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Characterization of Immobilized Denitrifying Bacteria Isolated from Municipal Sewage

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Abstract As a component for a recirculating aquaculture system, a new strain of denitrifying bacterium was isolated from municipal sewage. The isolate was motile by means of one polar flagellum, catalase-positive, and a Gram-negative rod-shaped cell measuring 0.5–0.6 µm in width and 1.3–1.9 µm in length. The isolate was identified as *Pseudomonas fluorescens* and produced dinitrogen gas via the reduction of nitrate. The optimal growth conditions (pH, temperature, carbon source, and C/N ratio) of the isolate were found to be 6.8, 30°C, malate, and 3, respectively. Under optimal growth conditions of *P. fluorescens*, dinitrogen gas was first detected in the exponential growth phase, then a small amount of nitrite was developed and converted to dinitrogen gas in the stationary phase. *Pseudomonas fluorescens* cells were immobilized in modified polyvinyl alcohol (PVA) gel beads, and the maximum denitrification rate was measured as 36.6 µl N_2 h^{-1} per bead with an optimum cell loading of 20 mg μl^{-1} and 2% sodium alginate added to the PVA gel. The operating stability of the modified PVA gel beads remained unchanged for up to 43 repeated batches.

Key words: Nitrate removal, denitrification, cell immobilization, PVA beads, *Pseudomonas fluorescens*

Nitrogenous compounds are major pollutants of water and occur in domestic waste, agricultural waste, and aqueous waste [18]. In a recirculating aquaculture system, nitrate accumulates through nitrification, and high nitrate concentrations should be avoided due to possible nitrite accumulation and environmental restrictions regarding the discharge of nitrate-rich effluent water [1, 2]. It is known that nitrates can cause infant methemoglobinemia [6] and lead to the possible formation of nitrosoamines, which are known to be carcinogens [19].

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Available methods for nitrate treatment include anion exchange, electrodialysis, biological denitrification, reverse osmosis, distillation, and possibly chemical reduction [17, 25, 26]. When compared with physical and chemical techniques, biological denitrification is known to be a relatively inexpensive and reliable method of nitrate removal [16]. Biological denitrification has been used successfully for the removal of nitrate from wastewater, with the advantage that nitrates are converted into harmless dinitrogen gas and low concentrations of nitrous and nitric oxides [10].

Cell immobilization has also attracted attention due to several advantages, including the easy separation of liquid and solid in a settling tank, a high microbial content, the preservation of microorganisms from the external environment, and the prevention of microbial wash-out [13, 15, 20, 23, 24]. Materials such as polyacrylamide, sodium alginate, agar, K-carrageenan, and polyvinyl alcohol (PVA) have found extensive application as carrier materials for cell immobilization. Of these materials, PVA shows particular promise due to its low production cost and lack of antimicrobial effects [3]. In addition, PVA beads have the elasticity and high mechanical strength that is theoretically adequate for high shear stresses. However, PVA beads gradually expand and in the long run float to the solution surface. Accordingly, it has been suggested that modification of the carrier structure to increase the gel voidness may offer a promising approach to improve its gas permeability, thereby ultimately solving the effect of the beads floating to the surface [9].

The use of a high density, water re-use system is one alternative to conventional pond production systems [30]. Yet, one of the major problems and requirements of this type of system is the removal of nitrite and nitrate. As such, an anaerobic denitrification unit has to be integrated into the recirculating system [22]. So far, few studies on nitrate removal from intensive fish culture systems have been conducted since nitrate is considered to be relatively harmless to fish [4, 14, 31]. Consequently, in this study, a

new strain of denitrifying bacterium as a component of a recirculating aquaculture system was isolated from municipal sewage and the characteristics of its immobilization were investigated with a modified PVA gel.

MATERIALS AND METHODS

Bacterial Cultures and Media

The sludge was obtained from a municipal sewage treatment plant in Pusan, Korea. The sludge sample was first agitated to obtain homogeneous suspensions in sterile 0.2% NaCl. One milliliter was pipetted into a 10-ml screw-cap tube. Then, the tube was completely filled with a modified malate-basal medium (Table 1) and closed tightly in order to selectively enrich facultatively anaerobic bacteria. The pH of the medium was adjusted to 6.8 before autoclaving, and the medium was sterilized at 121°C for 15 min. After 3 days of incubation at 30°C, the cells were first spread with a platinum loop on a modified malate-basal agar medium containing 1.5% nutrient agar, and then covered with sterile agar, which was gelled at 45°C. The cells were then grown between the agar layers. A purified isolate was obtained by repeated streaking on fresh agar plates. Each pure culture was maintained on an agar plate at 4°C and transferred to a fresh agar plate every month.

For flask cultures, an isolated colony was first transferred with a platinum needle into a screw-cap tube under aseptic conditions. The tube was completely filled with a modified malate-basal medium and closed tightly, incubated at 30°C and rotated at 120 rpm. The cells were harvested at the end of the exponential growth phase, and a 5% cell volume was used as the inoculum for the flask cultures. The Hungate technique was used to create anaerobic conditions inside the flask through which O_2-free argon gas was flushed [7]. In order to determine the optimum culture conditions for the isolated denitrifier, the flasks were incubated under growth conditions of various pHs (6, 6.5, 6.8, 7, and 7.5), temperatures (20, 25, 30, and 35°C), C-sources (acetate, ethanol, fructose, malate, and methanol), and C/N ratios (1, 2, 3, and 4). In all the denitrification flask cultures, 0.2% KNO_3 was added to the modified malate-basal medium.

Table 1. Composition of modified malate-basal medium.

Component	Amount (l^{-1})
Malate (g)	2.5
Yeast extract (g)	1
$(NH_4)_2 \cdot SO_4$ (g)	1.25
$MgSO_4 \cdot 7H_2O$ (g)	0.2
$CaCl_2 \cdot 2H_2O$ (g)	0.07
$FeSO_4 \cdot 7H_2O$ (g)	0.01
Ethylenediamine tetraacetic acid (g)	0.02
KH_2PO_4 (g)	0.6
K_2HPO_4 (g)	0.9
Trace element solution[1] (ml)	1
[1]Trace element solution	
$FeSO_4 \cdot 7H_2O$ (g)	3
H_3BO_3 (g)	0.01
$Na_2MoO_4 \cdot 2H_2O$ (g)	0.01
$MnSO_4 \cdot H_2O$ (g)	0.02
$CuSO_4 \cdot 5H_2O$ (g)	0.01
$ZnSO_4$ (g)	0.01
Ethylenediamine tetraacetic acid (g)	0.05

Test of Capacity for Denitrification and Taxonomic Identification

Under anaerobic conditions, 50-ml glass syringes served as the growth vessels in order to screen for denitrifying bacteria. The syringe was completely filled with 20 ml of the inoculated medium under aseptic conditions and placed upside down. The syringe needle was then flamed and stabbed into a solid rubber stopper. In the syringe, the gas produced by the cells accumulated and displaced the plunger upwards as the cells grew. The modified malate-basal medium containing 0.2% KNO_3 was used, and the syringe prepared in this way was incubated at 30°C.

For taxonomic identification, Gram staining and catalase tests were performed. The cell size, motility, and morphology were determined microscopically (1,000×). The isolate was studied by transmission electron microscopy (TEM) to observe its cell wall, membrane, and flagellum. Detailed identification was performed with API 20NE (bioMerieux).

Cell Immobilization Within the Modified PVA Gel

The isolated denitrifying cells were harvested in the late exponential phase of growth by centrifugation at 6,000 rpm for 10 min. The pellet was washed and resuspended in sterile distilled water, and the resulting dense cell suspension was used for immobilization. Different amounts (0, 1, 2, and 3%, w/v) of sodium alginate were first mixed with PVA-HC to determine the optimum concentration of sodium alginate to be added to the PVA gel. One portion of a mixture of sodium alginate and a PVA-HC aqueous solution (18%, w/v) was mixed thoroughly with different amounts (5, 10, 20, and 30 mg ml^{-1}) of cells to determine the optimal cell loading inside the PVA gel beads. The final concentration of PVA-HC was adjusted to 12% (w/v) with 1/15 M phosphate buffer. The resulting mixture was dropped into a saturated boric acid solution containing 0.1% concentration of $CaCl_2$ and gently stirred for 1h to form spherical beads. These modified PVA gel beads were then placed in a 0.5 M sodium phosphate solution (pH 7) for 50 min to simultaneously enforce the gel structure and disintegration of the Ca-alginate binding [9]. The resulting

beads within 4.5±0.5 mm diameters were washed with sterilized distilled water and then subjected to a series of experiments.

Analyses

Samples from the anaerobic flask cultures were analyzed to measure the cell density of denitrifying bacteria. The cell density was measured spectrophotometrically at 440-nm wavelength using a 752 UV Grating spectrophotometer. The samples were diluted in order to confine the absorption readings to a range of 0.1–0.7 optical densities (OD). To relate the measured OD to the dry-cell weight of the bacteria (DCW), the 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 5-ml sample of culture broth at 6,000 rpm for 10 min, and then by decanting the supernatant after washing twice with distilled water. A linear correlation between the DCW and the OD was obtained. The number of viable cells sampled from the anaerobic flask cultures and the extent of cell leakage out of the beads were calculated by counting the colonies formed on an agar plate after considering a dilution factor.

The accumulated gases in the syringe were identified by gas chromatography using 100/120 Carbosieve S-II. Helium gas flowing at 30 ml min^{-1} was used as the carrier gas, and the temperatures of the injector and thermal conductivity detector were 100°C and 150°C, respectively. The column temperature was 35°C at the beginning and increased from 35°C to 70°C at a rate of 8°C min^{-1} after 7 min. The concentrations of nitrate and nitrite were determined by ion chromatography using a DX-120 (Dionex Co.) with an IonPac AS14 column. All measurements were performed in triplicate.

RESULTS

Isolation and Identification of the Denitrifying Isolate

The denitrifying bacteria grown under anaerobic conditions were isolated. Colonies of various colors developed on the surface of the modified malate-basal agar plates after 5 days of incubation at 30°C. Five different colonies were purified by repeated colony selection. The denitrification capacities of the isolates were tested by the use of the syringe technique. Four isolates evolved small amounts of gas, although their growth in the syringe was heavy. One of the isolates produced 5 ml of gas after 15 h of incubation at 30°C. The gas trapped in the syringe was identified by gas chromatography. According to a gas chromatographic analysis, the gas consisted mostly of dinitrogen gas (Fig. 1).

The isolate was motile by means of one polar flagellum (Fig. 2). The strain was a catalase-positive, Gram-negative rod measuring 0.5–0.6 µm in width and 1.3–1.9 µm in length. Multiplication occurred by binary fission. The cells usually occurred singly or in pairs. Using the API 20NE test, the isolate was clearly identified as a member of the species *Pseudomonas fluorescens*.

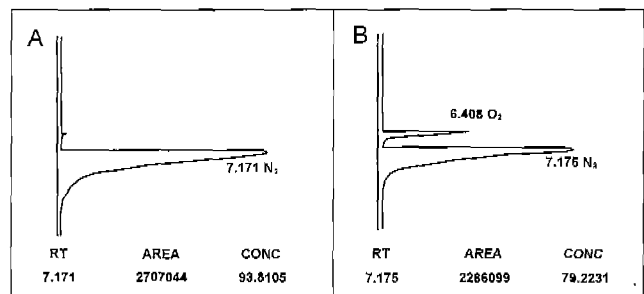

Fig. 1. GC analyses for gas produced by isolate (A) and for air as control (B).

Optimum Growth Conditions of the Isolate

Anaerobic flask cultures were conducted at different pHs and temperatures. The results in Table 2 represent average values for three determinations. The best denitrifying growth of the isolate, *P. fluorescens*, was obtained at pH 6.8 and 30°C.

The denitrifying ability of *P. fluorescens* was tested in a syringe (as the growth vessel) with various organic substrates. As seen in Table 2, the denitrifier used all the substrates tested as electron donors to denitrify nitrate. Of these substrates, malate produced the best result of 555 µl $N_2 \cdot h^{-1}$, and methanol gave the lowest denitrification rate. Since full denitrification was achieved above a C/N ratio of 3 (w/w), the optimum C/N ratio in this study was considered to be 3 (Table 2).

Fig. 2. TEM micrograph of the isolate.

Table 2. The maximum specific growth rate (μ_{max}) and gas production rate of the isolate at various culture conditions.

Culture type	Culture conditions		μ_{max} (h^{-1})	Culture type	Culture conditions		Maximum gas production rate (μl N_2h^{-1})
Flask	pH[1]	6	0.23±0.01	Syringe	C-source	Acetate	250±7
		6.5	0.25±0.01			Ethanol	266±5
		6.8	0.26±0.01			Fructose	286±6
		7	0.25±0.01			Malate	500±10
		7.5	0.14±0.02			Methanol	160±8
	Temperature[2] (°C)	20	0.15±0.01		C/N Ratio[3]	1	119±9
		25	0.19±0.02			2	375±7
		30	0.26±0.01			3	500±10
		35	0.22±0.01			4	500±5

[1]Under 30°C on the malate medium with C/N ratio of 3. [2]Under pH 6.8 on the malate medium with C/N ratio of 3. [3]Under 30°C and pH 6.8 on the malate medium.

Anaerobic Flask Culture of Isolate

The growth of the isolate *P. fluorescens*, under optimal growth conditions, was characterized in anaerobic flask cultures (Fig. 3A). The growth reached a stationary phase after 15 h of cultivation. The number of cells doubled within 2.1 h after a lag of 1 h, and the maximum specific growth rate was estimated to be 0.32 h^{-1}. The maximum number of viable cells was 8×10^9 cfu ml^{-1} with 0.68 g l^{-1} as the DCW.

In the exponential growth phase, dinitrogen gas was detected and a small amount of nitrite accumulated (Fig. 3B). The nitrate was completely converted to dinitrogen gas by the stationary phase.

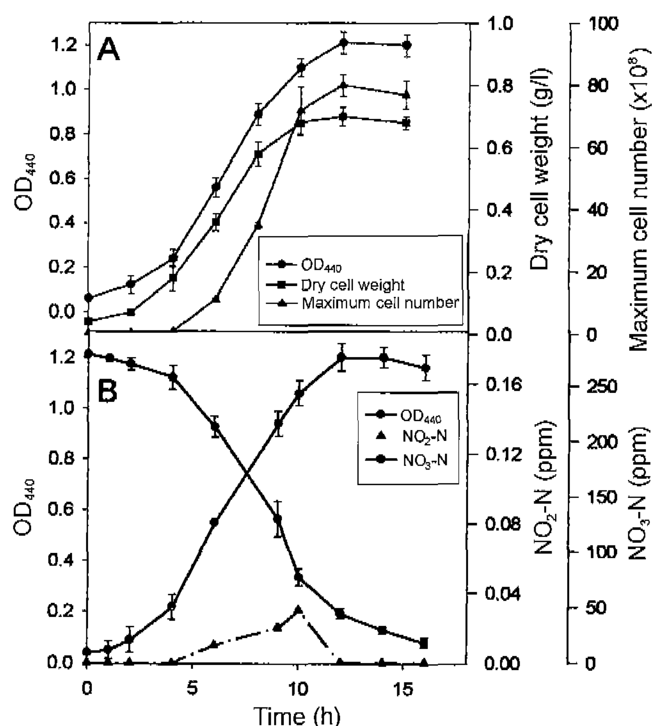

Fig. 3. Profiles of growth (A) and substrate utilization (B) of *P. fluorescens* cultivated in anaerobic flasks.
Error bars, ±0.1 S.D.

Characteristics of Immobilized Isolate

The effect of the sodium-alginate concentration on the denitrification rate in the preparations of PVA gel beads was examined by the use of the syringe technique. The results in Fig. 4 represent the average values for three determinations. The beads in the syringe were transferred under aseptic conditions into another sterilized syringe containing 20 ml of a new modified malate-basal medium after the denitrification finished in each batch. The optimal concentration of sodium alginate added to the PVA gel was found to be 2% (Fig. 4). Without the addition of sodium alginate to the PVA gel, the beads swelled as the denitrification started, then almost all the beads floated up to the plunger. However, the ratio of floating to nonfloating beads decreased with an increase in the concentration of sodium alginate. As a result, the denitrification rate increased up to 2% of sodium alginate added to the PVA gel, however, it decreased above 2% due to cell leakage. Consequently, the maximum denitrification rate was estimated to be 36.6 μl N_2 h^{-1} per bead with 2% sodium alginate in the PVA gel beads.

The optimal cell loading in the PVA gel beads was investigated by the use of the syringe technique. The

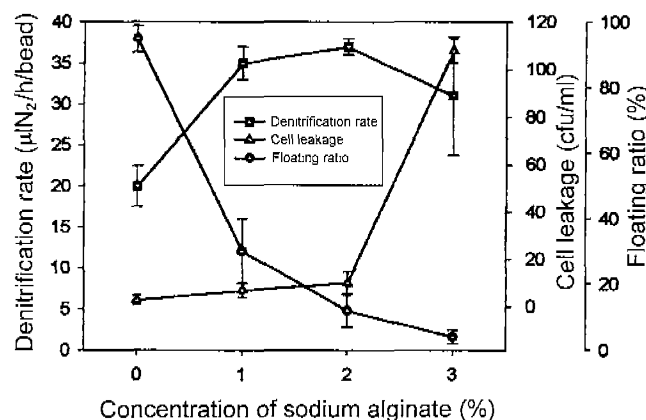

Fig. 4. Characteristics of immobilized *P. fluorescens* with various concentrations of sodium alginate added to polyvinyl alcohol gel beads.
Error bars, ±0.1 S.D.

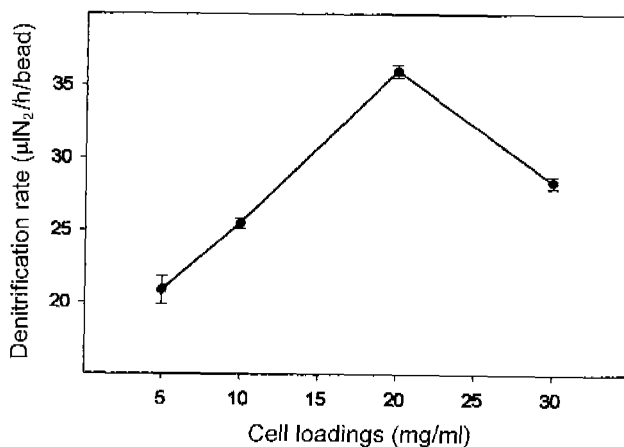

Fig. 5. Denitrification rate with various cell loadings of *P. fluorescens* inside PVA gel beads.
Error bars, ±0.1 S.D.

denitrification rate increased as the cell loading increased (Fig. 5). The maximum denitrification rate was estimated to be 36.6 μl N_2 h^{-1} per bead at the initial cell concentration of 20 mg dry cell weight ml^{-1} in the beads. Above this cell loading, the denitrification rate decreased.

The operating stability of the modified PVA gel beads was tested in a syringe. The beads in the syringe were repeatedly transferred under aseptic conditions into another sterilized syringe containing 20 ml of a new modified malate-basal medium. Figure 6 demonstrates that the denitrification ability of the immobilized isolate gradually improved during the first 4 batches and then remained unchanged for up to 43 batchwise operations. The full capacity of denitrification at a rate of 36.6 μl N_2 h^{-1} per bead was retained by the beads over 43 batch experiments. Thereafter, the denitrification rate decreased, while the apparent mechanical strength of the PVA beads seemed to be maintained.

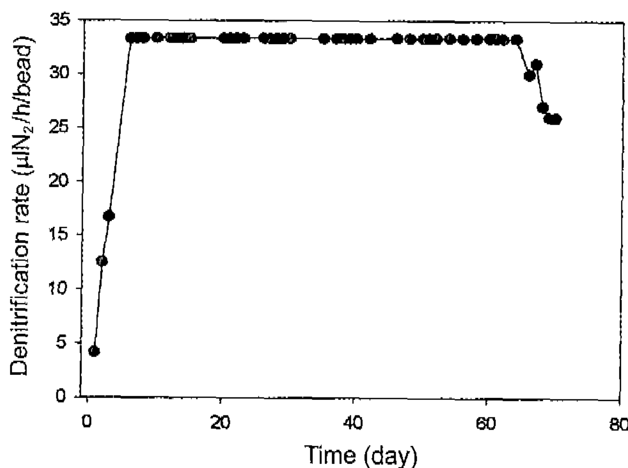

Fig. 6. Repeated batchwise operation of immobilized *P. fluorescens*.

DISCUSSION

In screening denitrifying bacteria, only one isolate produced 5 ml of gas in the syringe, whereas the other isolates produced only an insignificant amounts of gas. Stoichiometric calculations of the denitrification from nitrate (0.2% KNO_3) to dinitrogen gas indicated that the maximum quantity of dinitrogen gas that could be produced in a syringe with a working volume of 20 ml was about 5 ml of gas. Accordingly, this result shows that the isolate exhibited a full capacity for denitrification. The isolate was clearly identified as a member of the species *Pseudomonas fluorescens*, which is known to use nitrate as an alternate electron accepter, thereby growing anaerobically [5].

Among the substrates tested as electron donors to denitrify nitrate, malate produced the best result of 555 μl N_2 h^{-1}. Malate is known to be an excellent carbon source for denitrifying bacteria [28]. However, most denitrification systems currently use methanol as the carbon source for economical and operational (low solid production) reasons [10]. However, methanol resulted in the lowest denitrification rate in our study. The maximum denitrification rate of 555 μl N_2 h^{-1} is equivalent to 40.8 μL N_2 h^{-1} per mg of dry cells. It was previously reported that *Pseudomonas stutzeri*, cultivated on a modified Luria-Bertani (LB) broth, produced only dinitrogen without any nitrous oxide and had a maximum denitrification rate of 57.5 μl N_2 h^{-1} per mg of dry cells with transient accumulation of a small amount of nitrite [8]. This difference may have resulted from the culture conditions and denitrification pathway. In our previous study [14], the maximum denitrification rate was found to be 9.35 μl N_2 h^{-1} per mg of dry cells with photosynthetic bacterium *Rhodopseudomonas palustris*.

The optimum C/N ratio was determined to be 3 (w/w) (Table 2). Respiratory denitrification relies on a carbon source as an electron donor, however, since the available carbon source is not always sufficient in wastewater treatment, an external carbon source may be needed [11]. Nevertheless, an overdose of carbon above the stoichiometric requirement for denitrification can result in high effluent organics. To prevent this problem, the appropriate C/N ratio between the carbon source and the nitrate must be considered. It has been previously reported that a C/N ratio of carbon (glucose) to nitrate of 3–4 is optimum for denitrification by *Pseudomonas* species [29]. In all denitrification capacity tests, 0.2% of KNO_3 was added to the modified malate-basal medium, since nitrate-nitrogen at levels of 181 mg of NO_3-N l^{-1} has been reported to be toxic to fish [12].

The growth of the isolate, *P. fluorescens*, under optimal growth conditions in anaerobic flask cultures showed that dinitrogen gas was detected and a small amount of nitrite accumulated in the exponential growth phase (Fig. 3). The nitrate was completely converted to dinitrogen gas by the stationary phase. This finding agreed with the result by Carlson

and Ingraham [8]. Nitrite is an intermediate in the reduction of nitrate, and a variety of incomplete denitrification pathways also exist. Nitrite accumulation should be avoided due to its toxicity. Accordingly, since the isolate appears to have a complete denitrification pathway, *P. fluorescens* is better suited for use in a recirculating aquaculture system.

It was observed that the PVA gel beads swelled as the cells started denitrification, and almost all the beads thereafter floated up to the plunger. However, the ratio of floating to nonfloating beads decreased with the addition of sodium alginate to the PVA gel. The optimal concentration of sodium alginate in the PVA gel was found to be 2% (Fig. 4). It is presumed that the PVA gel itself had poor gas permeability due to the bead structure and that a loose structure was created in the PVA gel beads after the addition of sodium alginate [9]. Consequently, the denitrification rate increased with the addition of up to 2% sodium alginate in the PVA gel, however, it decreased above 2% due to cell leakage, indicating that addition of sodium alginate to the PVA gel resulted in not only improvement of gas permeability but also increase of cell leakage.

The denitrification rate increased by raising the cell loading (Fig. 5). The maximum denitrification rate was estimated to be 36.6 μl N_2 h^{-1} per bead at an initial cell concentration of 20 mg dry cell weight ml^{-1} in the beads. This rate was almost the same as that obtained from the suspended cells. Above this cell loading the denitrification rate decreased, probably due to diffusional limitation. Such a drop of the denitrification rate by diffusional limitation has also been reported to occur during a denitrification process with immobilized cells in alginate gels [20].

During the first 4 batches in repeated batchwise operation (Fig. 6), it is presumed that the improvement in denitrification resulted from adaptation and growth of the isolate, *P. fluorescens*, inside the beads. After 43 batchwise operations, the denitrification rate decreased, while the apparent mechanical strength of the PVA beads was seemingly maintained. This probably occurred due to a decrease in the cell activity and the weakness of the matrix inside the beads [21, 27]. Accordingly, this study proved that immobilized *P. fluorescens* could be a good vehicle for the removal of nitrate in a recirculating aquaculture system.

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