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ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF AN IMMOBILIZED BACTERIUM PRODUCING N_2 FROM NH_4^+ UNDER AN AEROBIC CONDITION

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Abstract : To treat wastewater efficiently by a one-step process of nitrogen removal, a new bacterial strain producing (N_2) gas from NH_4^+ under an aerobic condition was isolated and identified. The cell was motile and a Gram-negative rod, and usually occurred in pairs. By 16S-rDNA analysis, the isolated strain was identified as *Enterobacter asburiae* with 96% similarity. The isolate showed that the capacity of N_2 production under an oxic condition was approximately three times higher than that under an anoxic condition. Thus, the consumption of NH_4^+ by the isolate was significantly different in the metabolism of N_2 production under the two different environmental conditions. The optimal conditions of the immobilized isolate for N_2 production were found to be pH 7.0, 30°C and C/N ratio 5, respectively.

Under all the optimum reaction conditions, N_2 production by the immobilized isolate resulted in reduction of ORP with both the consumption of DO and the drop of pH. The removal efficiencies of COD_{Cr} and TN were 56.1 and 60.9%, respectively. The removal rates of COD_{Cr} and TN were the highest for the first 2.5 hrs with the removal COD_{Cr}/TN ratios of 32.1, and afterwards the rates decreased as reaction proceeded. For application of the immobilized isolate to a practical process of ammonium removal, a continuous operation was executed with a synthetic medium of a low C/N ratio. The continuous bioreactor system exhibited a satisfactory performance at 12.1 hrs of HRT, in which the effluent concentrations of NH_4^+-N was measured to be 15.4 mg/L with its removal efficiency of 56.0%. The maximum removal rate of NH_4^+-N reached 1.6 mg NH_4^+-N/L/hr at 12.1 hrs of HRT (with N loading rate of 0.08 Kg-N/m^3-carrier/d). As a result, the application of the immobilized isolate appears a viable alternative to the nitrification-denitrification processes.

Key Words : Wastewater treatment, One-step process of ammonium removal, Aerobic ammonium removal, Immobilized cell, *Enterobacter*

INTRODUCTION

The presence of nitrogenous substances in wastewater discharges has attracted much attention because of the role of nitrogen in eutrophication of receiving waters. Nitrogen removal is an important aspect of wastewater treatment processes while biological nitrification-denitrification poses one of the most economical processes for nitrogen removal from municipal wastewaters.[1] The nitrogenous substances in

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municipal wastewater is mostly in the form of ammonium ion requiring a treatment process involving the biological oxidation of NH_4^+ into NO_3^- (nitrification) followed by the biological reduction of NO_3^- into N_2 (denitrification). It has commonly been accepted that nitrification and denitrification require aerobic and anoxic conditions, respectively. However, there have been many precedent reports of anaerobic ammonium oxidation (anammox)[2-3] and aerobic denitrification.[4-7] The nitrification-denitrification process has been also challenged by a one-step process in which ammonium is oxidized directly to N_2.[8-9] Recently, researches focus on nitrite nitrification, which might be a short cut process for savings in oxygen for nitrification and carbon requirements for denitrification.[10-11]

A high cell concentration is possible with immobilization, and thus the volumetric efficiency is greatly increased. This can lead to relatively small reactors.[12] The immobilized cells may afford protection from adverse conditions by creating micro-environments within the gel matrix, which would help maintain year round treatment.[13] In response to the need for the development of a more compact and an efficient system for treatment of wastewater, immobilized-cells processes have been receiving increasing attention in the field of wastewater biodenitrification recently.[14-18] As widely recognized, entrapment of cells in a proper support matrix is an effective means for cell immobilization. Compared to commonly used polymeric substances such as acrylamide, κ-carrageenan, Ca-alginate and agar, polyvinyl alcohol (PVA) has some advantages: cheap chemical cost required for cell immobilization and strong gel strength.[19] In addition, PVA gel beads would not float upward to the solution surface by N_2 production, due to their good gas permeability.[20] For this reason, cell immobilization using PVA has been reported to be successfully applied to the immobilization of denitrifying sludge in the denitrification process.[21]

So far, there has been little research on a one-step process of nitrogen removal in which ammonium is oxidized directly to N_2. In this study, microorganisms producing nitrogen gas from ammonium ion under an aerobic condition were isolated and identified, and the characteristic of the immobilized isolate was investigated in a five-neck flask. To apply the immobilized isolate to a practical process of nitrogen removal, a continuous stirred bioreactor was also executed with a synthetic medium that simulated municipal wastewater.

MATERIALS AND METHODS

Bacterial Culture and Medium

The sludge was obtained from a municipal sewage treatment plant in Busan, Korea. A sludge sample was agitated to obtain homogeneous suspensions in sterile 0.2% NaCl. One milliliter was injected using pipette into a 10 mL screw-cap tube. The tube was filled with a synthetic medium which contained: 1.05 g/L of glucose, 0.38 g/L of NH_4Cl, 0.1 g/L of yeast extract, 0.07 g/L of KH_2PO_4, and 1 mL/L of mineral solution. The mineral solution contained: 3 g/L of $FeSO_4 \cdot 7H_2O$, 0.01 g/L of H_3BO_3, 0.01 g/L of $Na_2MoO_4 \cdot 2H_2O$, 0.02 g/L of $MnSO_4 \cdot H_2O$, 0.01 g/L of $CuSO_4 \cdot 5H_2O$, 0.01 g/L of $ZnSO_4$, and 0.5 g/L of ethylenediamine tetraacetic acid. The pH of the culture medium was adjusted to 7.0 before autoclaving, and the culture medium was sterilized at 121°C for 15 min. After one day of incubation at 30°C, cells were first spread with a platinum loop on a solid agar plate which contained the culture medium and 1.5% nutrient agar. A purified isolate was then obtained by repeated streaking on the fresh agar plates. Each pure culture was maintained on the agar plate at 4°C until use and transferred to a fresh agar plate every month.

Identification of Isolate

Identification of the isolate was carried out using 16S ribosomal DNA (rDNA) analysis. DNA was extracted from cells grown in the given medium with the DNA extraction kit

(Bioneer Accuprep, Genomic DNA extraction kit, Korea), according to the manufacturer's instructions. PCR amplification of the DNA using the EUB1 (5'-ACGCTGGCGGCAGGCCT AACAG-3') and EUB2 (5'-ATTACTAGCGATT CCGTCTTC-3') were performed with a Gene-Amp PCR system 2400 (Perkin Elmer applied Biosystems, USA). Reaction mixture contained 10 pmol of each primer, 2.5mM dNTP, 10x buffer, 5U Taq polymerase (G-tag), 1μg DNA template and sterilized water to achieve a final volume of 20$\mu\ell$. PCR was performed under the following conditions: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 10 min. Five microliter of amplification products were separated by electrophoresis on a 1% agarose in 0.5% TAE buffer at 100V for 10 min. Gels were stained with ethidium bromide and photographed under UV light. Gel images were recorded using a digital camera.

The band excised from the gel and DNA recovered by the GENE ALL (Generalbiosystem, USA). The purified products were ligated into TOPO®-cloning kit (Invitrogen) and then transformed into *E.coli* TOP 10F' Competent Cells according to manufacturer's instructions (Promega). Colonies were bule/white screened on LB agar with Ampicillin (Sigma), X-gal and IPTG (Promega). White colonies were randomly chosen, cultivated and stored in freezing medium at -80°C.

The 5'- end and 3' - ends of the constructs were sequenced using M13 primers flanking the cloning sites. These partial sequences were searched against GenBank (National Center for Biotechnology Information, Rockville Pike, Bethesda, MD) using the Advanced BLAST similarity search option[22] accessible from the homepage at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). All nucleotide sequences were determined using a DNA sequencer, model 377 (ABI 100). Bio-Edit Sequence Alignment Editor version 5.0.9[23] was used to check alignment and remove all positions with gaps before calculating distances with DNAdist programme in PHYLIP (version 3.5c; distributed by J. Felsenstein, University of Washington, Seattle).

The Gram staining was made in order to obtain microscopic features of the isolate. Morphology of the isolate such as cell size, motility and formation of chain was also determined microscopically (1,000×).

Test of Capacity for N_2 Production from NH_4^+

Test of capacity for N_2 production from NH_4^+ was executed in a 100 mL- syringe that served as the reaction vessel. Tygon tubing was put on the syringe needle and clamped at its end. Under an aseptic condition, 0.86 g (wet weight basis) of pure cells harvested at the end of the exponential growth phase was suspended in the syringe with 20 mL of the culture medium. Oxygen gas was supplied into the syringe with 40 mL of sterile oxygen (80% purity) at once. The syringe prepared in this way was incubated in a shaking incubator at 30°C and 180 rpm. The gas produced by the isolate during incubation was sampled through the tygon tubing by the use of a Hamilton gastight syringe and analyzed by gas chromatography (GC). At the same time, liquid broth was sampled from the syringe and the concentration of NH_4^+ was analyzed by ion chromatography (IC). Thus, the ability of N_2 production from NH_4^+ was verified by measuring both N_2 gas production and the conversion of NH_4^+.

Cell Immobilization

The isolated cells were harvested in the late exponential phase of growth by centrifuge at 7,000 rpm for 10 min. The pellet was washed and resuspended in sterile distilled water (DW), and the resulting dense cell suspension was used for cell immobilization. The cell was immobilized in phosphorylated PVA gel beads according to the method of Chen et al.[14] A mixture containing concentrated cell of 200 mg/mL was thoroughly mixed with an equal

volume of PVA (18% w/v; Kuraray PVA-HC, Kuraray Co. Ltd., Osaka, Japan). This cell-PVA mixture was dropped into a saturated solution of boric acid through the hole of a needle and gently stirred for 1 hr to form spherical beads. The formed labile beads were then transferred to a 0.5 M sodium phosphate solution for 1 hr for complete gelation by esterification of PVA with phosphate. The subsequent beads of 7 mm diameter were washed with sterile DW, and the beads were acclimated until use. The specific gravity of beads was approximated to 1.08.

Characteristics of the Immobilized Isolate

In order to determine optimum culture conditions of immobilized isolate, the 100 mL-syringes were incubated under various growth conditions of pH (5, 6, 7 and 8), temperatures (15, 20, 25 and 30°C) and C/N ratios (1, 3, 5 and 7). Under an aseptic condition, 10% (v/v) of gel beads were suspended in the syringe with 20 mL of the culture medium. Oxygen gas was supplied into the syringe with 40 mL of sterile oxygen (80% purity). The amounts of gases produced by beads at various conditions were measured, and the maximum N_2 production rates of beads were calculated after analyzing the gas by GC.

Under the optimum conditions, the characteristic of the immobilized N_2-producing isolate was examined in a five-neck flask of 1L (working volume) in three replicates. The dissolved oxygen (DO) probe with a thermometric sensor, the oxidation-reduction potential (ORP) probe and the pH probe were inserted in three necks of the flask, respectively. On the other two necks, a silicon rubber with two stainless-steel tubings for inlet and outlet of oxygen gas and a silicon rubber with a 2N-NaOH supplementing stainless-steel tubing for pH adjustment were equipped, respectively. A sampling port was set up at the same neck in which the pH probe was inserted. Gel beads (10% packing: equivalent volume of 100 mL) were suspended into the culture medium. The flask prepared in this way was executed in a hot-stirring bath system (Eyela, Japan) and maintained at 30±0.2°C. The Variomag Telesystem (H+P Labortechnik AG, Germany) was equipped under the bath system in order to provide for adequate mixing inside the flask.

During reaction in the five-neck flask, the changes of DO, pH and ORP were obtained by real-time measurement. During the experiment, the concentration of DO and pH were manually controlled between 1-3 mg/L and 6.5-7.0, respectively. The agitation was created using a magnetic bar inside the flask and its speed was set at 700 rpm. Liquid broth was sampled from the flask by a peristaltic pump using Tygon tubing and the concentrations of nitrate, nitrite and ammonium ions were analyzed. Except for sampling time, the sampling tubing whose one end was immersed in liquid broth inside the flask was clamped and the other end was immersed in 95% ethyl alcohol always. To obtain the liquid broth present in the flask, approximately 5 mL- broth that was remained in the tubing after sampling was wasted from the second sampling. The ability of N_2 production from NH_4^+ by the isolate was verified by measuring the removal of NH_4^+.

The application of the gel beads (immobilized isolate) to a bioreactor system was executed in a 1L- continuous stirred bioreactor (Marubishi, Japan). Gel beads (10% packing) were suspended in the bioreactor with 700 mL of the reduced culture medium containing glucose, 0.208 g; NH_4Cl, 0.134 g; yeast extract, 0.020 g; KH_2PO_4, 0.066 g; and mineral solution, 1 mL per liter of DW (pH 7), which simulated typical characteristics of municipal wastewater in Korea.[24] The characteristics of the synthetic medium are presented in Table 1. The continuous operations at four different hydraulic retention times (HRT) were initiated at a stationary phase of batch culture. The pH was adjusted to 7 by using 1N-HCl and 2N-NaOH, and agitation speed was set at 200 rpm. The concentrations of DO were controlled at a range of 1-3 ppm, and the feed medium was always maintained fresh, which pumped into the

bioreactor by a two-way Masterflex peristaltic pump.

Table 1. Characteristics of the synthetic medium

Constituents	Concentration (mg/L)
COD_{Cr}	252
BOD_5	198
TKN	37
NH_4^+-N	35
NO_3^--N	0
NO_2^--N	0
TN	37
TP	15

Analytical Methods

The concentrations of nitrite, nitrate and ammonium ions were estimated by ion chromatography (Metrohm 792 Basic IC, Switzerland). The columns used in these analyses were Metrosep Supp 5-150 (150×4.0 mm) and Metrosep C2-150 (150×4.0 mm) for anion and cation, respectively. Chemical oxygen demand (COD) and total nitrogen (TN) concentrations were analyzed by the Water-quality Analyzer (Humas Co., Ltd, Korea). The five days biological oxygen demand (BOD_5) and total Kjeldahl nitrogen (TKN) was analyzed by the OxiDirect BOD-System (Lovibond, Germany) and by the 2100 Kjeltec system (Foss, Sweden), respectively. With a proper dilution, the numbers of viable cells sampled from the 1-L bioreactor were measured by counting colonies formed on the plate of the culture medium containing 1.5% (w/v) agar. The dry-cell weight (DCW) was determined by weighing the cell pellet after being dried in an oven at 100°C for 12 h. The cell pellet was prepared by centrifuging a 20 mL sample of broth culture at 7,000 rpm for 10 min and then by decanting the supernatant after washing twice with DW.

For determination of nitrogen and carbon dioxide gases, 20 $\mu\ell$ samples (injection volume) were taken by a Hamilton gastight syringe for GC/TCD (Perkin Elmer Instruments, USA) analysis. The columns used were a 'molecular sieve 13X' (stainless steel, mesh 80/100, 6 ft×1/8 in) and 'carboxen 1000' (stainless steel, mesh 60/80, 6 ft×1/8 in) for nitrogen and carbon dioxide, respectively. The carrier gas was helium at a flow rate of 30 mL/min, and the injector and the detector temperatures were 100 and 200°C for the both gases. The oven temperature for nitrogen gas was 40°C, and that for carbon dioxide gas was 40°C for 3 min initially then increased to 170°C with a rate of 30°C/min. The amounts of nitrogen and carbon dioxide were calculated by applying the ideal gas law. All measurements were performed in three replicates.

Statistical Analyses

Statistical analyses were done with measurements obtained from this study. Since the sample observations were not arranged in a frequency distribution, the standard deviations were calculated by the following procedures: each deviation was squared, the sum of the squares was divided by (n-1), one less than the sample size (n), (this resulted in the sample variance) and finally extraction of the square root recovered the original scale of measurement. Comparisons of means were performed by the Tukey method[25] using the SAS program, since all sample sizes were equal. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Identification of Isolate

The N_2-producing bacteria from NH_4^+ under an aerobic condition were isolated. Various types of colonies developed on the surface of the agar plate after 3 days of incubation at 30°C. Seven different colonies were purified by repeated colony selection. The N_2-producing capacities of the isolates were tested by the use of the syringe technique,[18] and only two isolates evolved gases under the aerobic condition. The gases trapped in the syringe were identified by GC. According to the GC analysis, the gases consisted mostly of carbon dioxide and nitrogen gases.

The two isolates looked similar under the

microscope. The cells were motile and Gram-negative rods, and usually occurred in pairs (Table 2). Species-specific identification can be derived, since all bacterial species possess one or more unique 16S-rDNA nucleotide regions. For each species, 1,319 bp sized- fragment band was confirmed by electrophoresis after performance of PCR. The 497 bp from 5'-end and 552 bp from 3'-end are presented in Figure 1. Between the two 16S-rDNA sequences, only 4 bp were different. When these partial sequences were searched against GenBank, species of *Enterobacter* or *Klebsiella* had the highest similarity (more than 90%). According to the Bergey's Manual,[26] the similarity between the two species is approximately 30-60% and one of different characteristics is the existence of motility. Based upon this information, the isolates were identified to be *Enterobacter* as seen in Table 2. Among the species, *E. asburiae* was the highest similarity (96%). Interestingly, it has not reported that this species could produce N_2

(a)

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KJ1 GGCGGCAGGCCTAACACATGCAAGTCGAACGGTAGCACAGAGGAGCTTGCTC T TCGGGTG  60
KJ2 GGCGGCAGGCCTAACACATGCAAGTCGAACGGTAGCACAGAGGAGCTTGCTC C TCGGGTG  60

KJ1 ACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGGGATAACTACT  120
KJ2 ACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGGGATAACTACT  120

KJ1 GGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTC  180
KJ2 GGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTC  180

KJ1 TTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGC  240
KJ2 TTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGC  240

KJ1 GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAG  300
KJ2 GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAG  300

KJ1 ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA  360
KJ2 ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCA  360

KJ1 A GCCGCGTGTGTGAAGAAGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCAGT  420
KJ2 T GCCGCGTGTGTGAAGAAGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCAGT  420

KJ1 ACGGGTAATAACCGTGTTGATTGACGTTCCCGCAGAAAAACACCCGGCTAACTTCGTGCC  480
KJ2 ACGGGTAATAACCGTGTTGATTGACGTTCCCGCAGAAAAACACCCGGCTAACTTCGTGCC  480

KJ1 AGCAGCCCGCGGTAATA 497
KJ2 AGCAGCCCGCGGTAATA 497
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(b)

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KJ1 TGGTAGTCCACGCCGTAAACGATGTCGACTTTGAGGTTGTGCCTTGAGGCGTGGTTCCGG  60
KJ2 TGGTAGTCCACGCCGTAAACGATGTCGACTTTGAGGTTGTGCCTTGAGGCGTGGTTCCGG  60

KJ1 AGCTAACGCGTTAAGTCGCCCGCGTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA  120
KJ2 AGCTAACGCGTTAAGTCGCCCGCGTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA  120

KJ1 TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAAC  180
KJ2 TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAAC  180

KJ1 CTTACCTGGTCTTGACATCCACGGAATTCGGCAGAGATGCCTTAGTGCCTTCGGGAACCG  240
KJ2 CTTACCTGGTCTTGACATCCACGGAATTCGGCAGAGATGCCTTAGTGCCTTCGGGAACCG  240

KJ1 TGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG  300
KJ2 TGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG  300

KJ1 CAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGT CC GGCCGGGAACTCAAAGGAGACT  360
KJ2 CAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGT TA GGCCGGGAACTCAAAGGAGACT  360

KJ1 GCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCA  420
KJ2 GCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCA  420

KJ1 GGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGG  480
KJ2 GGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGG  480

KJ1 ACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGACGGA  540
KJ2 ACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGACGGA  540

KJ1 ATCGCTAGTAAT 552
KJ2 ATCGCTAGTAAT 552
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Figure 1. Comparison of 16S-rDNA. (a) 497 bp from 5'-end; and (b) 552 bp from 3'-end.

Table 2. Characteristics of two isolated bacteria producing N_2 from NH_4^+ under an aerobic condition

Isolate	KJ1	KJ2
Size[1] (μm)	L:2.0, W:1.0	L:1.5, W:1.2
Gram reaction	-	-
Color of colony	white	white
Formation of chain	pair was common, 4, 6 & 8 chains were also seen	pair was common, 4, 6 & 8 chains were also seen
Motility	Very active	Very active
Agar-stab culture	facultatively anaerobic with deep growth,	facultatively anaerobic with deep growth,

[1]L and W mean the length and the width of the each bacterium, respectively.

from NH_4^+, yet.[8-9] Bacterially mediated nitrification-denitrification processes do not exclude alternative pathways to the formation of N_2, and there is some evidence suggesting that alternative pathways may be important.[27] Only one species was used in the later experiments, since the two species had very similar characteristics and 16S-rDNA sequence.

Capacity of N_2 Production from NH_4^+ by the free Isolate

Test of capacity for N_2 production from NH_4^+ was executed with the free isolate in a 100 mL-syringe. Comparison of capacity for N_2 production from NH_4^+ between oxic and anoxic conditions is presented in Table 3. After 40 hrs experiment, the final pHs were 5.6 and 4.8 under oxic and anoxic conditions, respectively. The concentrations of COD_{Cr} and TKN removed were significantly different between the two conditions and were found to be 539.7 and 37.3 mg/L (under oxic condition) and 146.1 and 8.7 mg/L (under anoxic condition), respectively. The isolated bacteria removed NH_4^+-N by 28.3 and 9.8 mg/L under oxic and anoxic conditions and produced 171.3 and 57.8 µmoles of N_2, respectively with very low productions of NO_2^--N and NO_3^--N at the both conditions. When the gas composition of O_2 in the syringe was maintained at less than 40%, the N_2 production by the isolate was active. A similar result can be found in the study of simultaneous aerobic nitrification-denitrification,[28] in which maximum removal of NH_4^+ and cell production occurred under the similar condition of DO. Percentages of CO_2 and N_2 found in the gas, which was produced in the syringe, were between 25-35 and 52-60% (the rest was oxygen not consumed) under an oxic condition, and between 74-80 and 15-20% (with trace of H_2) under an anoxic condition, respectively. Therefore, the concentration of DO influenced N_2 production of the isolate significantly, and similar results have been reported in the study of simultaneous nitrifi-

Table 3. Comparison of experimental results between oxic and anoxic conditions[1]

Measurement	Initial	Final[2]	
		Oxic	Anoxic
pH	7.0±0.2[a]	5.6±0.1[b]	4.8±0.1[c]
COD_{Cr} (mg/L)	2509.3±11.6[a]	1969.6±22.8[c]	2363.2±17.8[b]
TKN (mg/L)	188.2±8.6[a]	108.9±6.3[c]	128.5±4.7[b]
NH_4^+-N (mg/L)	121.1±2.5[a]	92.8±4.8[c]	111.7±3.1[b]
NO_2^--N (mg/L)	0.0±0.0[b]	0.6±0.3[a]	0.2±0.1a[b]
NO_3^--N (mg/L)	0.0±0.0[a]	0.3±0.2[a]	0.1±0.1[a]
N_2 (µmole)	0.0±0.0[c]	171.3±7.6[a]	57.8±2.6[b]
DCW (mg/mL)	2.1±0.1[b]	2.8±0.1[a]	2.6±0.1[a]

[1]Values (mean±S.D. of three replicates) in the same row not sharing a common superscript are significantly different ($P < 0.05$).
[2]Experiments were executed on the culture medium (initial pH=7 and C/N= 3) at 30°C for 40 hours.

cation-denitrification.[29,30)]

All these results indicate that the capacity of N_2 production under an oxic condition was significantly different from that under an anoxic condition. The capacity was approximately three times higher, and this resulted in lower pH drop than that under an anoxic condition. The removed COD_{Cr}/TN ratios under oxic and anoxic conditions at the final state were approximately 19.1 and 14.9, respectively. For 40 hrs reaction, the concentration of dry cell in the syringe increased by 0.7 and 0.5 mg/mL under oxic and anoxic conditions, respectively. This result suggests that the cell synthesis by the isolate with the consumption of NH_4^+ was not significantly different between the two conditions. But, the consumption of NH_4^+ by the isolate was significantly different in the metabolism of N_2 production under the two different environmental conditions.[28)]

Optimum Conditions of the Immobilized Isolate for N_2 Production

Cultures in 100 mL- syringes were conducted at various pHs, temperatures and C/N ratios to obtain optimum reaction conditions of PVA beads (the immobilized isolate) for N_2 production. The results are represented in Table 4, and with the maximum N_2 production rate of 4.8 mL/L/h, the best result was obtained at pH 7.0, 30°C and C/N ratio of 5. The maximum N_2 production rates were not significantly different in a pH range of 5.0-8.0 and in a temperature range of 20-40°C as well. But, the maximum N_2 production rate was significantly different at 15°C, although the immobilized cells may afford protection from adverse conditions by creating micro-environments within the beads.[13)] The values were also significantly different among C/N ratio of 1, 3 and 5. A similar result by the effect of C/N ratio on N_2 production can be found in the study of simultaneous nitrification-denitrification.[28,31)]

Table 4. The maximum N_2 production rates of beads at various culture conditions[1]

Culture condition		Maximum N_2 production rate (mL/L/h)
pH[2]	5	3.4±0.1[b]
	6	3.7±0.1[b]
	7	3.8±0.1[b]
	8	3.4±0.2[b]
Temperature[3] (°C)	15	0.5±0.1[d]
	20	3.7±0.2[b]
	25	3.7±0.1[b]
	30	3.8±0.1[b]
C_{glu}/N_{NH_4} Ratio[4]	1	1.0±0.3[c]
	3	3.8±0.1[b]
	5	4.8±0.1[a]
	7	4.6±0.1[a]

[1]Values (mean±S.D. of three replicates) in the same column not sharing a common superscript are significantly different ($P < 0.05$).
[2]Under 30°C on the culture medium with C/N ratio of 3.
[3]Under pH 7.0 on the culture medium with C/N ratio of 3.
[4]Various ratios of glucose (C-source) and NH_4Cl (N-source) under 30°C and pH 7.0 on the culture medium.

Characteristics of the Immobilized Isolate

Under all the optimum reaction conditions, the characteristics of the immobilized isolate were investigated in a five-neck flask, and the results are shown in Figure 2. As shown in Figure 2(a), pH was adjusted to 7 whenever it dropped up to 6.5. Frequent adjustment of pH was accomplished for the first several hours. Since then this phenomenon was hardly seen and the final pH increased a little bit to 7.2. The concentration of DO decreased gradually by the uptake of the immobilized isolate, and it was adjusted to 3 mg/L by the supplement of O_2 into the flask whenever it dropped up to 1 mg/L. Frequent adjustment of DO was also accomplished for the first several hours, and this phenomenon was gradually reduced since then. ORP that showed 170 mV at the beginning reduced to -165 mV within 6.5 hrs, then increased slowly, and finally stayed at approximately -90 mV. These results suggest that N_2 production by the immobilized isolate resulted in reduction of ORP with both the consumption of DO and the drop of pH.

The concentration of COD_{Cr} decreased gradually and its removal for 25 hrs was 2,541.5 mg/L (Figure 2(b)). The removal efficiency of COD_{Cr} reached 56.1%. The removal rates of

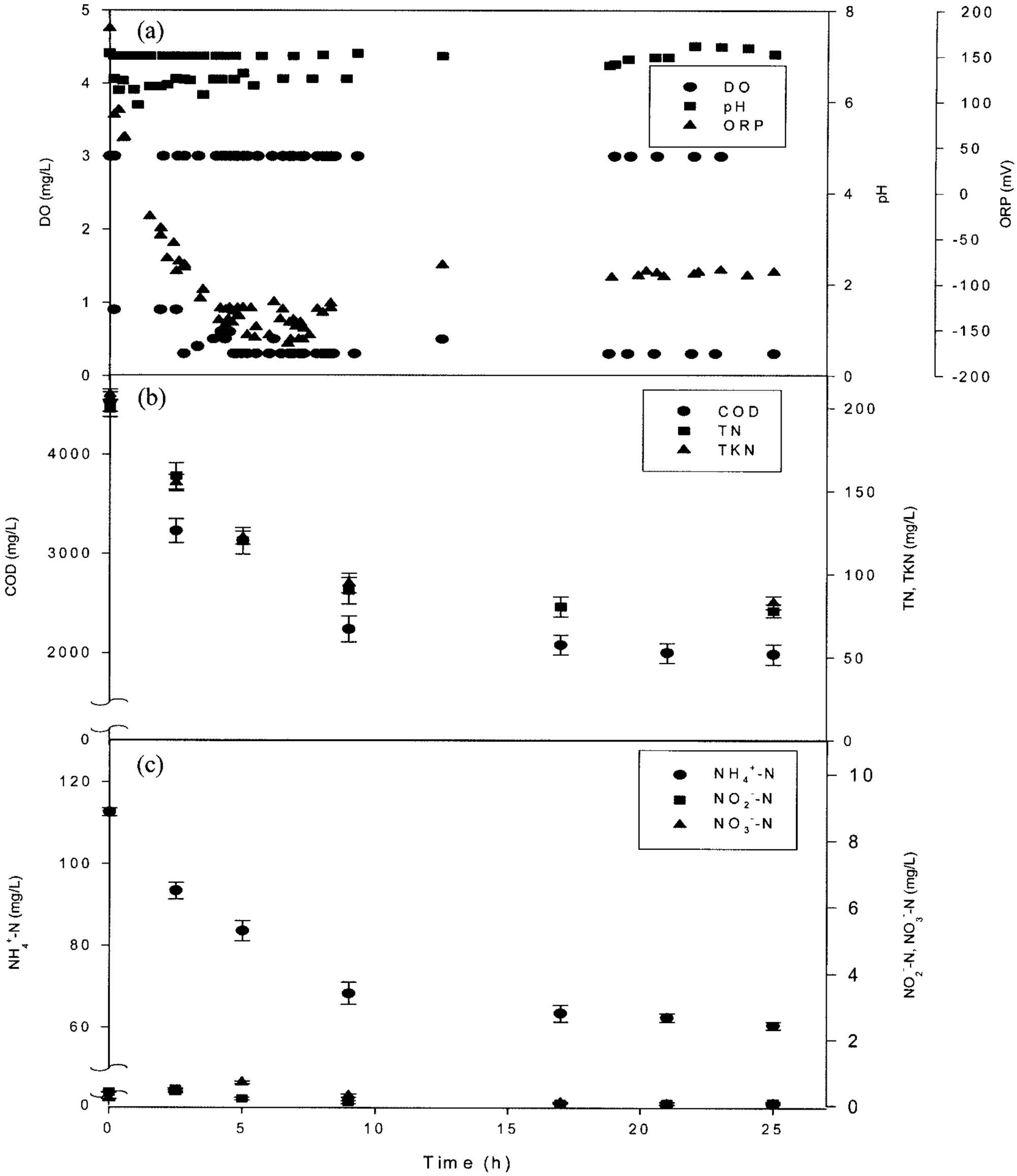

Figure 2. Changes of various parameters in ammonium removal by the immobilized isolate. Error bars: mean±S.D of three replicates. (a) DO, pH and ORP; (b) COD_{Cr}, TN, and TKN; and (c) NH_4^+-N, NO_2^--N and NO_3^--N.

COD_{Cr} were 520.1 mg/L/h for the first 2.5 hrs and 151.8 mg/L/h for the second 6.5 hrs, respectively, and afterwards the rate decreased as reaction proceeded. The concentration of TN removed was 121.5 mg/L for 25 hrs, and its removal efficiency reached 60.9%. During the first 2.5 hrs and the second 6.5 hrs, the removed COD_{Cr}/TN ratios were approximately 32.1 and 25.3, respectively, and the ratio decreased afterwards. This result was better in ammonium removal with higher removal ratio of COD_{Cr}/TN due to the adjustment of DO during the experiment, compared to that obtained from the experiment of syringe. From all these data,

it is known that the best N_2 production from ammonium by the isolate took place under appropriate DO and C/N ratio as the reaction of simultaneous nitrification and denitrification.[28-31] The concentration of organic nitrogen removed for 25 hrs experiment was calculated to be 69.5 mg/L from the TKN measurement, and TN levels decreased with the decrease of ammonium levels, which can be also seen in the study of simultaneous nitrification and denitrification.[32]

In Figure 2(c), changes in concentrations of NH_4^+-N, NO_2^--N and NO_3^--N were seen. Neither nitrite nor nitrate was almost accumulated in this experiment. Nitrite accumulation has been reported in shortcut biological nitrogen removal from ammonium, in which nitrite is an intermediary compound.[30,33] However, that kind of phenomenon was not found in this study. This indicates that the isolate did not follow the metabolism of shortcut biological nitrogen removal, although the N_2 production took place directly from ammonium. The NH_4^+-N was removed by 52.0 mg/L with the maximum removal rate of 7.7 mg/L/hr. The rate gradually decreased as mentioned.

Application of the Immobilized Isolate to a Bioreactor System

The continuous operations in 1L- bioreactor were initiated at a stationary phase of batch culture. In batch operation, the immobilized isolate could remove 17.5 mg NH_4^+-N/L of the medium in 20 hrs with a very small accumulation of nitrite and nitrate (Figure 3). Scarce detection of nitrite and nitrate as intermediate products may therefore be useful in attempts to enhance the ammonium removal from wastewater.[28] During this period of operation, approximately 120.6 and 19.5 mg/L of COD_{Cr} and TN were removed, respectively. The maximum removal rate of COD_{Cr} was 49.4 mg/L/hr, and the concentration of final TN maintained stably below 20 mg/L. The maximum removal rate and the removal efficiency of NH_4^+-N were 6.0 mg/L/hr and 50.5%, respectively. The removal ratio of COD_{Cr}/TN for 20 hrs experiment was 6.2, which was lower than that obtained from the experiment of five-neck flask. This result is not surprising because C/N ratio in the synthetic medium used in this experiment was much lower than that used in five-neck flask experiment, which is consistent with the C/N ratio test (Table 4). Similar results can be found in other studies.[28-31] As experiment proceeded, cell leaking increased. This phenomenon took place because some cells leaked when N_2 gas

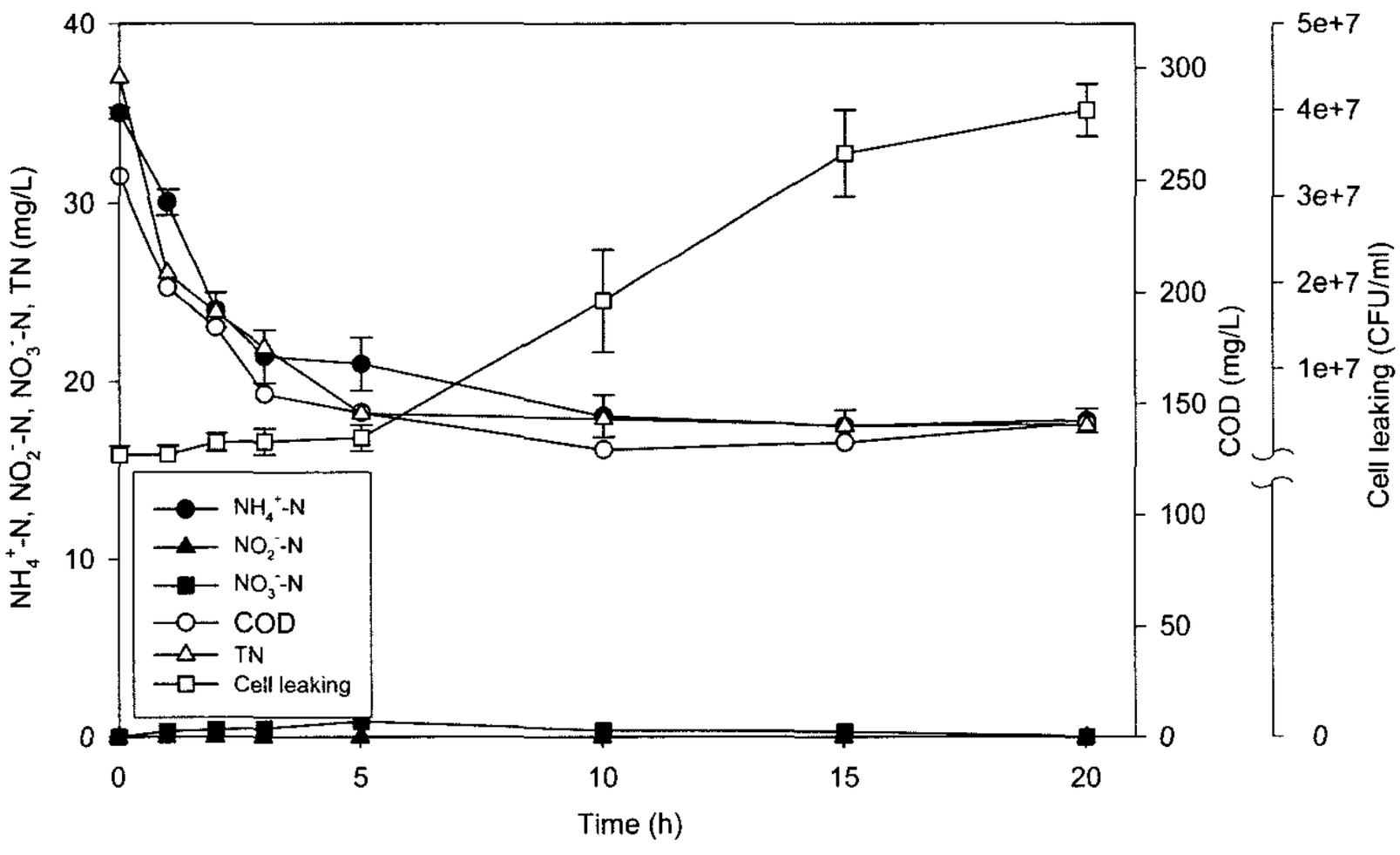

Figure 3. Profiles of concentrations of NH_4^+-N, NO_2^--N, NO_3^--N, COD_{Cr}, TN and cell leaking during batch operation at 30°C and pH 7. Error bars: mean±S.D of three replicates.

produced inside the beads squeezed out. It reached up to 4.0×10^7 CFU/mL maximally in the bioreactor. It is presumed that the organic nitrogen was utilized mostly by free cells leaked out of the bio-beads for their growth.[12)]

The results of continuous operations at four different HRTs are shown in Figure 4. A steady state was reached at each dilution rate after three bioreactor volumes had passed through the system, and the effluent concentrations of NH_4^+-N at HRTs of 15.5, 12.1, 8.6 and 5.6 hrs were 16.0, 15.4, 17.8 and 21.6 mg/L, respectively. Thus, the ammonium removal efficiencies at HRTs of 15.5, 12.1, 8.6 and 5.6 hrs were calculated to be 54.3, 56.0, 49.1 and 38.3% with removal COD_{Cr}/TN ratios of 8.7, 8.7, 7.0 and 7.2, respectively. Nitrite and nitrate were scarcely detected at all the HRTs. The maximum removal rate of NH_4^+-N reached 1.6 mg NH_4^+-N/L/hr at 12.1 hrs of HRT (with N loading rate of 0.08 Kg-N/m^3-carrier/d), and the poorer performance of bioreactor was obtained at the lower HRTs due to the short contact time.[16)] Approximately $5\text{-}6\times10^5$ CFU/mL of cells leaked out of beads at HRTs of 15.5 and 12.1 hrs, but cell leaking decreased at the lower HRTs due to lower removal rate of NH_4^+-N. At 12.1 hrs of HRT, the bioreactor exhibited a satisfactory performance with the synthetic medium of low C/N ratio.

CONCLUSIONS

Microorganisms producing nitrogen gas from ammonium ion under an aerobic condition were isolated and identified to develop a one-step process of nitrogen removal. From the strain screening, only two isolates evolved N_2 gas under an aerobic condition. The cells were motile and Gram-negative rods, and usually occurred in pairs. By the 16S-rDNA analysis, it was known that only 4 bp were different between the two 16S-rDNA sequences, and the two isolate were identified as *Enterobacter asburiae* with 96% similarity.

Test of capacity for N_2 production from NH_4^+ was carried out with the free isolate in a 100 mL- syringe. The isolated bacteria removed NH_4^+-N by 28.3 and 9.8 mg/L under oxic and anoxic conditions and produced 171.3 and 57.8 μmoles of N_2, respectively. Therefore, the capacity of N_2 production under an oxic condition was approximately three times higher, and this resulted in lower pH drop than that under an anoxic condition. In the experiment using 100 mL- syringes to obtain optimum reaction conditions of the immobilized isolate, the best result was obtained at pH 7.0, 30°C and C/N ratio 5.

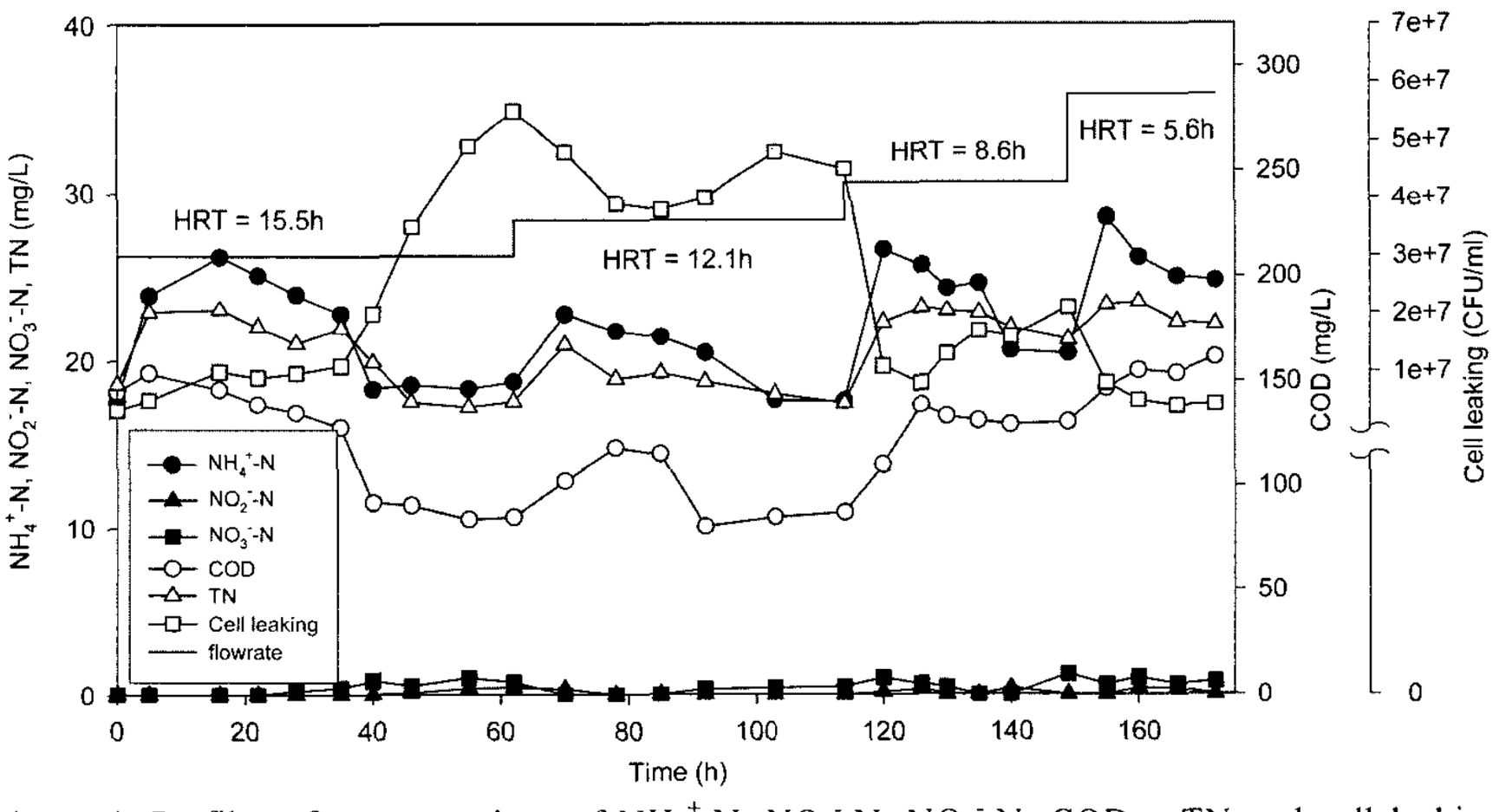

Figure 4. Profiles of concentrations of NH_4^+-N, NO_2^--N, NO_3^--N, COD_{Cr}, TN and cell leaking during continuous operation at 30°C and pH7.

Under all optimum reaction conditions with appropriate oxygen supplement, the characteristics of the immobilized isolate were investigated in a five-neck flask. N_2 production by the immobilized isolate resulted in reduction of ORP with both the consumption of DO and the drop of pH. The removal efficiency of COD_{Cr} and TN reached 56.1 and 60.9%, respectively. The NH_4^+-N was removed by 52.0 mg/L with the maximum removal rate of 7.7 mg/L/hr, and neither nitrite nor nitrate was almost accumulated.

In batch operation, the immobilized isolate could remove 17.5 mg NH_4^+-N/L of the medium in 20 hrs with a very small accumulation of nitrite and nitrate. During this period of operation, approximately 120.6 and 19.5 mg/L of COD_{Cr} and TN were removed, respectively. The maximum removal rate of COD_{Cr} was 49.4 mg/L/hr, and the concentration of final TN maintained stably below 20 mg/L. The maximum removal rate and the removal efficiency of NH_4^+-N were 6.0 mg/L/hr and 50.5%, respectively. The continuous operations showed that the effluent concentrations of NH_4^+-N at HRTs of 15.5, 12.1, 8.6 and 5.6 hrs were measured to be 16.0, 15.4, 17.8 and 21.6 mg/L with their removal efficiencies of 54.3, 56.0, 49.1 and 38.3%, respectively. Nitrite and nitrate were scarcely detected at all the HRTs. The maximum removal rate of NH_4^+-N reached 1.6 mg NH_4^+-N/L/hr at 12.1 hrs of HRT (with N loading rate of 0.08 Kg-N/m^3-carrier/d). At 12.1 hrs of HRT, the bioreactor exhibited a satisfactory performance with the synthetic medium of low C/N ratio. As a result, the development of a one-step process of ammonium removal by the immobilized isolate could be a viable alternative to the nitrification-denitrification processes.

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