85	7.82	-0.03	0.03		8.15	8.28	8.22	-0.06	0.06
140	7.26	7.94	7.60	-0.34	0.34		7.74	7.84	7.79	-0.05	0.05		8.13	8.27	8.20	-0.07	0.07
210	6.54	7.43	6.99	-0.45	0.45		7.69	7.77	7.73	-0.04	0.04		8.13	8.22	8.18	-0.04	0.04
280	6.22	6.92	6.57	-0.35	0.35		7.64	7.72	7.68	-0.04	0.04		8.09	8.06	8.08	0.01	0.01
350	6.07	6.71	6.39	-0.32	0.32		7.59	7.64	7.62	-0.02	0.02		8.06	8.00	8.03	0.03	0.03
420	5.84	6.43	6.14	-0.30	0.30		7.56	7.61	7.59	-0.03	0.03		8.06	7.99	8.03	0.04	0.04
490	5.31	5.98	5.65	-0.34	0.34		7.52	7.58	7.55	-0.03	0.03		8.05	8.02	8.04	0.02	0.02
560	4.99	5.78	5.39	-0.40	0.40		7.47	7.49	7.48	-0.01	0.01		8.02	8.03	8.03	-0.01	0.01
630	4.52	5.41	4.97	-0.45	0.45		7.45	7.47	7.46	-0.01	0.01		8.00	8.02	8.01	-0.01	0.01
700	4.16	4.95	4.56	-0.40	0.40		7.43	7.43	7.43	0.00	0.00		8.00	8.01	8.01	-0.01	0.01
770	3.63	4.25	3.94	-0.31	0.31		7.40	7.31	7.36	0.05	0.05		8.01	8.04	8.03	-0.02	0.02
840	3.29	3.87	3.58	-0.29	0.29		7.39	7.21	7.30	0.09	0.09		7.98	8.01	8.00	-0.01	0.01
910	2.79	3.37	3.08	-0.29	0.29		7.38	7.18	7.28	0.10	0.10		7.98	8.00	7.99	-0.01	0.01
980	2.49	3.00	2.75	-0.26	0.26		7.38	7.13	7.26	0.13	0.13		7.98	7.99	7.99	0.00	0.00
1050	2.22	2.76	2.49	-0.27	0.27		7.37	7.09	7.23	0.14	0.14		8.00	7.98	7.99	0.01	0.01
1120	1.87	2.46	2.17	-0.30	0.30		7.37	7.06	7.22	0.16	0.16		7.99	7.96	7.98	0.01	0.01
1190	1.74	2.38	2.06	-0.32	0.32		7.34	6.99	7.17	0.18	0.18		7.97	7.94	7.96	0.01	0.01
1260	1.66	2.33	2.00	-0.34	0.34		7.33	6.98	7.16	0.18	0.18		7.96	7.94	7.95	0.01	0.01
1330	1.65	2.33	1.99	-0.34	0.34		7.32	6.97	7.15	0.18	0.18		7.96	7.94	7.95	0.01	0.01

Table B.5 Dissolved oxygen concentration from the large PVA-entrapped cells

Time (min)	0 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|	0.5 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|
1	7.55	7.34	7.45	0.11	0.11	7.55	7.57	7.56	-0.01	0.01
25	7.34	7.16	7.25	0.09	0.09	7.40	7.42	7.41	-0.01	0.01
50	7.15	6.99	7.07	0.08	0.08	7.11	7.07	7.09	0.02	0.02
75	6.98	6.83	6.91	0.08	0.08	6.72	6.69	6.71	0.01	0.01
100	6.68	6.57	6.63	0.05	0.05	6.43	6.40	6.42	0.01	0.01
125	6.38	6.30	6.34	0.04	0.04	5.92	5.93	5.93	0.00	0.00
175	6.11	6.04	6.08	0.04	0.04	5.68	5.71	5.70	-0.01	0.01
200	5.98	5.94	5.96	0.02	0.02	5.27	5.35	5.31	-0.04	0.04
225	5.57	5.59	5.58	-0.01	0.01	5.09	5.18	5.14	-0.04	0.04
250	5.29	5.34	5.32	-0.03	0.03	4.90	5.01	4.96	-0.05	0.05
275	4.99	5.09	5.04	-0.05	0.05	4.47	4.58	4.53	-0.05	0.05
300	4.51	4.71	4.61	-0.10	0.10	4.21	4.35	4.28	-0.07	0.07
325	4.14	4.36	4.25	-0.11	0.11	4.05	4.20	4.13	-0.08	0.08
350	4.01	4.23	4.12	-0.11	0.11	3.73	3.90	3.82	-0.09	0.09
375	3.87	4.09	3.98	-0.11	0.11	3.55	3.74	3.65	-0.10	0.10
400	3.43	3.64	3.54	-0.11	0.11	3.15	3.36	3.26	-0.11	0.11
425	3.20	3.39	3.30	-0.10	0.10	2.98	3.21	3.10	-0.12	0.12
450	3.08	3.26	3.17	-0.09	0.09	2.82	3.04	2.93	-0.11	0.11
475	2.90	3.09	3.00	-0.09	0.09	2.71	2.93	2.82	-0.11	0.11
500	2.29	2.39	2.34	-0.05	0.05	2.61	2.82	2.72	-0.11	0.11

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Time (min)	1 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|	5 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|
1	7.58	7.68	7.63	-0.05	0.05	7.49	7.42	7.46	0.04	0.04
25	7.42	7.51	7.47	-0.04	0.04	7.32	7.27	7.30	0.03	0.03
50	7.20	7.33	7.27	-0.06	0.06	7.03	6.97	7.00	0.03	0.03
75	7.03	7.19	7.11	-0.08	0.08	6.70	6.64	6.67	0.03	0.03
100	6.89	7.09	6.99	-0.10	0.10	6.56	6.51	6.54	0.03	0.03
125	6.76	6.96	6.86	-0.10	0.10	6.48	6.41	6.45	0.04	0.04
175	6.65	6.85	6.75	-0.10	0.10	6.33	6.25	6.29	0.04	0.04
200	6.75	6.52	6.64	0.12	0.12	6.21	6.12	6.17	0.04	0.04
225	6.64	6.40	6.52	0.12	0.12	6.05	5.98	6.02	0.04	0.04
250	6.56	6.31	6.44	0.13	0.13	5.93	5.83	5.88	0.05	0.05
275	6.21	6.47	6.34	-0.13	0.13	5.78	5.65	5.72	0.06	0.06
300	6.12	6.39	6.26	-0.14	0.14	5.62	5.47	5.55	0.08	0.08
325	6.01	6.29	6.15	-0.14	0.14	5.45	5.30	5.38	0.08	0.08
350	5.88	6.18	6.03	-0.15	0.15	5.27	5.11	5.19	0.08	0.08
375	5.76	6.06	5.91	-0.15	0.15	5.09	4.89	4.99	0.10	0.10
400	5.66	5.95	5.81	-0.15	0.15	4.96	4.76	4.86	0.10	0.10
425	5.55	5.87	5.71	-0.16	0.16	4.80	4.56	4.68	0.12	0.12
450	5.43	5.75	5.59	-0.16	0.16	4.63	4.37	4.50	0.13	0.13
475	5.31	5.66	5.49	-0.18	0.18	4.39	4.11	4.25	0.14	0.14
500	5.20	5.54	5.37	-0.17	0.17	4.29	4.01	4.15	0.14	0.14

Table B.6 Dissolved oxygen concentration from the small CA-entrapped cells

Time (min)	0 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|	0.5 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|
1	7.58	8.52	8.05	-0.47	0.47	8.18	9.05	8.62	-0.44	0.44
40	7.54	8.23	7.89	-0.35	0.35	8.11	8.97	8.54	-0.43	0.43
120	7.53	8.16	7.85	-0.32	0.32	8.05	8.76	8.41	-0.35	0.35
160	7.41	7.86	7.64	-0.23	0.23	7.99	8.60	8.30	-0.31	0.31
200	7.35	7.75	7.55	-0.20	0.20	7.82	8.16	7.99	-0.17	0.17
240	7.27	7.73	7.50	-0.23	0.23	7.67	7.90	7.79	-0.12	0.12
280	7.05	7.45	7.25	-0.20	0.20	7.48	7.56	7.52	-0.04	0.04
320	6.98	7.39	7.19	-0.20	0.20	6.87	6.67	6.77	0.10	0.10
360	6.84	7.32	7.08	-0.24	0.24	6.60	6.33	6.47	0.14	0.14
400	6.21	6.77	6.49	-0.28	0.28	6.37	6.02	6.20	0.18	0.18
440	5.90	6.50	6.20	-0.30	0.30	6.05	5.62	5.84	0.22	0.22
480	5.30	6.29	5.80	-0.50	0.50	5.70	5.16	5.43	0.27	0.27
520	4.68	5.99	5.34	-0.66	0.66	5.44	4.97	5.21	0.24	0.24
560	4.00	5.58	4.79	-0.79	0.79	4.97	4.46	4.72	0.26	0.26
600	3.04	4.92	3.98	-0.94	0.94	4.80	4.24	4.52	0.28	0.28
640	2.39	4.42	3.41	-1.02	1.02	4.53	4.02	4.28	0.26	0.26
680	1.25	3.19	2.22	-0.97	0.97	4.31	3.84	4.08	0.23	0.23
720	0.95	2.78	1.87	-0.92	0.92	3.94	3.52	3.73	0.21	0.21
760	0.53	2.09	1.31	-0.78	0.78	3.78	3.36	3.57	0.21	0.21

Time (min)	1 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|		5 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|
1	9.37	9.05	9.21	0.16	0.16		8.29	8.46	8.38	-0.09	0.09
40	9.05	8.79	8.92	0.13	0.13		8.15	8.48	8.32	-0.16	0.16
120	8.62	8.62	8.62	0.00	0.00		8.08	8.39	8.24	-0.16	0.16
160	8.04	8.30	8.17	-0.13	0.13		8.00	8.18	8.09	-0.09	0.09
200	7.59	7.99	7.79	-0.20	0.20		7.90	8.07	7.99	-0.09	0.09
240	7.28	7.75	7.52	-0.23	0.23		7.79	7.94	7.87	-0.08	0.08
280	6.87	7.33	7.10	-0.23	0.23		7.48	7.58	7.53	-0.05	0.05
320	6.58	7.03	6.81	-0.23	0.23		7.15	7.24	7.20	-0.04	0.04
360	5.86	6.34	6.10	-0.24	0.24		6.95	7.01	6.98	-0.03	0.03
400	5.66	6.16	5.91	-0.25	0.25		6.73	6.82	6.78	-0.04	0.04
440	5.32	5.83	5.58	-0.26	0.26		6.21	6.25	6.23	-0.02	0.02
480	4.86	5.34	5.10	-0.24	0.24		5.38	5.27	5.33	0.05	0.05
520	4.17	4.51	4.34	-0.17	0.17		5.17	5.04	5.11	0.07	0.07
560	3.76	3.98	3.87	-0.11	0.11		5.00	4.83	4.92	0.09	0.09
600	3.19	3.31	3.25	-0.06	0.06		4.81	4.61	4.71	0.10	0.10
640	2.96	3.00	2.98	-0.02	0.02		4.33	4.12	4.23	0.11	0.11
680	2.46	2.36	2.41	0.05	0.05		4.06	3.76	3.91	0.15	0.15
720	2.17	2.04	2.11	0.06	0.06		3.80	3.44	3.62	0.18	0.18
760	2.00	1.83	1.92	0.09	0.09		3.33	2.97	3.15	0.18	0.18

Table B.7 Dissolved oxygen concentration from the large CA-entrapped cells

Time (min)	0 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|	0.5 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|
1	7.00	7.22	7.11	-0.11	0.11	8.05	8.11	8.08	-0.03	0.03
25	6.81	6.90	6.86	-0.04	0.04	7.78	7.87	7.83	-0.04	0.04
50	6.50	6.76	6.63	-0.13	0.13	7.18	7.26	7.22	-0.04	0.04
75	6.20	6.45	6.33	-0.13	0.13	6.98	7.09	7.04	-0.05	0.05
100	5.86	6.30	6.08	-0.22	0.22	6.17	6.35	6.26	-0.09	0.09
125	5.62	5.95	5.79	-0.17	0.17	5.79	6.01	5.90	-0.11	0.11
175	5.24	5.75	5.50	-0.26	0.26	5.00	5.31	5.16	-0.16	0.16
200	4.94	5.44	5.19	-0.25	0.25	4.68	5.03	4.86	-0.18	0.18
225	4.64	5.25	4.95	-0.31	0.31	4.13	4.51	4.32	-0.19	0.19
250	4.06	4.93	4.50	-0.44	0.44	3.83	4.25	4.04	-0.21	0.21
275	3.52	4.41	3.97	-0.45	0.45	3.18	3.65	3.42	-0.24	0.24
300	3.04	3.90	3.47	-0.43	0.43	2.91	3.38	3.15	-0.24	0.24
325	2.56	3.40	2.98	-0.42	0.42	2.01	2.47	2.24	-0.23	0.23
350	2.13	2.93	2.53	-0.40	0.40	1.80	2.25	2.03	-0.23	0.23
375	1.75	2.48	2.12	-0.37	0.37	1.39	1.81	1.60	-0.21	0.21
400	1.41	2.07	1.74	-0.33	0.33	1.27	1.68	1.48	-0.21	0.21
425	1.12	1.71	1.42	-0.30	0.30	1.08	1.40	1.24	-0.16	0.16
450	0.89	1.41	1.15	-0.26	0.26	1.00	1.30	1.15	-0.15	0.15
475	0.68	1.16	0.92	-0.24	0.24	0.89	1.14	1.02	-0.13	0.13
500	0.51	0.94	0.73	-0.22	0.22	0.82	1.05	0.94	-0.12	0.12

Time (min)	1 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|	5 mg/L of AgNPs (mg-O$_2$/L)		Average (mg-O$_2$/L)	Difference	|Difference|
1	8.30	8.14	8.22	0.08	0.08	8.20	8.29	8.25	-0.04	0.04
25	8.10	7.93	8.02	0.09	0.09	8.10	8.25	8.18	-0.07	0.07
50	7.98	7.80	7.89	0.09	0.09	7.95	8.10	8.03	-0.07	0.07
75	7.24	7.06	7.15	0.09	0.09	7.33	7.54	7.44	-0.11	0.11
100	6.94	6.72	6.83	0.11	0.11	7.03	7.25	7.14	-0.11	0.11
125	6.54	6.32	6.43	0.11	0.11	6.87	7.11	6.99	-0.12	0.12
175	5.91	5.68	5.80	0.12	0.12	6.27	6.56	6.42	-0.15	0.15
200	5.20	4.97	5.09	0.12	0.12	6.00	6.30	6.15	-0.15	0.15
225	4.99	4.79	4.89	0.10	0.10	5.48	5.81	5.65	-0.17	0.17
250	4.24	4.04	4.14	0.10	0.10	5.11	5.47	5.29	-0.18	0.18
275	4.05	3.86	3.96	0.10	0.10	4.96	5.34	5.15	-0.19	0.19
300	3.87	3.69	3.78	0.09	0.09	4.63	5.01	4.82	-0.19	0.19
325	3.40	3.22	3.31	0.09	0.09	4.30	4.69	4.50	-0.20	0.20
350	3.13	2.96	3.05	0.09	0.09	4.11	4.51	4.31	-0.20	0.20
375	3.00	2.85	2.93	0.07	0.07	3.84	4.24	4.04	-0.20	0.20
400	2.85	2.71	2.78	0.07	0.07	3.41	3.82	3.62	-0.21	0.21
425	2.45	2.34	2.40	0.06	0.06	3.18	3.60	3.39	-0.21	0.21
450	2.20	2.10	2.15	0.05	0.05	2.85	3.27	3.06	-0.21	0.21
475	1.87	1.78	1.83	0.05	0.05	2.54	2.96	2.75	-0.21	0.21
500	1.78	1.71	1.75	0.04	0.04	2.32	2.74	2.53	-0.21	0.21

BIOGRAPHY

Miss Chutima Ploychankul was born on December 24th, 1983 in Bangkok, Thailand. She enrolled Biochemistry class at Chulalongkorn University in 2003. During the time in, she has been joined in the International Association for the Exchange of Student for Technical Experience (IAESTE), Thailand for internship at the Max-Planck Institute of Molecular Plant Physiology, Potsdam, Germany for 6 months. After recieving B.Sc. degree in Biochemistry, she pursued her Master degree in international program in the field of Environmental and Hazardous Waste Management, Chulalongkorn University in May 2008 to October 2010. During the period, she got a chance joining an international conference, Asian-Pacific Regional Conference on Practical Environmental Technology (APRC 2010), to present her research entitled "INFLUENCE OF SILVER NANOPARTICLE ON NITRIFICA PERFORMANCE IN WASTEWATER TREATMENT SYSTEM".