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Characteristics of Immobilized PVA Beads in Nitrate Removal

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Abstract Before applying PVA bio-beads to practical biological treatment of nitrate-containing wastewater, their characteristics were examined. PVA bio-beads could steadily produce nitrogen gas from nitrate for 28 batches with 0.04 ml/l/h of the maximum gas production rate; however, the maximum gas production rate dropped remarkably thereafter with apparent deformation of beads. Addition of 2.2% solution containing 1% casamino acid, 1% yeast extract, 0.1% mineral solution, and 0.1% vitamin solution to the culture medium resulted in not only recovery of activity of deactivated beads, but also a higher rate of gas production. Calculation of economic benefit for the use of bio-beads in a long-run operation indicated that reactivation of bio-beads by chemicals had economical advantages over packing new bio-beads in the system. The continuously stirred bioreactor exhibited a satisfactory performance at HRT of 20.0 h. With a 9.5 mg NO_3^--N/l/h nitrate removal rate, nitrate could completely be removed without nitrite accumulation. The use of PVA bio-beads in nitrate removal appears very promising.

Key words: PVA bio-beads, activity of deactivated beads, economic calculation, nitrate removal

Nitrogenous compounds are major pollutants of water and occur in domestic, agricultural, and aqueous wastes from industries. The presence of nitrogenous substances in wastewater discharges has attracted a great deal of attention, because of the role of nitrogen in eutrophication of receiving waters. To prevent eutrophication, biological denitrification has successfully been used in removing nitrates from wastewater [9, 13, 17, 21]. In the biological denitrification processes, denitrifying bacteria convert nitrates into harmless nitrogen gas commonly under anoxic conditions.

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A high cell concentration is possible with immobilization, thereby greatly increasing the volumetric efficiency. This can lead to relatively small reactors [18]. The immobilized cells may be protected from adverse conditions by creating microenvironments within the gel matrix, which would help maintain year-round treatment [23]. In an effort to develop a more compact and efficient system for treatment of wastewater, immobilized-cells processes in the field of wastewater biodenitrification have recently received increasing attention [2, 3, 7, 24]. As widely recognized, entrapment of cells in a proper support matrix is an effective means for cell immobilization. Compared with commonly used polymeric substances such as acrylamide, κ-carrageenan, Ca-alginate, and agar, polyvinyl alcohol (PVA) has some advantages: cheap cost for chemicals required for cell immobilization and strong gel strength [4]. In addition, PVA gel beads would not float upward to the solution surface by N_2 production, due to their good gas permeability [1]. Thus, cell immobilization using PVA has been reported to be successfully applied to the immobilization of denitrifying sludge in the denitrification process [20]. However, there is scarce information on the characteristics of immobilized PVA beads for application to practical wastewater treatment. Therefore, some characteristics of the PVA beads, such as durability of the beads, recovery of deactivated beads in repeated batchwise operation, economics for long-term operation, and application in a continuous operation in nitrate removal, were studied.

Materials and Methods

Microorganism and its Identification

The cells used for immobilization were obtained from a sewage treatment plant in City of Busan, where active denitrification took place. The samples were first agitated

to obtain homogeneous suspensions in sterile 0.1% peptone. One ml of the suspended liquid was pipetted into 10 ml of medium [KNO_3, 2 g; glucose, 2 g; yeast extract, 1 g; $(NH_4)_2SO_4$, 1.25 g; KH_2PO_4, 0.6 g; K_2HPO_4, 0.9 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.07 g; EDTA, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; and trace element, 1 ml in 1 l of deionized water (D.W.), pH 7.0]. After 5 days of incubation at 30°C and 150 rpm, cells were spread with a platinum loop on the plate of the same medium containing 1.5% (w/v) agar and incubated until colonies were visible. With each colony with different type, the capacity of N_2 production from NO_3^- was tested in a 100-ml syringe using the culture medium. The gas produced by the cell was confirmed by gas chromatography (GC) analysis. The purified isolate that was able to produce N_2 gas by the reduction of nitrate was obtained by repeated streaking on the fresh agar plates.

To obtain microscopic features of the isolate, the Gram staining and catalase test were performed. Cell size, motility, and morphology of the isolate were also determined microscopically (1,000×). Taxonomic identification of the isolate was performed by the VITEK system (Biomerieux Industry, France).

Cell Immobilization

The isolated denitrifying cells were harvested in the late exponential phase of growth by centrifuging at 6,000 rpm for 10 min. The pellet was washed and resuspended in sterile D.W., and the resulting dense cell suspension was used for cell immobilization. The cells were immobilized in phosphorylated PVA gel beads according to the method of Chen *et al.* [2]: A mixture containing concentrated cells of 200 mg/ml was mixed thoroughly with an equal volume of PVA (18% w/v; Kuraray PVA-HC, Kuraray Co. Ltd., Osaka, Japan). With addition of 1% sodium alginate, this cell-PVA mixture was dropped into a saturated boric acid solution through the hole of a needle and gently stirred for 1 h to form spherical beads. The formed labile beads were then transferred to 0.5 M sodium phosphate solution for 1 h for complete gelation by esterification of PVA with phosphate. The subsequent beads of 7 mm diameter were washed with sterile D.W. The specific gravity of the beads was approximated to 1.07. After complete adaptation, features of cells attached on the inside surface of the PVA gel beads were examined by scanning microscopy.

Denitrification by PVA Bio-Beads

The characteristics of denitrification by PVA bio-beads were examined in a repeated batchwise operation, and the beads were applied in a continuous operation. The repeated batchwise operation was carried out 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 aseptic condition, gel beads (15% packing) were suspended in the syringe with 50 ml of the culture medium containing 0.1 g KNO_3, 0.1 g glucose, 0.05 g yeast extract, 0.0625 g $(NH_4)_2SO_4$, 0.03 g KH_2PO_4, 0.045 g K_2HPO_4, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.0035 g $CaCl_2 \cdot 2H_2O$, 0.001 g EDTA, 0.0005 g $FeSO_4 \cdot 7H_2O$, and 0.05 ml of trace element (pH 7.0). The syringe prepared in this way was incubated in a shaking incubator at 30°C and 150 rpm. The gas produced by the PVA beads during incubation was sampled through the tygon tubing by the use of a Hamilton gas-tight syringe and analyzed by GC. At the same time, liquid broth was sampled from the syringe, and the concentration of nitrate was analyzed by ion chromatography (IC). Thus, the ability of denitrification was verified by measuring both N_2 gas production and nitrate reduction. To investigate the durability of the PVA beads, the beads were separated at the end of each run, washed with sterile D.W., and suspended in a fresh medium for subsequent reaction. In experiments to test the effect of chemicals on the activity of deactivated bio-beads, the chemicals added to the given culture medium included 1% casamino acid, 0.1% mineral solution, 1% yeast extract, and 0.1% vitamin solution, and they were reagent grade. To obtain optimum reaction conditions for gas production, the experiments were also performed in a 100-ml syringe.

The denitrification activity of the PVA bio-beads was observed in a 1-l continuous stirred bioreactor (Marubishi, Japan) after optimum reaction conditions of the PVA beads were found. Gel beads (15% packing) were suspended in the bioreactor with 800 ml of the culture medium containing the same composition of that used in the repeated batchwise operation. The continuous operations at three different hydraulic retention times (HRT) were initiated at a stationary phase of batch culture. The pH was adjusted to 7 by 1 N HCL and 1 N NaOH, and agitation rate was 200 rpm. A steady state at each dilution rate was considered to have been reached after four bioreactor volumes had passed through the system. The feed medium was always maintained fresh and pumped into the bioreactor by a two-way Masterflex peristaltic pump. The ability of denitrification was verified by measuring the nitrate reduction rate of the PVA beads.

Analytical Methods

The concentrations of nitrite and nitrate were estimated by IC (Metrohm 792 Basic IC, Switzerland). Chemical oxygen demand (COD) and total nitrogen (TN) concentrations were analyzed by the Water-quality Analyzer (Humas Co., Ltd., Korea). The number of viable cells was sampled from the culture broth and, with a proper dilution, cell leaking was measured by counting colonies formed on the plate of the culture medium containing 1.5% (w/v) agar.

For determination of nitrogen, 20 μl samples (injection volume) were taken by a Hamilton gas-tight syringe for GC/TCD (Perkin Elmer Instruments, U.S.A.) analysis. The column used was a molecular sieve 13X (stainless steel,

mesh 80/100, 6 ft×1/8 in) with helium as carrier gas at a flow rate of 20 ml/min. The column and detector temperatures were 70°C and 120°C, respectively. The amount of nitrogen was calculated by applying the ideal gas law.

RESULTS AND DISCUSSION

Preliminary Experiments

The isolate that formed a white colony on the agar plate was a motile, catalase-positive, and Gram-negative rod measuring 0.4–0.5 μm in width and 1.5–2.0 μm in length. Multiplication occurred by binary fission, and the cells were usually present singly or in a pair. Gas analysis revealed that more than 93% of the gas released by the isolate was nitrogen with 0.2% KNO_3 as the terminal electron acceptor for denitrification. Using the VITEC system, the isolate was identified as a member of the species *Pseudomonas stutzeri* with 90% similarity.

In the repeated batchwise experiment in a 100-ml syringe, the gas production rate of the newly prepared bio-beads gradually increased up to the fourth batch, and this indicates that the entrapped microorganism grew at the inside of beads during the repeated batch operation [1]. After the bio-beads were newly prepared, four batches of adaptation were then required to obtain stable denitrification ability. A scanning electron micrograph of the inside of the bio-beads after complete adaptation is shown in Fig. 1. Cells were scarcely attached on the surface of PVA at the shallow inside (approximately 0.5 mm from the exterior), whereas many cells attached at the deep inside (approximately 3 mm from the exterior), so that dissolved oxygen (DO) hardly diffused, indicating that the isolated microorganism preferred to reside under anaerobic condition [8].

To obtain optimum cell loading of the bio-beads, various cell concentrations in a range of 50–200 mg/ml, which were mixed with 18% PVA solution in the process of making beads, were preliminarily tested: The gas production rate of the beads increased with the increase of cell loading into PVA, and no more increase was observed at 200 mg/ml. This indicates that the cells at this concentration fully occupied an appropriate position in the beads. Three different sizes (5, 6, and 7 mm diameter) of beads were made and tested at 200 mg/ml cell loading concentration in order to obtain optimum bead size for gas production. The maximum gas production rate was obtained with the bead of 7 mm, but it was not much different from those of other bead sizes. Thus, 7 mm beads were used in the following experiments.

Repeated Batchwise Operation

Durability of Bio-Beads. Batchwise operation in a 100-ml syringe was repeated after adaptation of four batches in order to find the durability of PVA bio-beads, since the stability during long-term operation is an essential factor for practical application of an immobilized cell system. To exclude the possibility of leaked cell attachment on the surface of the bio-beads, the bio-beads were washed with sterile D.W. after separation at the end of each run and resuspended in a fresh medium for subsequent reaction. As seen in Fig. 2, the volume of gas produced by the PVA gel beads was maintained almost constant up to the twenty-eighth batch, which was almost the same as that from the theoretical stoichiometric equation for conversion of NO_3^- to N_2. During this operation period, the maximum rate of gas production and the amount of cell leaking fluctuated up

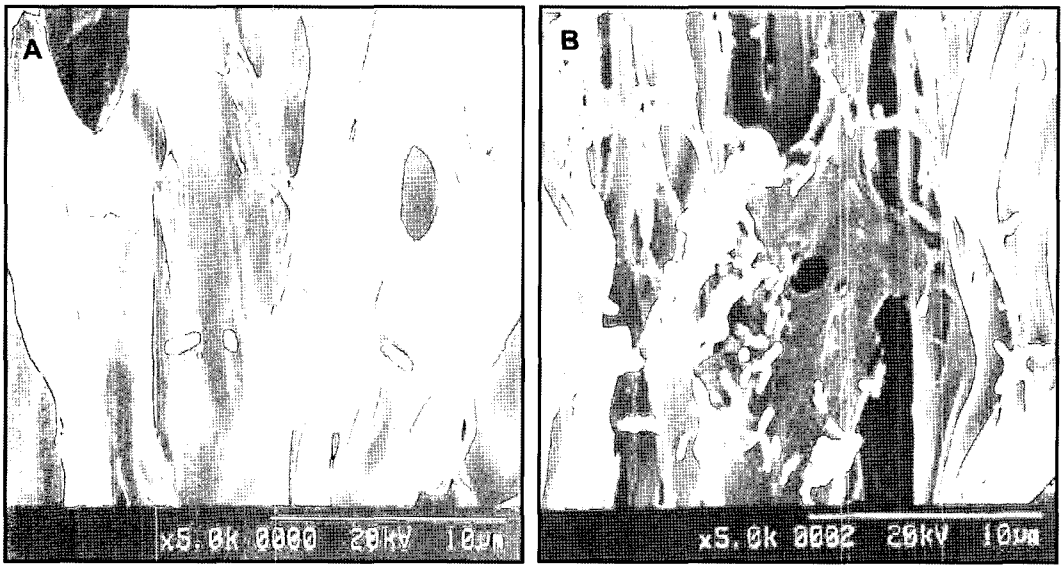

Fig. 1. Scanning electron micrographs (5,000×) of shallow inside (**A**) and deep inside (**B**) of the PVA gel beads.

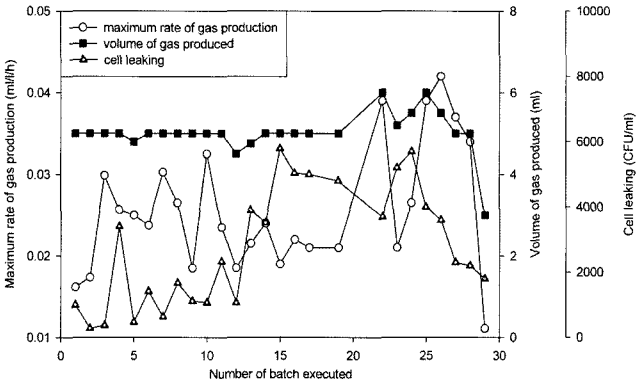

Fig. 2. Changes of maximum rate of gas production and cell leaking during repeated batchwise operation of immobilized denitrifying heterotroph.

and down, but cell leaking tended to increase whenever the gas production rate was high. The maximum rate of gas production was 0.04 ml/l/h, and the average gas production rate was 0.03 ml/l/h during 28 batches. After the twenty-eighth batch, the volume of gas and its maximum production rate dropped remarkably, and the shape of the beads started to slightly deform, while the apparent mechanical strength of the PVA beads was seemingly maintained. It is highly likely that this phenomenon happens particularly by gas produced at the inside of bio-beads: The bio-beads swell when the gas cannot easily squeeze out, because of its poor permeability, resulting in increase of cell leaking, decrease of bio-beads activity, and weakness of the matrix of bio-beads as well [8, 15, 22].

Effect of Chemicals on the Activity of Bio-Beads

To recover the activity of PVA gel beads, four chemicals that have been known as additives in biological wastewater treatment were tested in a 100-ml syringe with deactivated bio-beads [5, 11, 12, 16], and the result is shown in Fig. 3. The bio-beads used in this experiment had approximately half of the initial capacity of denitrification at the beginning, but they were deactivated slowly as the batchwise operation was repeated continuously. Except for the addition of 2.2% mixed solution of all four chemicals, a rather lower activity was obtained by the addition of each chemical to the culture medium: With the 2.2% mixed solution, the volume of gas produced in the syringe was almost doubled. It has been reported that the rate of nitrogen removal increased with the addition of casamino acids [16], denitrification yield increased by the addition of yeast extract [11], and microbial activity was stimulated with the addition of trace metals [5]. In the present study, however, the effects of 1% casamino acid, 1% yeast extraction, or 0.1% mineral solution alone on the activity of the deactivated bio-beads were found to be weak. To find chemicals effective for recovery of deactivated bio-beads activity, three kinds of mixed solutions

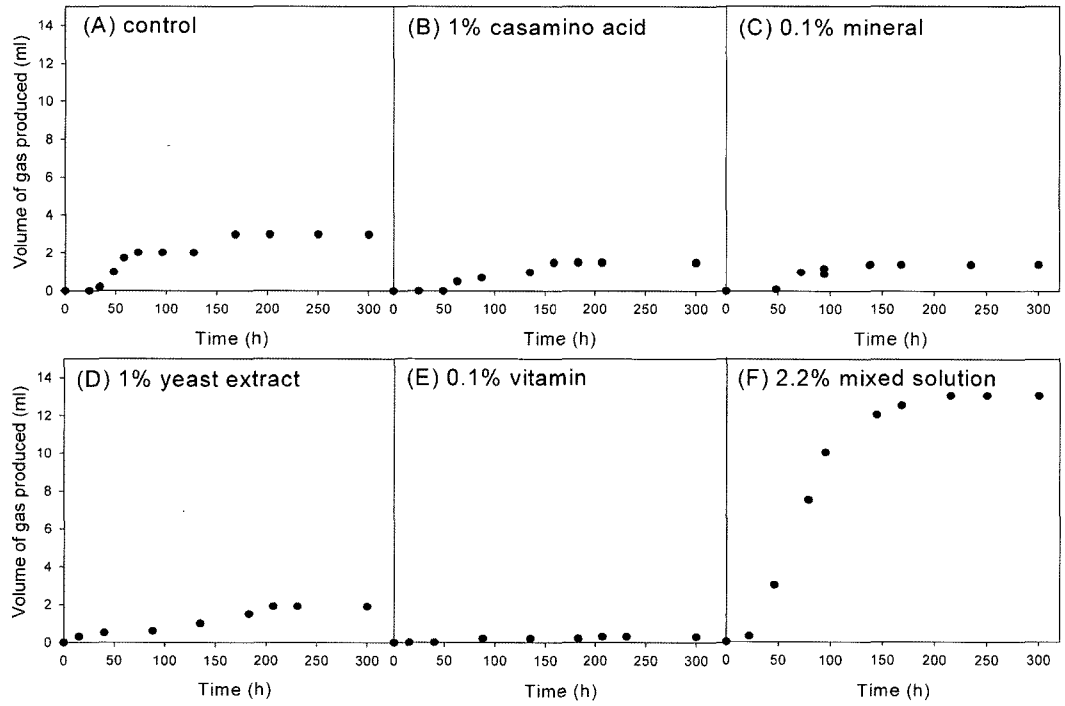

Fig. 3. The effects of chemicals on the activity of deactivated bio-beads.
(A) control; (B) casamino acid; (C) mineral; (D) yeast extract; (E) vitamin; and (F) all mixed solution. The experiment was executed sequentially from (A) to (F).

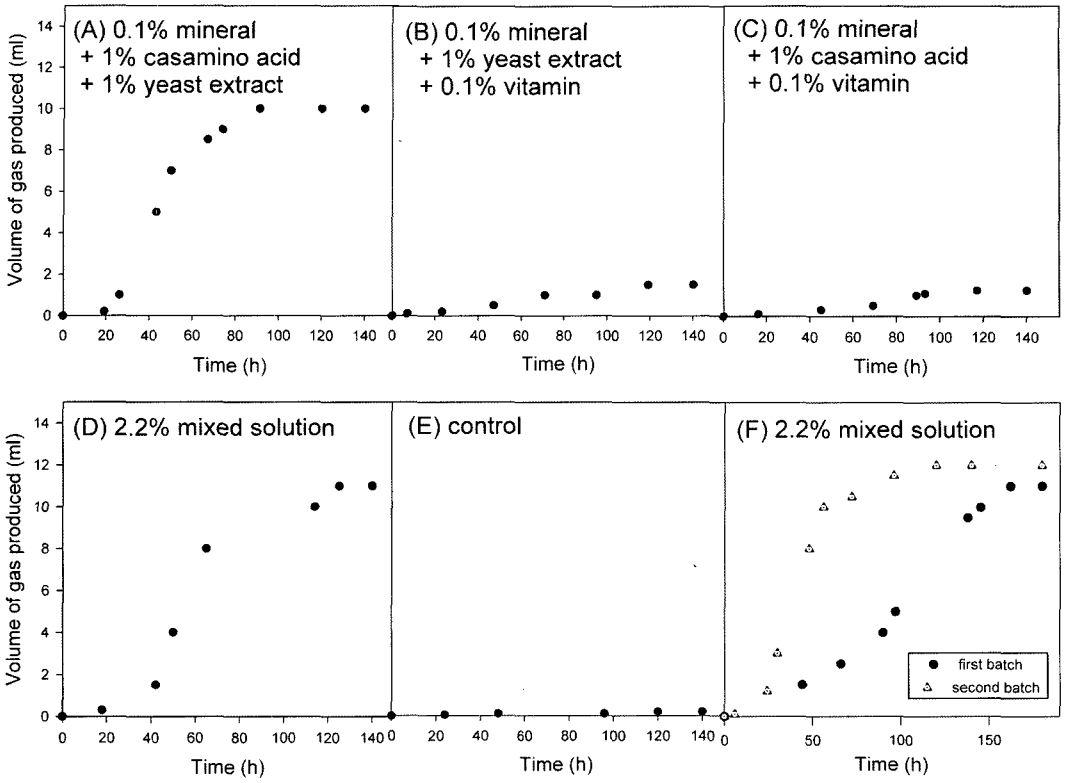

Fig. 4. The effects of chemicals on the activity of deactivated bio-beads.
(A) M+C+Y; (B) M+Y+V; (C) M+C+V; (D) 2.2% all mixed solution; (E) control; and (F) 2.2% all mixed solution. The experiment was executed sequentially from (A) to (F).

mixed with two chemicals (0.1% mineral solution+1% casamino acid, 0.1% mineral solution+1% yeast extract, and 0.1% mineral solution+0.1% vitamin) were tested. Each mixed solution contained 0.1% mineral solution, since it gave slightly better activity than the other two. However, results showed that their activity was very low (data not shown).

Again, three kinds of mixed solutions mixed with three chemicals (0.1% mineral solution+1% casamino acid+1% yeast extract, 0.1% mineral solution+1% yeast extract+ 0.1% vitamin solution, and 0.1% mineral solution+1% casamino acid+0.1% vitamin) were tested. As seen in Fig. 4, only the mixed solution of 0.1% mineral solution+1% casamino acid+1% yeast extract showed approximately 90% recovery of the activity of the bio-beads, compared with the volume of gas produced in the 2.2% mixed solution. Thus, it is likely that nitrate reductase for denitrification was activated by the presence of all three chemicals, and that the vitamin solution had an effect only in the presence of all three chemicals. On the other hand, the mixed solution could also be a growth factor for the cells in the inside beads [12].

To further confirm the data of bio-beads reactivation by the mixed chemicals, experiments were sequentially performed again with the deactivated bio-beads. The data could be reproduced, except that the deactivated bio-beads were not completely reactivated by the addition of 2.2% mixed solution in the first batch experiment owing to very low activity of the deactivated beads (Fig. 4). However, it was completely recovered after the second batch: The maximum gas production rate was 0.07 ml/l/h, and the average gas production rate was 0.05 ml/l/h. Thus, it is noteworthy that the addition of 2.2% mixed solution to the culture medium resulted in not only the recovery of activity of the bio-beads, but also a higher rate of gas production.

Economic Consideration of Bio-Beads

Operation costs for long-run wastewater treatment were compared between the use of new bio-beads after every 28 batches (since bio-beads were durable up to 28 batches) and deactivated bio-beads continuously reactivating with activating chemicals. Total working volume and percentage of the bio-beads suspended in liquid volume were assumed to be 1 l and 15%, respectively, in the economic calculation of the bio-beads. Without considering the maintenance cost of machine, the total costs for making the bio-beads were estimated, based on the prices of substrates for active PVA bead-making, labor, and power (Table 1). The total costs for making bio-beads and for recovery of deactivated

Table 1. Costs for making bio-beads.[a]

	Materials	Cost
Support[b]	PVA (27.0 g)	27.0 g×\$0.09/g=\$2.43
(total 150 ml)	Sodium alginate (1.5 g)	1.5 g×\$0.23/g=\$0.345
Cell culture[b]	Nutrient (50.88 g) (DIFCO)	50.88 g×\$0.13/g=\$6.614
(for harvest of 6.36 g wet cells)	KNO_3 (12.72 g)	12.72 g×\$0.06/g=\$0.763
	Glucose (31.8 g)	31.8 g×\$0.008/g=\$0.254
Bead formation[b]	Boric acid (160 g) (industrial chemical)	160 g×\$0.02/g=\$3.2
(total 2 l)	$CaCl_2$ (13.25 g)	13.25 g×\$0.05/g=\$0.663
* P-buffer	NaH_2PO_4 (60.9 g)	60.9 g×\$0.05/g=\$3.045
(total 2 l)	Na_2HPO_4 (70.065 g)	70.065 g×\$0.05/g=\$3.603
Adaptation[b]	Peptone (20 g)	20 g×\$0.15/g=\$3
(total 4 l for 4 batches)	Yeast extract (12 g)	12 g×\$0.04/g=\$0.48
	KNO_3 (8 g)	8 g×\$0.06/g=\$0.48
Labor[c]	Dissolving PVA	2 h×\$2.6/h=\$5.2
	Cultivating cells	4 h/d×4 d×\$2.6/h=\$41.6
	Making bio-beads	4 h×\$2.6/h=\$10.4
	Adapting bio-beads	4 h/d×4 d×\$2.6/h=\$41.6
Power[d]	Basic cost	\$3.53
	Operating shaking incubator	1 KW×250 h×\$0.05/KW=\$12.5
	Operating clean bench	500 W×8 h×\$0.05/KW=\$0.2
	Pumping	264 W×1 h×\$0.05/KW=\$0.0132
	Operating hot plate	575 W×16 h×\$0.05/KW=\$0.46
	Total	\$140.38

[a]15% packing of the bio-beads was assumed to be used in 1-l working volume.
[b]Based on selling prices of year 2004 (in Korea).
[c]Based on Korean wages of year 2004.
[d]Based on Korea electricity rates of year 2004.

bio-beads were estimated to be \$140.38 and \$127.96, respectively (Tables 1 and 2). The cost of the latter was lower than that of the former and, what is more, the latter could considerably save time. This kind of economic calculation has not been reported before, and the results revealed that the use of chemicals for reactivation of deactivated bio-beads seemed to have economic advantages in long-run operation for nitrate removal.

Optimum Conditions of Bio-Beads

Optimum reaction conditions of PVA beads for gas production were investigated in a 100-ml syringe for their application in a continuous operation, and the results are shown in Fig. 5. The optimum pH was found to be 7.0, although the production rates were not much different in the pH range of 6.0–8.0. Experiments to obtain optimum temperature were performed in the range of 20–40°C, and the maximum gas production rate was found at 35°C. These results indicate that the immobilized cells may be protected from adverse conditions by creating microenvironments within the beads [23].

Effects of C/N ratio and C:N:P ratio on the gas production rate of bio-beads were investigated. The optimum ratios of the two were 3 and 30:10:1, respectively. A similar result of C/N ratio was found in the study of immobilized denitrifying bacterium *Pseudomonas fluorescens* [8] and *Pseudomonas stutzeri* [7]. Since organic carbonaceous compound is used as an electron donor in denitrification, the C/N ratio can affect the conversion rate of nitrate [6]. Under the optimum reaction conditions described above, the effect of initial concentration of KNO_3 on gas production was examined, and the results are tabulated in Table 3: The highest gas production rate was obtained at 0.2% KNO_3, more than 93% of the gas released was nitrogen, and the composition of N_2 in the released gas increased as the initial concentration of KNO_3 increased. This implies that the concentration of electron acceptor in denitrification can change the metabolic pathway of denitrification [10, 19].

Continuous Operation

The continuous operations in a 1-l bioreactor were initiated at a stationary phase of batch culture. In batch culture, PVA bio-beads could completely remove 189 mg of NO_3^--N/l of the medium without accumulation of nitrite in 27 h. Nitrite accumulation in denitrification has been reported

Table 2. Costs for recovery of deactivated bio-beads.[a]

	Materials	Cost
Chemicals[b]	Casamino acid (10 g)	10 g×\$0.14/g=\$1.4
	Yeast extract (10 g)	10 g×\$0.04/g=\$0.4
	Vitamin solution (per l)	
	nicotinic acid (0.2 g)	0.2 g×\$0.12/g=\$0.024
	thiamine-HCl (0.4 g)	0.4 g×\$2.04/g=\$0.816
	nicotinamine (0.2 g)	0.2 g×\$0.1/g=\$0.02
	biotin (0.008 g)	0.008 g×\$121/g=\$0.968
	Mineral solution (per l)	
	ferrous sulfate (3 g)	3 g×\$0.05/g=\$0.15
	boric acid (0.01 g)	0.01 g×\$0.22/g=\$0.0022
	sodium molybdate (0.01 g)	0.01 g×\$0.36/g=\$0.0036
	magnesium sulfate (0.02 g)	0.02 g×\$0.06/g=\$0.0012
	copper sulfate (0.01 g)	0.01 g×\$0.11/g=\$0.0011
	zinc sulfate (0.01 g)	0.01 g×\$0.16/g=\$0.0016
	EDTA (0.5 g)	0.5 g×\$0.21/g=\$0.105
	Subtotal	\$3.92/batch⇒\$109.76 for 28 batches
Labor[c]	Preparing 2.2% mixed solution	0.25 h×\$2.6/h=\$0.65/batch⇒\$18.2 for 28 batches
	Total	\$127.96 for 28 batches

[a]Chemicals were assumed to be required in 1-l working volume.
[b]Based on selling prices of year 2004 (in Korea).
[c]Based on Korean wages of year 2004.

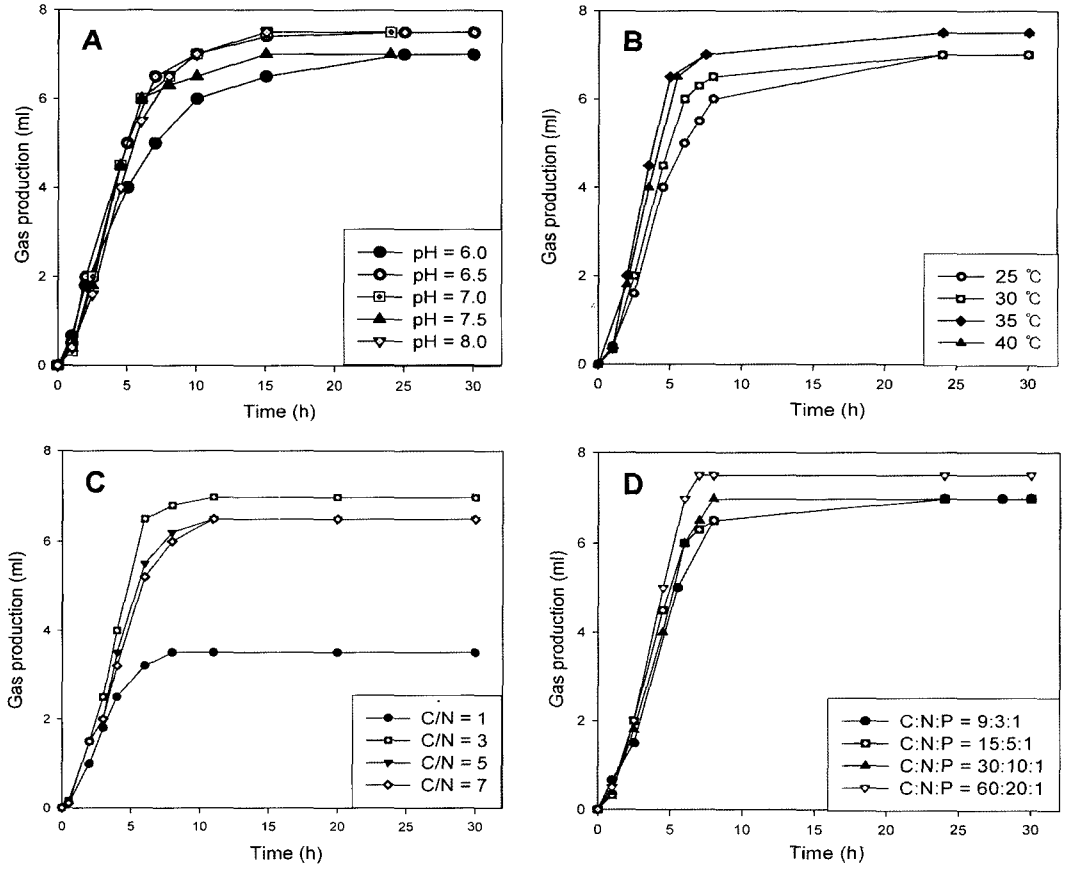

Fig. 5. Results of experiments to obtain optimum reaction conditions of the bio-beads.
(**A**) pH; (**B**) temperature; (**C**) C/N ratio; and (**D**) C:N:P ratio.

Table 3. The effect of initial concentration of KNO_3 on gas production.[a]

KNO_3 (%)	Max. vol. of gas (ml)	Gas production rate (ml/l/h)	% N_2 on GC	μmoles of N_2, calculated
0.05	10	0.04	85.88	216.93
0.1	13	0.04	90.99	276.14
0.2	14	0.10	93.12	367.38
0.4	15	0.09	95.38	510.76

[a]The reaction conditions of pH, temperature, and ratio of C:N:P were 7, 30°C, and 30:10:1, respectively.

to be due to the inhibitory effect of PVA on nitrite reductase in PVA immobilization of cells [14]. However, this phenomenon was not found in our present study, and no build-up of nitrite as an intermediate product might therefore be useful in attempts to enhance the removal of nitrate from wastewater. During this period of operation, approximately 147 and 257 mg/l of COD and TN were removed, respectively. The discrepancy between the amounts of TN and nitrate removed reflects the amount of organic nitrogen removed (approximately 68 mg/l). These nitrogen losses could occur because of nitrogen incorporated into the heterotrophic biomass during growth [18]. As nitrate removal rate increased, cell leaking increased, maximally reaching up to 2.8×10^7 CFU/ml in the bioreactor. Because of the more active N_2 production by the PVA beads under better reaction condition, the number of cells leaked in this experiment was much higher than that occurred in the repeated batch experiment. It is likely that organic nitrogen was utilized mostly by free cells that came out of the bio-beads.

The results of continuous operations at three different HRTs are shown in Fig. 6. Under the steady state, the nitrate removal efficiencies at HRTs of 11.1, 20.0, and 15.5 h were 21, 100, and 68.4%, respectively. At HRT of 11.1 h, the bio-beads drastically lost their performance. The nitrate concentration increased up to approximately 150 mg/l with accumulation of nitrite concentration (approximately 34 mg/l). The poor performance of the bioreactor at this HRT is due to short contact time and possibility of high concentration of DO carried by the feed stream [7]. At the HRT of 20.0 h, the bioreactor performed satisfactorily: It could completely remove the nitrate without leaving any nitrite in the effluent. The nitrate removal rate at this HRT was estimated to be 9.5 mg NO_3^--N/l/h. This value is close to that (12.79 mg NO_3^--N/l/h) obtained from continuous operation in a packed-bed bioreactor using *Pseudomonas stutzeri* [7]. The discrepancy might have resulted from the type of bioreactor, medium used in cell immobilization, species of microorganism, and nitrate loading rate. When the HRT was lowered to 15.5 h, approximately 60 mg of NO_3^--N/l remained in the bioreactor, and a small amount of nitrite was accumulated. Approximately 6×10^5 CFU/ml of cells leaked out of bio-beads at HRTs of 11.1 and 15.5 h, but cell leaking increased up to 2.5×10^7 CFU/ml at HRT of 20.0 h, possibly due to a higher gas production rate.

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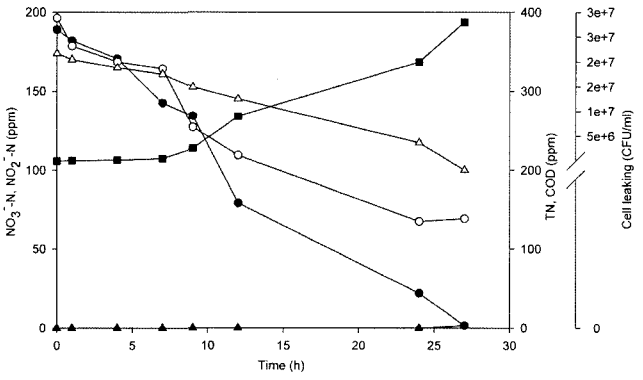

Fig. 6. Profiles of concentrations of NO_3^--N (●), NO_2^--N (▲), COD (△), TN (○), and cell leaking (■) during batch operation at 30°C and pH 7.

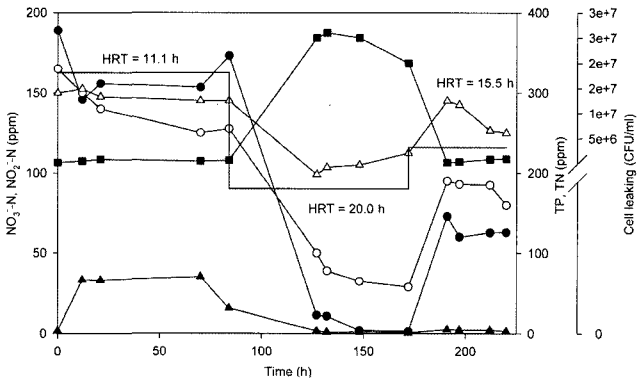

Fig. 7. Profiles of concentrations of NO_3^--N (●), NO_2^--N (▲), COD (△), TN (○) and cell leaking (■) during continuous operation at 30°C and pH 7: flow rate (—).

REFERENCES

1. Chen, K.-C., S.-J. Chen, and J.-Y. Houng. 1996. Improvement of gas permeability of denitrifying PVA gel beads. *Enzyme Microb. Technol.* **18:** 502–506.
2. Chen, K.-C., S.-C. Lee, S.-C. Chin, and J.-Y. Houng. 1998. Simultaneous carbon-nitrogen removal in wastewater using phosphorylated PVA-immobilized microorganisms. *Enzyme Microb. Technol.* **23:** 311–320.
3. Chen, K.-C. and Y. F. Lin. 1994. Immobilization of microorganisms with phosphorylated polyvinyl alcohol (PVA) gel. *Enzyme Microb. Technol.* **16:** 79–83.
4. Hashimoto, S. and K. Furukawa. 1987. Immobilization of activated sludge by PVA-boric acid method. *Biotechnol. Bioeng.* **30:** 52–59.
5. Jefferson, B., J. E. Burgess, A. Pochon, J. Harkness, and S. J. Judd. 2001. Nutrient addition to enhance biological treatment of greywater. *Water Res.* **35:** 2702–2710.
6. Jun, B.-H., K. Miyanage, Y. Tanji, and H. Unno. 2003. Removal of nitrogenous and carbonaceous substances by a porous carrier-membrane hybrid process for wastewater treatment. *Biochem. Eng. J.* **14:** 37–44.
7. Kariminiaae-Hamefaani, H.-R., K. Kanda, and F. Kato. 2004. Denitrification activity of the bacterium *Pseudomonas* sp. ASM-2-3 isolated from the Ariake Sea Tideland. *J. Biosci. Bioeng.* **97:** 39–44.
8. Kim, J. K., S.-K. Kim, and S.-H. Kim. 2001. Characterization of immobilized denitrifying bacteria isolated from municipal sewage. *J. Microbiol. Biotechnol.* **11:** 756–762.
9. Kim, S. H., S. H. Song, and Y. J. Yoo. 2004. The pH as control parameter for oxidation reduction potential on the denitrification by *Ochrobacterum anthropi* SY509. *J. Microbiol. Biotechnol.* **14:** 639–642.
10. Koike, I. and A. Hattori. 1975. Growth yield of a denitrifying bacterium, *Pseudomonas denitrificans*, under aerobic and denitrifying conditions. *J. Gen. Microbiol.* **88:** 1–10.
11. Lee, N. M. and T. Welander. 1996. The effect of different carbon sources on respiratory denitrification in biological wastewater treatment. *J. Ferment. Bioeng.* **82:** 277–285.
12. Lemmer, H., G. Lind, G. Metzner, L. Nitschke, and M. Schade. 1998. Vitamin addition in biological wastewater treatment. *Water Sci. Technol.* **37:** 395–398.
13. Matsumura, M., H. Tsubota, O. Ito, P.-C. Wang, and K. Yasuda. 1997. Development of bioreactors for denitrification with immobilized cells. *J. Ferment. Bioeng.* **84:** 144–150.
14. Nagadomi, H., T. Hiromitsu, K. Takeno, M. Watanabe, and K. Sasaki. 1999. Treatment of aquarium water by denitrifying photosynthetic bacteria using immobilized polyvinyl alcohol beads. *J. Biosci. Bioeng.* **87:** 189–193.
15. Nussinovich, A., Y. Aboutboul, Z. Gershon, and J. van Rijn. 1996. Changes in mechanical, structural, and denitrifying properties of entrapped *Pseudomonas stutzeri* bacteria preparations. *Biotechnol. Prog.* **12:** 26–30.
16. Pai, S.-L, N.-M. Chong, and C.-H. Chen. 1999. Potential applications of aerobic denitrifying bacteria as bioagents in wastewater treatment. *Bioresour. Technol.* **68:** 179–185.
17. Park, E.-J., J.-K. Seo, J. K. Kim, K.-H. Suh, and S.-K. Kim. 2000. Denitrification characteristics and microorganism composition of acclimated denitrifier consortium. *J. Microbiol. Biotechnol.* **10:** 410–414.
18. Rostron, W. M., D. C. Struckey, and A. A. Young. 2001. Nitrification of high strength ammonia wastewaters: Comparative study of immobilisation media. *Water Res.* **35:** 1169–1178.
19. Schulthess, R., M. Kuhni, and W. Gujer. 1995. Release of nitric and nitrous oxides from denitrifying activated sludge. *Water Res.* **29:** 215–226.
20. Shen, J. and O. Hirayama. 1993. Denitrification of PVA-immobilized denitrifying photosynthetic bacterium, *Rhodobacter sphaeroides*. *J. Ferment. Bioeng.* **75:** 43–47.
21. Song, S. H., S. H. Yeom, S. S. Choi, and Y. J. Yoo. 2003. Effect of oxidation-reduction potential on denitrification by *Ochrobacterum anthropi* SY509. *J. Microbiol. Biotechnol.* **13:** 473–476.
22. Tartakovsky, B., L. Petti, J. Hawari, and S. R. Guiot. 1998. Immobilization of anaerobic sludge using chitosan crosslinked with lignosulfonate. *J. Indust. Microbiol.* **20:** 45–47.
23. Wijffels, R. H., G. Englund, J. H. Hunik, E. J. T. M. Leenan, A. Bakketun, A. Gunther, J. M. Obon de Castro, and J. Tramper. 1995. Effects of diffusion limitation on immobilized nitrifying micro-organisms at low temperatures. *Biotechnol. Bioeng.* **45:** 1–9.
24. Wijffels, R. H., G. C. Schukking, and J. Tramper. 1990. Characterization of a denitrifying bacteria immobilized in *k*-carrageenan. *Appl. Microbiol. Biotechnol.* **34:** 399–403.