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Hydrogenotrophic denitrification with immobilized *Alcaligenes eutrophus* for drinking water treatment

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Abstract

This paper used a new entrapment method for cell immobilization to elucidate the rate of autotrophic denitrification and obtain the appropriate operating conditions for drinking water treatment. *Alcaligenes eutrophus*, a hydrogenotrophic denitrifier, was immobilized in polyacrylamide and alginate copolymer to evaluate denitrification in continuous mode and batch mode in a fluidized-bed reactor. The total nitrogen removal rate in a continuous test was increased with operation time and reached a maximum rate (0.6–0.7 kg-N/m^3/day) after 6 days. The dissolved hydrogen concentration had a significant effect on denitrification. In batch test, nitrite reductase was inhibited when the dissolved hydrogen concentration fell below 0.2 mg/l, while nitrate reductase was inhibited at a concentration below 0.1 mg/l. The phosphate concentration also affected denitrification, especially in the accumulation of nitrite. The bacteria have a good ability to adapt to a shock nitrate loading. A high nitrite concentration was found when nitrate was first added to the reactor in batch mode. The nitrite concentration, however, decreased significantly with the second and third addition of nitrate after its degradation in the first addition.

Keywords: *Alcaligenes eutrophus*; Biological denitrification; Autotrophic; Drinking water; Nitrate; Nitrite; Immobilization; Polyacrylamide; Alginate

1. Introduction

Biological denitrification with heterotrophic microorganisms has been widely and successfully applied to wastewater treatment, but the residual carbon sources from these processes cause many problems in drinking water treatment. In this paper, an autotrophic bacterium was used to resolve these problems. There are four groups of autotrophic denitrifiers (Mateju et al., 1992; Rott and Lamberth, 1992): hydrogen oxidation bacteria, reduced sulfur oxidation bacteria, ferrous oxidation bacteria, and chloric compound oxidation bacteria. Hydrogen oxidation bacteria remove nitrates and nitrites by autotrophic denitrification, using hydrogen as an energy source and carbon dioxide or bicarbonate as the carbon source. In this reaction, nitrate or nitrite is the electron acceptor and hydrogen is the electron donor. The major pathway of denitrification may be: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. It is generally simplified into two steps (Kurt et al., 1987; Dries et al., 1988; Germonpre et al., 1992; Gros et al., 1988)

$$2NO_3^- + 2H_2 \rightarrow 2NO_2^- + 2H_2O$$

$$2NO_2^- + 3H_2 + 2H^+ \rightarrow N_2 + 4H_2O$$

The total reaction is:

$$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O$$

The equation shows that the pH will increase after the reaction, because 1 mole of H^+ is used when 1 mole of NO_2^- is converted to nitrogen gas. One milligram of NO_3^-–N would, therefore, use 0.357 mg of hydrogen gas theoretically. Autotrophic denitrification by hydrogen oxidation bacteria is the most suitable process for drinking water treatment, because it has several advantages: (1) this process has a highly selectivity for nitrate removal and the reaction byproducts are harmless to humans; (2) the substrate for hydrogen oxidation bacteria is hydrogen gas, which is harmless to humans, so the residual hydrogen gas would not cause

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problems; (3) this process uses inorganic carbon sources and thereby removes any problems that are caused by residual organic carbons. Three hydrogen oxidation bacteria have been reported (Mateju et al., 1992; Rott and Lamberth, 1992): *Paracoccus denitrificans*, *Alcaligenes eutrophus*, and *Pseudomonas pseudoflava*. Most papers on autotrophic denitrification report using a mixed-culture. In this paper, *Alcaligenes eutrophus* was immobilized in polyacrylamide and alginate copolymer for the denitrification study, comparison with mixed-culture processes, and obtaining the appropriate operating conditions.

2. Methods

2.1. Microorganism and growth medium

A. eutrophus (CCRC13036) was obtained from the Food Industry Research and Development Institute in Hsinchu, Taiwan. The growth medium contained KNO_3 (2 g/l), KH_2PO_4 (2 g/l), $NaHCO_3$ (2 g/l), and trace element solution (1 ml/l). The trace element solution was composed of $MgSO_4 \cdot 7H_2O$ (10.0 g/l), $ZnSO_4 \cdot 7H_2O$ (2.2 g/l), $CaCl_2 \cdot 2H_2O$ (7.3 g/l), $MnCl_2 \cdot 4H_2O$ (2.5 g/l), $CoCl_2 \cdot 6H_2O$ (0.5 g/l), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.5 g/l), $FeSO_4 \cdot 7H_2O$ (5.0 g/l), and $CuSO_4 \cdot 5H_2O$ (0.2 g/l) and adjusted to pH = 7.0.

2.2. Cell immobilization

A. eutrophus was entrapped in polyacrylamide and alginate copolymer as follows: 20 g acrylamide (Sigma), 4 g sodium alginate with medium viscosity (Sigma), and 2 g N,N′-methylenebisacrylamide (Sigma) were diluted with deionized water to 100 ml and heated untill dissolved. The solution was cooled down and then mixed with 100 ml of *A. eutrophus* suspension ($8.7 \pm 0.3 \times 10^8$ CFU/ml) and 1.5 ml of β-(dimethylamino)-propionitrile. The mixture was extruded as drops into a solution of $K_2S_2O_8$ (2%) and $CaCl_2$ (1%) and immersed for 1 h to form the copolymerized spherical beads (3–5 mm). The beads were washed with tap water and stored in deionized water at 4°C until use in the experiment.

2.3. Continuous experiment

A fluidized-bed reactor, 42-cm high and 5-cm internal diameter, was used in the experiments (Fig. 1). The total reactor volume was 1000 ml, and 20% of the reactor volume was occupied by the immobilized beads. The influent water was prepared as described in Table 1. System 1 was prepared with deionized water and System 2 was prepared with tap water. The composition of the trace element solution was the same as mentioned before. The hydraulic retention times of Systems 1 and 2 were 400 and 53 min, respectively. The recycling rate of both was 175 ml/min. The flow rate of hydrogen gas was controlled by a flow meter to adjust the dissolved hydrogen concentration in the reactor.

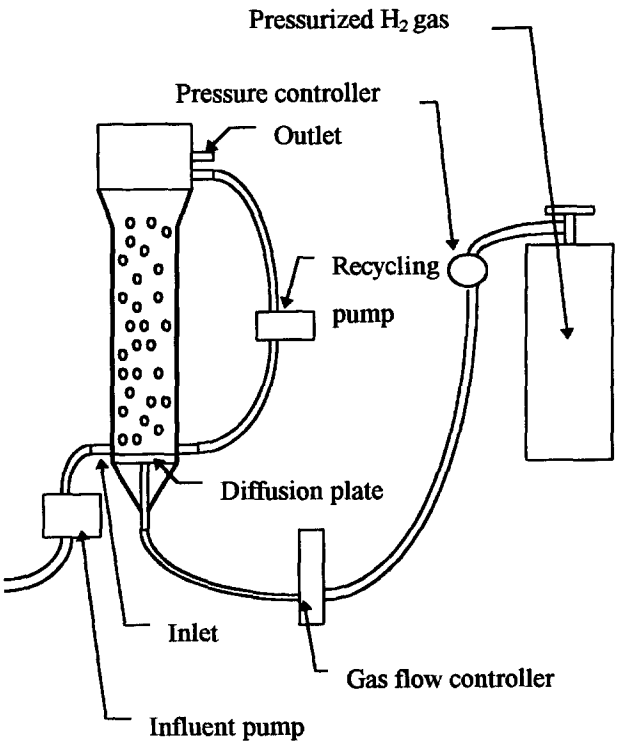

Fig. 1. Diagram of the reactor.

Table 1
Compositions of influent water in continuous experiments

	KNO_3 (g/l)	$NaHCO_3$ (g/l)	KH_2PO_4 (mg/l)	Trace element (ml/l)	pH
System 1 (phosphate deficient)	0.33	1.33	Not added	0.1	Adjusted with CO_2 to pH = 7.0
System 2 (phosphate enriched)	0.16	0.16	8.3	0.1	Adjusted with CO_2 to different pH

2.4. Batch experiment

The reactor described above was used for batch experiments. The initial conditions of each batch test are listed in Table 2, with the operating conditions the same as those for System 2 of the continuous test. In the shock nitrate loading test, nitrate was added in three consecutive runs (Table 2). Run A corresponds to the first addition of nitrate to the reactor, and runs B and C to the second and third additions. In runs B and C, nitrate was added after the compound from the previous run was completely degraded.

2.5. Analytical methods

The pH was measured with a pH meter (WTW pH537). The nitrate, nitrite, and phosphate concentrations were determined by ion chromatography (Dionex QIC). In each analysis (four samples), at least a sample was duplicated and the deviation between the two samples was always less than 5%. The dissolved hydrogen concentration was measured with a hydrogen analyzer (Orbisphere laboratories 3600). Bacteria concentration was analyzed by a spread plate method (APHA, 1995) for seven replications.

3. Results and discussion

3.1. Cell immobilization

Polyacrylamide has been widely used in immobilization of microorganisms and enzymes (Axelsson et al., 1994; Cottenceau et al., 1990; Pizarro et al., 1997; Sadhukhan et al., 1993; Seip and Cosimo, 1992; White and Thomas, 1990). Its use in cell immobilization has many advantages, but spherical beads are difficult to produce. The present study used a new method for cell immobilization with polyacrylamide. Sodium alginate was added to polyacrylamide to improve the formation, porosity and diffusion properties of the beads. Five different sodium alginate concentrations (1%, 2%, 3%, 4% and 5%) were tested (data not shown). The optimal concentration of sodium alginate in the copolymer solution was 2%. Decreasing the sodium alginate concentration made formation of spherical beads difficult, but increasing the concentration reduced the strength of the beads and caused them to break easily.

3.2. Continuous experiments

3.2.1. Process performance of System 1

The reactor was operated in a phosphate-deficient condition in System 1. The composition of the influent water is given in Table 1. In this reactor system, nitrate was converted to nitrite gradually in the first 6 days; after 6 days, the nitrate removal rate decreased rapidly (Fig. 2). The process of denitrification was not complete, and the total nitrogen removal rate was very low. These phenomena may be due to the phosphate deficiency because when the reactor operated in a phosphate enriched condition (System 2) the removal rate increased rapidly. A similar phenomenon was found by Dries et al. (1988), who added soil extract solution in influent water as the nutrient source. Withholding soil extract solution from the influent caused a 25–30% decrease in nitrate removal, whereas replacing the soil extract solution with 1.1 mg-P/l produced good nitrate removal rates. Germonpre et al. (1992) found that 0.49 mg of phosphate was required for removal of 75 mg of nitrate. This shows that phosphorous is a necessary nutrient in this reaction system.

3.2.2. Process performance of System 2

In this system, the reactor was operated with a phosphate enriched environment. The composition of the influent is given in Table 1, and the results of System 2 are shown in Fig. 3. In the first week, the denitrification was not complete. The nitrate concen-

Table 2
Initial conditions for batch tests

	Run no.	NO_3 (mg-N/l)	pH	DH[a] (mg/l)	P (mg-P/l)	Trace element (ml/l)
Shock nitrate loading test	Run A	16.00	7.45	0.909	1.8	0.1
	Run B	24.68	7.54	1.097	1.8	0.1
	Run C	18.27	7.42	0.980	1.8	0.1
Phosphate concentration test	Run 1	25.67	7.34	1.174	0.5	0.1
	Run 2	23.74	7.40	1.296	2.9	0.1
	Run 3	23.86	7.36	1.269	7.0	0.1
	Run 4	21.73	7.08	1.034	16.3	0.1
Dissolved hydrogen concentration test		27.57	9.76	0.210	1.8	0.1

[a]DH: dissolved hydrogen concentration.

tration in the effluent water decreased rapidly, and the nitrite concentration increased gradually during this period. After 10 days, near-complete denitrification occured, when the reactor was operated at the appropriate conditions. Nitrate and nitrite concentrations in effluent water were both below 0.5 mg-N/l, and total nitrogen removal efficiency was about 95–100%. The maximum total nitrogen removal rate of System 2 was 0.6–0.7 kg-N/m^3/day, which was more rapid than System 1. Compared to previous studies (Table 3), this nitrate removal rate is only slower than that obtained by Germonpre et al. (1992). It seems that the nitrate removal rate of fixed bed reactors is greater than that of fluidized-bed reactors, because the filling ratio of fixed-bed reactors is often greater than that of fluidized-bed reactors.

The effect of dissolved hydrogen concentration on nitrate removal was significant (Fig. 3). The threshold concentration for dissolved hydrogen appeared to be 0.2 mg/l. Nitrate removal was nearly 100% when the concentration of dissolved hydrogen was greater than 0.2 mg/l. Incomplete denitrification occurred when the dissolved hydrogen concentration fell below 0.2 mg/l, during which the nitrite concentration increased. These results imply that nitrite reductase is more sensitive than nitrate reductase, because nitrite reductase was inhibited when the dissolved hydrogen concentration was lower than 0.2 mg/l, but nitrate reductase was not then inhibited. During this period the nitrite concentration increased (confirmed in batch tests, see Fig. 7).

The pH of the effluent water showed a good correlation with nitrogen removal efficiency when the pH of influent water was stable. This is a good and convenient index for monitoring incomplete denitrification. An unusual drop in pH suggested that incomplete denitrification had occurred (Fig. 3).

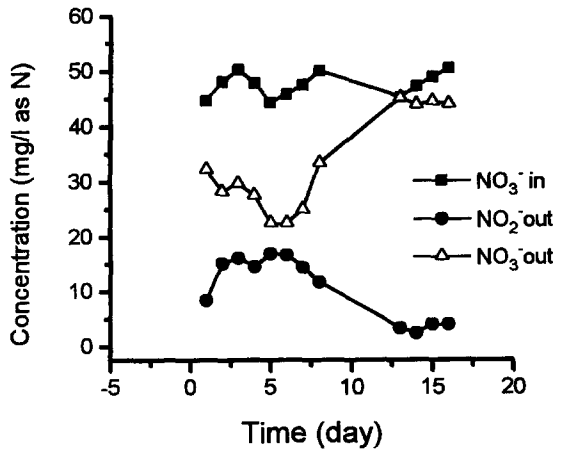

Fig. 2. Nitrate removal in continuous experiments (phosphate deficient).

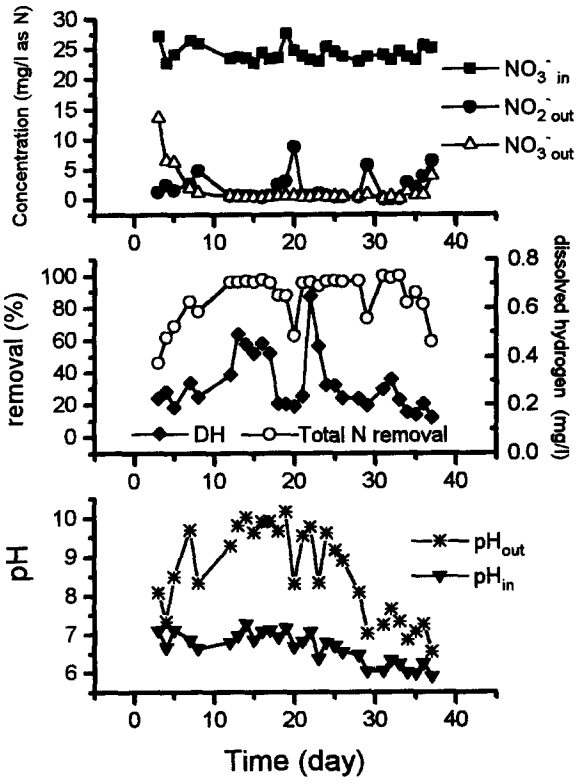

Fig. 3. Variation of nitrogen compounds in continuous experiments (phosphate enriched).

Table 3
Comparison of hydrogenotrophic denitrification systems

Microorganisms	Carriers	Filling ratio (%)	Reactor type	Removal rate (kg-N/m^3/day)	Influent concentration (mg-N/l)	Temperature (°C)	Reference
Mixed	Plastic	No[a]	Fixed	0.4	17	10.5	Gros et al. (1988)
Mixed	Sand	No	Fluidized	0.13	25	30	Kurt et al. (1987)
A. eutrophus	PU sponge cubes	40	Fixed	0.2	50	12–20	Dries et al. (1988)
Seeded	PU sponge strips	40	Fixed	0.5	15	12–20	
Mixed	Charcoal	No	Fixed	0.31–0.34	18–20	15	Ginocchio (1984)
Mixed	PU sponges	No	Fixed	1.35	17	20	Germonpre et al. (1992)
A. eutrophus	Polyacrylamide–alginate copolymer	20	Fluidized	0.6–0.7	22–25	30	This study continuous test

[a]No data.

3.3. Batch experiment

Diagrams of the batch tests results are shown in Fig. 4. Nitrate degradation was a first-order reaction; the first-order coefficient of this system was 0.15 min^{-1}, and the R^2 was 0.96. Nitrite concentration increased in the first 18 min and then decreased gradually. The dissolved hydrogen concentration curve was similar to the oxygen-sag-curve of a river, which showed depletion first and then gradual restoration to saturation. The dissolved hydrogen concentration curve proved that hydrogen gas was used by the bacteria in this system. The pH of the reactor continuously increased during the experimental period.

3.3.1. Effect of shock nitrate loading

This test was performed to understand what the conditions in the reactor would be if an unusually high concentration of nitrate was added. The degradation of total nitrogen in three runs (Table 2) did not differ significantly, but the degradations of nitrate and nitrite were obviously different (Fig. 5). The results showed that the bacteria have a good ability to adapt to a shock nitrate loading. Nitrite accumulated to a high concentration when nitrate was first added to the reactor, and decreased significantly in the second and third additions. Nitrite concentration in runs B and C did not show significant differences; this implies that nitrite reductase was already activated after nitrate was completely degraded in the first addition. The nitrate removal rate in run A was faster than those in runs B and C, but the removal rates in runs B and C did not show significant differences. The results of run A imply that nitrate reductase was activated before nitrite reductase, thereby the nitrate removal rate increased and the accumulation of nitrite in the reactor was high. The results also show that nitrate reductase competed with nitrite reductase for hydrogen gas, because the total nitrogen removal rates in the three runs were about the same, but the nitrate removal rate in runs B and C was slower than that in run A, and the accumulation of nitrite in runs B and C decreased.

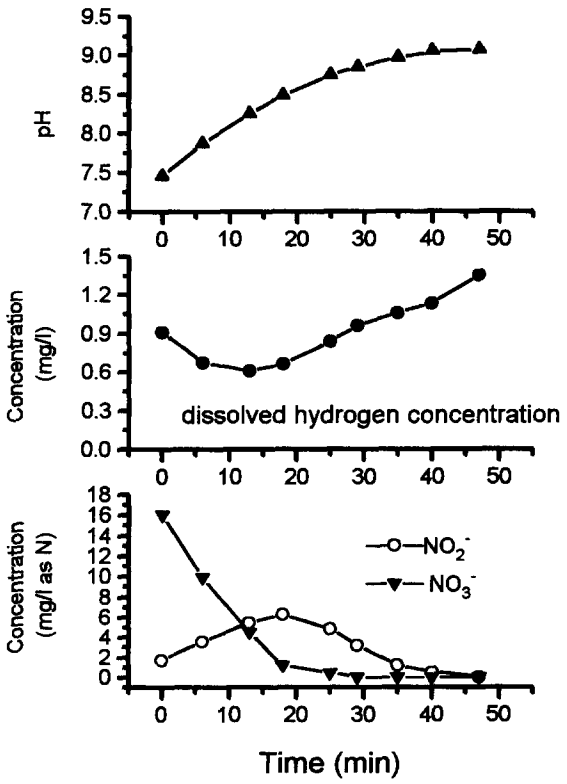

Fig. 4. Concentration diagrams of the batch tests.

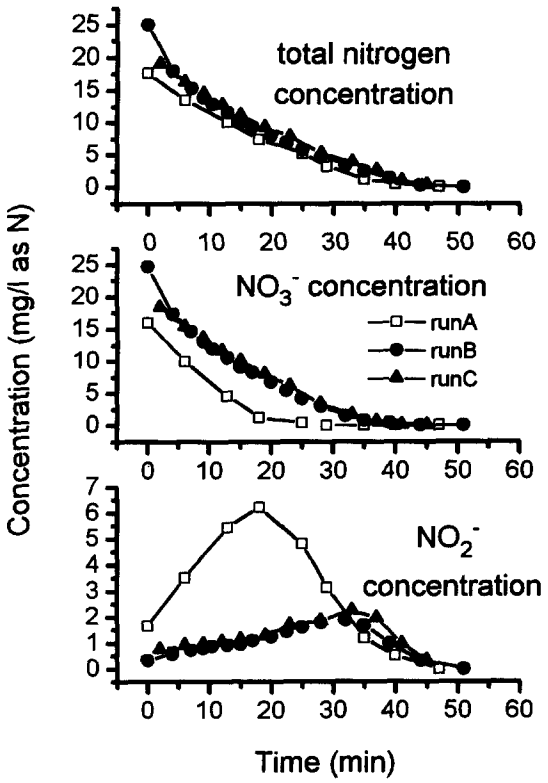

Fig. 5. Effect of shock nitrate loading.

3.3.2. Effect of phosphate concentration

Phosphate concentration (Table 2) did not have a significant effect on the nitrate removal rate but had a significant effect on the accumulation of nitrite (Fig. 6). As the concentration of phosphate increased, the accumulation of nitrite decreased. A relatively similar result was found in the continuous experiment. Combining the results from the batch and continuous experiments, it is evident that phosphorous is a necessary nutrient for this system. Phosphate had a significant effect on the accumulation of nitrite, but its effect on nitrate removal rate was small at a concentration greater than 0.5 mg-P/l.

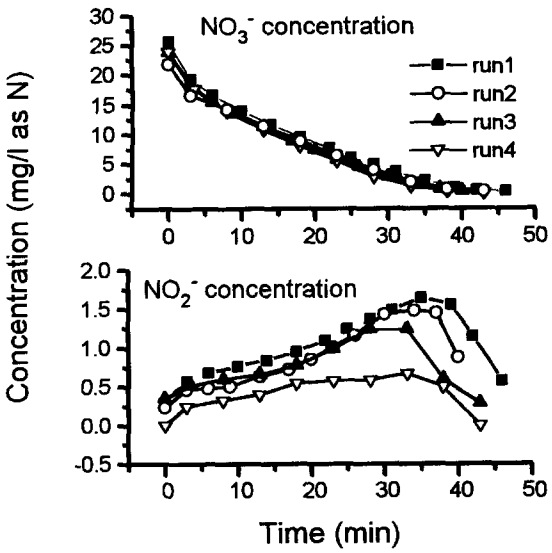

Fig. 6. Effect of phosphate concentration.

3.3.3. *Effect of dissolved hydrogen concentration*

The dissolved hydrogen concentration had a significant effect on nitrate and nitrite removal. Nitrite reductase was inhibited at a concentration lower than 0.2 mg/l and nitrate reductase was inhibited when the concentration was below 0.1 mg/l (Fig. 7). The results showed that nitrite reductase was more sensitive than nitrate reductase, often causing nitrite accumulation when the concentration of dissolved hydrogen fell below the threshold concentration. In order to allow complete denitrification and prevent the accumulation of nitrite, the concentration of dissolved hydrogen in the reactor should be maintained above 0.2 mg/l (confirmed in continuous tests, see Fig. 3).

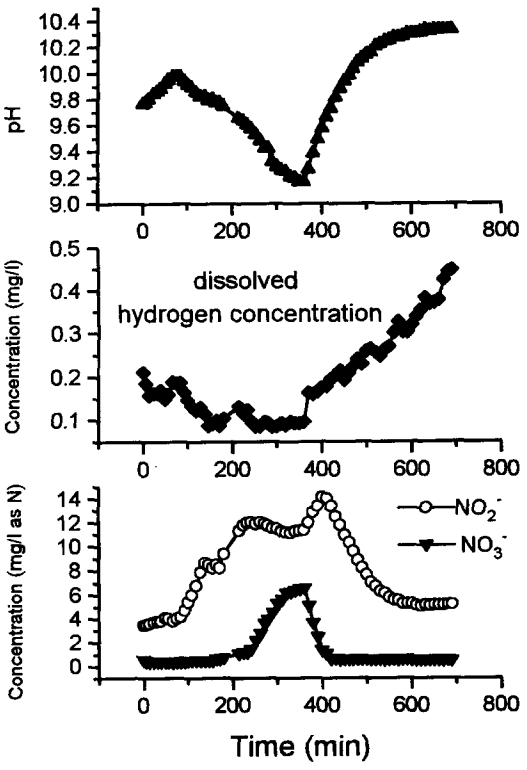

Fig. 7. Effect of dissolved hydrogen concentration.

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References

APHA, AWWA, WEF, 1995. Standard Methods for the Examination of Water and Wastewater, 19th ed. APHA, Washington.

Axelsson, A., Sisak, C., Westrin, B.A., Szajani, B., 1994. Diffusional characteristics of a swelling gel and its consequences for bioreactor performance. Chemical Engineering Journal 55, B35–B39.

Cottenceau, G., Dherbomez, M., Lubochinsky, B., Letellier, F., 1990. Immobilization and treatment of *Streptococcus faecalis* for the continuous conversion of arginine into citrulline. Enzyme Microb. Technol. 12, 355–360.

Dries, D., Liessens, J., Verstraete, W., Stevens, P., de Vos, P., de Ley, J., 1988. Nitrate removal from drinking water by means of hydrogenotrophic denitrifiers in a polyurethane carrier reactor. Water Supply 6, 181–192.

Germonpre, R., Liessens, J., Verstraete, W., Beernaert, S., 1992. Methylotrophic and hydrogenotrophic denitrification at the blankart plant. Water Supply 10, 53–64.

Ginocchio, J., 1984. Nitrate levels in drinking water are becoming too high. Water Treatment 88, 143–147.

Gros, H., Schnoor, G., Rutten, P., 1988. Biological denitrification process with hydrogen-oxidizing bacteria for drinking water treatment. Water Supply 6, 193–198.

Kurt, M., Dunn, I.J., Bourne, J.R., 1987. Biological denitrification of drinking water using autotrophic organisms with H_2 in a fluidized-bed biofilm reactor. Biotechnol. Bioeng. 29, 493–501.

Mateju, V., Cizinska, S., Krejci, J., Janoch, T., 1992. Biological water denitrification — a review. Enzyme Microb. Technol. 14, 170–183.

Pizarro, C., Fernandez-Torroba, M.A., Benito, C., Gonzalez-Saiz, J.M., 1997. Optimization by experimental design of polyacrylamide gel composition as support for enzyme immobilization by entrapment. Biotechnol. Bioeng. 53, 497–506.

Rott, U., Lamberth, B., 1992. Subterranean denitrification for the treatment of drinking water. Water Supply 10, 111–120.

Sadhukhan, R., Roy, S.K., Chakrabarty, S.L., 1993. Immobilization of α-amylase from *Myceliophthora thermophila* D-14 (ATCC 48104). Enzyme Microb. Technol. 15, 801–804.

Seip, J.E., Cosimo, R.D., 1992. Optimization of accessible catalase activity in polyacrylamide gel-immobilized *Saccharomyces cerevisiae*. Biotech. Bioeng. 40, 638–642.

White, G.F., Thomas, O.R.T., 1990. Immobilization of the surfactant-degrading bacterium Pseudomonas C12B in polyacrylamide gel beads: I. Effect of immobilization on the primary and ultimate biodegradation of SDS, and redistribution of bacteria within beads during use. Enzyme Microb. Technol. 12, 697–705.