Biotechnol. Bioprocess Eng. **1997**, 2, 77-81

Enzyme Immobilized Reactor Design for Ammonia Removal from Waste Water

Ju Yeong Song* and Soo Bae Chung

Department of Chemical technology, Changwon National University, Changwon 641-773, Korea

Removal of nitrogen compound from waste water is essential and often accomplished by biological process. To prevent washout and to develop an efficient bioreactor, immobilization of suitable microorganisms could be sensible approach. Strains and permeabilized cells encapsulated in cellulose nitrate microcapsules and immobilized on polystyrene films were prepared by the method described in the previous study. In the wastewater treatment system, nitrification of ammonia component is generally known as rate controlling step. To enhance the rate of nitrification, firstly nitrifying strains *Nitrosomonas europaea* (IFO14298), are permeabilized chemically, and immobilized on polystyrene films and secondly oxidation rates of strain system and permeabilized strain system are compared in the same condition. With 30 minute permeabilized cells, it took about 25 hours to oxidize 70% of ammonia in the solution, while it took about 40 hours to treat same amount of ammonia with untreated cells. All the immobilization procedures did not harm to the enzyme activity and no mass transfer resistance through the capsule wall was shown. In the durability test of immobilized system, the system showed considerable activity for the repeated operation for 90 days. With these results, the system developed in this study showed the possibility to be used in the actual waste water treatment system.

Key words: *Nitrosomonas europaea*, permeabilization, microencapsulation, immobilization

INTRODUCTION

The main sources of nitrogen are fertilizer, human wastes and industrial waste water, and 60% of nitrogen in the urban waste water is ammonia. Ammonia can cause eutrophication and consume much oxygen in the nitrification process [1]. There are several methods to remove ammonia from waste water, such as, breakpoint chlorination, ammonia stripping, selective ion exchange and biological treatment system. Recently, activated sludge system, one of biological treatment system, is widely used for the waste water treatment [2]. But, *Nitrosomonas europaea* (*N. europaea*), one of nitrifying strains, have very low growth rate and sometimes are washed out from the treatment reactor. These reasons make nitrifying step as a rate controlling step in the nitrogen removal system [3]. In the continuous biological treatment process for the nitrification, denitrification and phosphorus removal, the retention time of the whole system is strongly influenced by the nitrification process as shown in earlier study [5]. For the enhanced nitrification rate of ammonia, pure strains, *N. europaea*, are immobilized in several carriers and showed good results with physical and chemical stability [3, 4, 11]. And also, nitrifying strains, *N. europaea*, encapsulated in the cellulose nitrate microcapsules and immobilized on polystyrene film showed considerable activity and stability in the previous study.

The chemolithoautotrophic ammonia oxidizing bacterium obtains all of its energy for growth by the oxidation of ammonia to nitrite [13]. The initial oxidation of ammonia, which yields hydroxylamine as the product, is O_2 dependent reaction catalyzed by ammonia monooxygenase (AMO) which exist in plasma membrane:

$$NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O \quad (1)$$

Hydroxylamine is further oxidized to nitrite by hydroxylamine oxidoreductase (HAO) which exist in periplasma [7]:

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (2)$$

Two of the four electrons generated from the hydroxylamine oxidation are used to support the oxidation of additional ammonia molecules, while the other two electrons enter the electron transfer chain and are used to support CO_2 reduction and ATP biosynthesis. Despite the key role of AMO plays in initiating biological nitrification, the enzyme has not been purified, and little is known about its structure, enzyme mechanism, or the identity and the function of its cofactors. In the cell extraction process, the activity of AMO is reduced largely and most of the enzyme characteristics are disappeared in the presence of protease [8]. This can be largely attributed to the difficulty of working with and assaying the enzyme in cell-free systems. The most convenient assay for measuring AMO activity is to monitor the rate of ammonia consumption or nitrite production. These reasons forced to try to apply cell permeabilization technique to acquire AMO and HAO activity without complete cell extraction and purification [9]. In this study, ammonia oxidizing bacteria are permeabilized physically and chemically, and

* Corresponding author
Tel: 0551-79-7585 FAX: 0551-83-6465
e-mail: jusong@sarim.changwon.ac.kr

the activities of both system are compared with each other. The nitrifying ability of strains permeabilized and encapsulated in cellulose nitrate microcapsules, and strains permeabilized, encapsulated and immobilized on polystyrene film were also compared in the batch and continuous reactors.

MATERALS AND METHODS

Microorganism and Growth Conditions

Experiments were performed with *N. europaea* grown in a medium shown in Table 1 under controlled conditions of 30°C and pH 8 in a shaking incubator (VS 8480SR Vision Scientific Co.) for flask culture. For fermentor culture, 600 mL of flask cultured strain solution are added in the 3L of cultural media in a fermentor (BF-3000 Bock Sung Engineering Co.). Ten percent of K_2CO_3 solution is added intermittently to sustain pH 7.9 and 1.5 L/min of sterilized air is supplied to keep the dissolved oxygen concentration of 7-8 ppm. It takes about two months to harvest a considerable amount of strains to conduct experiment in the fermentor culture.

Chemical Permeabilization

Mechanical and ultrasonic disruption methods suffer from several drawbacks. Because cells are broken completely in the mechanical and ultrasonic homogenizing methods, all intracellular materials should be released. The product of interest must be separated from a complex mixture of proteins, nucleic acids, and cell wall fragments to apply the components to the treatment system. Culture broth was harvested after two months culture and separated under the controlled condition of 4°C and 15,000 rpm for 15 minutes centrifugation. After washing with sterilized saline solution, treat the strain solution by contacting 5% of acetone for 5 minutes and 30 minutes respectively. Two other samples were also prepared in the same procedure except acetone treatment to see the activity difference between permeabilized cells and normal cells. Keep all the samples in the 4°C of refrigerator for further use.

Quantitative Analysis of Protein

Lowry method is used for quantitative analysis of protein. This method is applied to validate the degree of permeabilization of *N. europaea*. Lowry test reagents are prepared according to the protocol and the absorbance of treated sample is measured at the optical density of 750 nm [12].

Nitrification Ability Test of Permeabilized *N. europaea*

Batch experiments were conducted with untreated strains, and treated strains with 5% of acetone for 5 and 30 minutes respectively. 10mL of 70 ppm ammonia solution is introduced into a 20 mL of batch reactor. The pH of reaction mixture was controlled to pH 8.1-8.3 by adding 0.075 g of $CaCO_3$. The biomass concentration of all three cases is 300 ppm by dry weight base. Aeration velocity was 1.11 mL/sec to sustain dissolved oxygen concentration of 6.6 to 7.3 ppm, and the temperature of reaction mixture was controlled to 30°C. Aliquots were taken at intervals to determine the concentration of ammonia in the reaction mixture.

Table 1. Nutrient composition of the culture media

$(NH_4)_2SO_4$	3 g
K_2HPO_4	0.5 g
$MgSO_4$	0.05 g
$CaCl_2$	4 mg
Fe/EDTA solution	0.1 mL
Cresol red (0.0005%)	25 mL
DW	1 L

Nitrification Ability Test of Microencapsulated Strains

The method of Chang *et al.* [14] was used to form cellulose nitrate microcapsules containing permeabilized *N. europaea*. Fig. 1 illustrates the experimental apparatus that was used for testing the nitrification ability of permeabilized strains and permeabilized microencapsulated strains. 40 mL of ammonia solution, which contain 11 mg of permeabilized *N. europaea* treated for 30 minutes and encapsulated in cellulose nitrate microcapsules, is introduced into a batch reactor in the same way mentioned in upper section. Air is supplied with a velocity of 4.5 mL/min to maintain dissolved oxygen concentration of 7 ppm. The result was compared to the result of the nitrification ability test of permeabilized *N. europaea* in the same condition.

Immobilization of Microcapsules on Polystyrene Films

One important aspect of the utilization of enzyme or permeabilized strains in water treatment applications is the means by which the biomass is immobilized such that it can be available to catalyze the desired reactions. In the case of enhancing the oxidation of ammonia in water, the permeabilized strains must be available to convert the ammonia to nitrate or nitrite in the solution, just as it is in the activated sludge system. Thus, a means was needed to immobilize the permeabilized and encapsulated *N. europaea* to the surface of suitable carriers. A 4 cm × 7 cm in segment of polystyrene film was cut and unfolded onto a glass plate. Pieces of 0.3 cm copper wire, 7 cm length, were placed on polystyrene film on both end. These acted as spacers upon which glass slides were placed to form a nar-

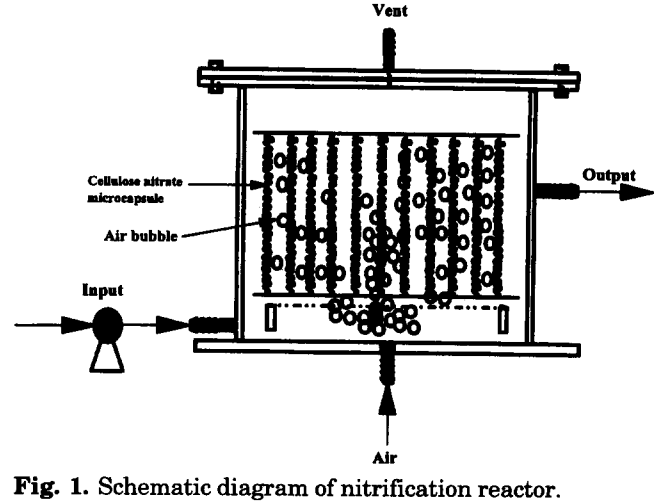

Fig. 1. Schematic diagram of nitrification reactor.

row gap above the polystyrene film. 10 mL of microcapsule suspension in sterilized saline solution was injected between the glass slide and polystyrene film and was left in the clean bench. As a result, starting with 60 mg of strains by dry weight, approximately 12 mg of permeabilized strains were immobilized on both side of polystyrene film of 10 sheets.

Nitrification Ability Test of Immobilized Strains

Nitrification process is generally known as a rate controlling step in the continuous biological waste water treatment system. The result of microencapsulated strain system also showed the same trend in the previous study [5]. Polystyrene films, which are treated as mentioned in the upper section, are introduced into the same reactor shown in Fig. 1 for batch and continuous operation of permeabilized, microencapsulated and immobilized strains. For continuous operation, 20, 40 and 70 ppm of initial concentration of ammonia solution were introduced into the reactor with the velocity of 2.67, 2.35 and 2.27 mL/hr respectively. These velocities are based on the result of batch operation. Operational conditions for batch and continuous system were basically same to the nitrification ability test condition of microencapsulated strains. The inlet and outlet solution of the shunt were analyzed in every hour until the outlet ammonia concentration gets the steady state condition. Retention time of each operation is also recorded when the system reached at steady state.

Analytical Methods

All reaction product were analyzed by Ion Chromatography (Shimadzu, LC10/M10A, Tokyo) using a Shimpack IC-C1 column and conductivity detector CDD-6A. The column temperature was maintained constant at 40°C. 5 mM of nitric acid was used as a mobile phase at a flow rate of 1.5 mL/min.

RESULTS AND DISCUSSION

Quantitative Analysis of Protein

Microorganism could be permeabilized in order to use enzymes without purification by various methods: treatment with organic solvents, or detergent, osmotic shock and enzymatic treatment [13]. In this work, *N. europaea* was permeabilized with organic solvent acetone. The result of Lowry test is shown in Fig. 2. This figure shows the obvious difference between control, 5 minute and 30 minute permeabilized strain solution. As the permeabilization time is increasing, more permeabilized strains were acquired. This result means that the substrate is more easily accessible to AMO and HAO in plasma membrane of *N. europaea*. 30 minute permeabilized strains with 5% acetone were chosen as the best condition in this system by the preliminary experiment.

Nitrification Ability Test of Permeabilized *N. europaea*

Nitrifying strain, *N. europaea*, is oxidizing ammonia to nitrate or nitrite in the aerobic atmosphere. But, the nitrifying step is figured out as a rate controlling step

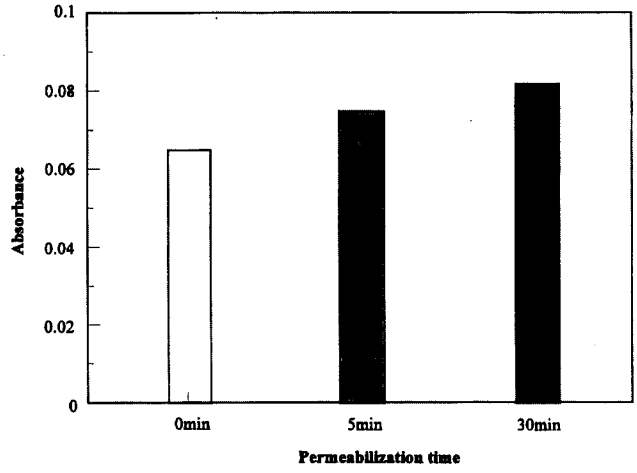

Fig. 2. Absorption difference between control and permeabilized cells.

in the continuous waste water treatment system in the previous study. And, as is known as, nitrification rate is under the control of the enzyme AMO and HAO in the plasma membrane of *N. europaea*. In this work, permeabilization method was applied to enhance the nitrification rate of ammonia, and showed the result in Fig. 3. With 30 minute treated cells, it took about 25 hours to oxidize 70% of ammonia in the solution, while it took about 28 hours and 40 hours to treat same amount of ammonia with the 5 minute treated cells and untreated cells respectively. This result showed the feasibility of permeabilized *N. europaea* to be used in the oxidation of ammonia.

To ensure the durable stability of nitrifying enzyme, 20 ppm of ammonia solution is added after the completion of the first trial. The second trial was conducted about 50 hours time elapse after the first trial. As shown in Fig. 4, the stability and durable effect of enzyme AMO and HAO in the plasma membrane are confirmed.

Nitrification Ability Test of Immobilized Strains

A typical particle size and capsule wall thickness of cellulose nitrate microcapsule are 11 μm and 4000Å

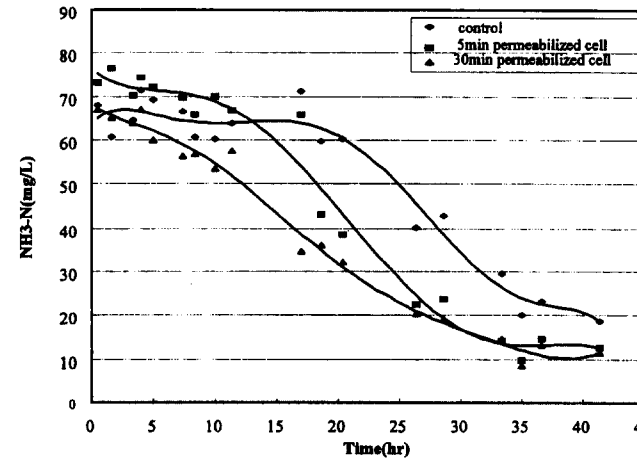

Fig. 3. Nitrification rate of normal cells and permeabilized cells in a batch reactor.

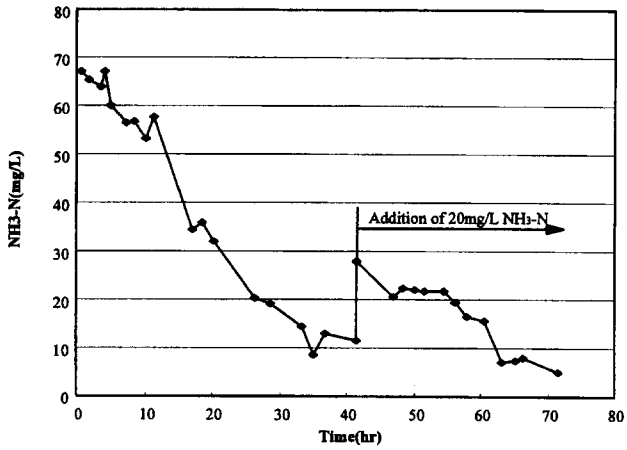

Fig. 4. Nitrification ability of permeabilized cells.

respectively [10]. Mass transfer resistance of microencapsulated biomass system is of obvious importance. Mass transfer resistance through the capsule wall is studied by comparing the nitrification ability of 30 minute permeabilized cells and 30 minute

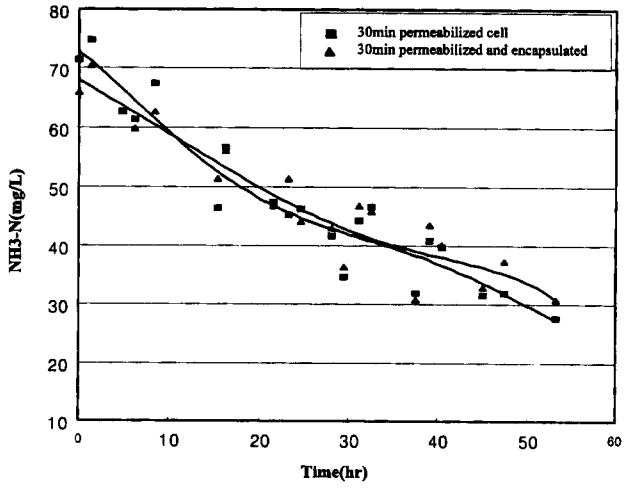

Fig. 5. Comparison of nitrification rate of 30 minute permeabilized cells and permeabilized microencapsulated cells.

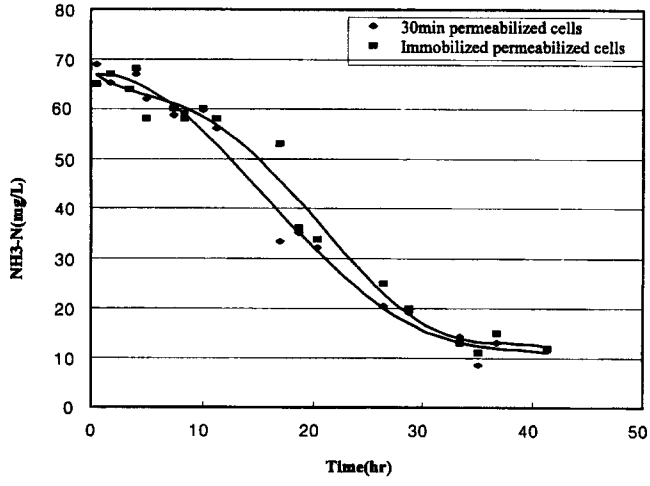

Fig. 6. Comparison of nitrification rate of 30 minute permeabilized cells and permeabilized immobilized cells.

Table 2. The result of nitrification rate of continuous operation

Input conc. (mg NH_3-N/L)	Output conc. (mgNH_3-N/L)	Retention time (hr)
20	3	15
40	5	17
70	5	22

permeabilized and encapsulated *N. europaea*. The biomass weight of two systems is the same and is about 12 mg in each case. In Fig. 5, no obvious difference is shown between two systems. This result means that there's no mass transfer resistance through the capsule wall.

30 minute permeabilized and immobilized cells on polystyrene film did not show the difference in the nitrification ability as compared with 30 minute permeabilized cells as shown in Fig. 6. This result also means that all the procedures of immobilization are no harm to the enzyme activity.

The result of continuous operation is given in Table 2. Various initial concentration of ammonia can be oxidized to under 5 ppm in the retention time of 20 hours. When we applied once immobilized *N. europaea* to the treatment of various concentration of ammonia solution, the strains still have considerable activity for the repeated operation for 90 days. In a conclusion, we got the possibility to apply the system developed in this study to the actual waste water treatment system.

Acknowledgment This work was funded by the Biochemical Engineering Research Fund (Project number: F-26, Ministry of Education of Korea). Authors deeply appreciate financial support.

REFERENCES

[1] Wisesmann, U. (1994) Biological Nitrogen Removal from Wastewater. *Advances in Biochem. Engineering Biotechnol.* 51: 114-119.

[2] Kim, C. W. (1995) N, P removal by continuous batch reactor and aeration methods. *J. of Environmental Hi-Technology*.2/12

[3] Wijffels, R. H. and J. Tramper (1989) Performances of growing *Nitrosomonas europaea* cells immobilized in k-carreginan. *Appl. Microbiol. Biotechnol.* 32: 108-112.

[4] Wijffels, R. H., G. Englund, and J. H. Hunik (1995) Effect of Diffusion Limitation on Immobilized Nitrifying Microorganisms at Low Temperatures. *Biotechnol. Bioengin.* 45: 1-9.

[5] Song J. Y. and Lee S. H. (1996) Biological removal of nitrogen and phosphorus from waste water by microencapsulated strains. *Korean J. of Biotechnol. Bioeng.* 12/3 269275.

[6] Alleman, J. E. (1985) Elevated nitrite occurrence in biological wastewater treatment. *Water Science and Technology*. 17: 409.

[7] Bergmann, D. J. and D. M. Arciero (1994) Organization of the hao Gene cluster of *N. europaea*: Genes for two tetraheme c cytochromes. *Journal of Bacteriology*. 176: 3148-3153.

[8] Hyman, M. R. and D. J. Arp (1995) Effect of ammonia on the De Novo synthesis of polypeptides in cells of *N. europaea* Denied Ammonia as an Energy source. *Journal of Bacteriology*. 177/17: 4971-

4979.
[9] Hansrued, F. (1982) Permeabilized cells. *Analytical Biochemistry*. 120: 211-234.
[10] Song, J. Y. (1991) Immobilized carbonic anhydrase in a membrane lung for enhanced carbon dioxide removal. Ph.D thesis, Dept. of Chemical Engineering, wayne state University, Mi., U.S.A.
[11] Kokufuta, E. and W. Matsumoto (1982) Immobilization of *N. somonas* cells with polyelectrolyte complex. *Biotechnol. And Bioengineer.* 23: 1591-1603.
[12] Korea Institute of Biochemistry (1997) Experiments in Biochemistry. 2nd ed. P201, Tam Ku Dang, Seoul.
[13] Kim, W. J., J. K. Park and H. S. Kim (1991) Continuous production of gluconic acid and sorbitol from glucose and fructose using permeabilized cells of *Zymomonas mobilis*. *Kor. J. Microbiol. Biotechnol.* 19/1: 70-75.
[14] Chang, T. M. S. (1966) Semipermeable aqueous microcapsules. *Canadian Journal of Physiology and Pharmacology*. 44: 115-128.