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Inhibitor Evaluation with Immobilized *Nitrobacter agilis* Cells

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***Nitrobacter agilis* was entrapped in calcium alginate beads and used as a floating bed supplied with a continuous flow of nitrite medium. Complete nitrite oxidation was achieved within 30 h, and the system could be maintained for at least 210 h. The immobilized *Nitrobacter* system was subjected to sulfur oxyanions, acidity, and metal ions. Thiosulfate and tetrathionate (up to 20 mM each) did not inhibit the nitrite oxidation activity. A low pH of 4.2 resulted in the complete cessation of nitrite oxidation, and the activity was not restored upon increasing the pH to 7. Nitrite oxidation by *N. agilis* was sensitive to 10 mM each Ni^{2+} and Al^{3+} but insensitive to 10 mM MoO_4^{2-}.**

Nitrification as a biological process is an important component of nitrogen cycling in the environment. Control of its rate is highly desirable in agriculture to prevent losses of nitrogen from the fertile root zone. Moreover, biological nitrification is of importance in the activated sludge process of wastewater treatment. Efforts to study the kinetics of ammonia and nitrite oxidation in relation to agricultural practices and wastewater treatment have included use of soil columns (6) and attached cell bioreactors (2, 9). The conversion of ammonia to nitrite has been characterized for entrapped *Nitrosomonas* systems (10, 12), whereas similar information on nitrite oxidation by entrapped *Nitrobacter* spp. has not been available.

In a previous study, the sensitivity of nitrite oxidation to metal ions, tetrathionate, and persulfate was determined by the measurement of oxygen uptake activities of washed cell suspensions of *Nitrobacter* spp. (11). This approach made it possible to estimate the effects of test compounds on the kinetic parameters of nitrite oxidation. In the present study, the work was expanded by establishing an immobilized cell system to determine the effects of several inorganic test compounds on the nitrite oxidation activity of *Nitrobacter agilis*. For this purpose, the bacteria were entrapped in a calcium alginate matrix and used as a floating-bed reactor to monitor the oxidation of nitrite under various experimental conditions.

MATERIALS AND METHODS

N. agilis was grown with 14.5 mM nitrite in a mineral salts medium at room temperature. The mineral salts medium was modified from that described by Bhuiya and Walker (4) by using iron chelate (1 mg/liter; GEIGY Agricultural Chemicals, Ardsley, N.Y.) as the source of iron.

Washed cell suspensions were prepared as previously described (11) and standardized by protein measurement (8). For immobilization, 35-ml samples containing 37.4 ± 3.7 mg of protein were mixed with equal volumes of 2% sodium alginate solution. The mixture was dropped into a stirred 0.1 M $CaCl_2$ solution through an 18-gauge, 0.5-in. (ca. 12.7 mm) needle to form 2-mm-diameter beads. After equilibration for 1 h in the calcium salt solution, the beads were rinsed with the mineral salts medium and packed in glass columns (40 by 330 mm) supplied with a continuous flow of nitrite medium. The total volume was 300 ml per column; the media were pumped from the bottom and removed from the top with the use of a peristaltic pump. The test compounds (1 mM each Al^{3+} [as $AlCl_3 \cdot 6H_2O$], Ni^{2+} [as $NiCl_2 \cdot 6H_2O$], and MoO_4^{2-} [as $Na_2MoO_4 \cdot 2H_2O$]; 10 mM each Al^{3+}, Ni^{2+}, MoO_4^{2-}, $S_2O_3^{2-}$ [as $Na_2S_2O_3$], and $S_4O_6^{2-}$ [as $K_2S_4O_6$]; and 20 mM each $S_2O_3^{2-}$ and $S_4O_6^{2-}$) were prepared in nitrite media. The low-pH media were adjusted with 0.1 N HCl. The test compound was introduced into the influent after complete nitrite oxidation was achieved in the column. The tests were carried out at room temperature. The flow rates and pH of effluents were recorded, and the residual nitrite in the effluent was determined by a colorimetric method (1). Thiosulfate and tetrathionate were determined colorimetrically (7). A control column which contained calcium alginate beads without *N. agilis* was also used. In a separate control experiment, it was established that *N. agilis* was not sensitive to chloride ions in the medium (30 mM KCl or NaCl).

RESULTS AND DISCUSSION

Experimental column design. In the initial experiments, tightly packed beads overlaid with glass wool in the columns were used with aeration at the bottom. This experimental column design resulted in incomplete utilization of nitrite and disintegration of beads as a result of formation of air pockets and breakage of beads. Complete nitrite oxidation in the column was not achieved by aerating the medium in the reservoir before pumping it to the packed-bed column. Complete oxidation was achieved with loose bead packing as a floating-bed system, with aeration (68 ml of air per min) introduced at the bottom of the column. The beads with entrapped cells as floating-bed columns retained the nitrite oxidation activity for at least 210 h, by which time the experiments were terminated. Nitrite oxidation did not take place in the control column in the absence of *N. agilis*. The flow rate, nitrite oxidation rate, and retention time of each immobilized *Nitrobacter* column are summarized in Table 1.

Material balance. To estimate the nitrite oxidation rate, a nitrite balance around the column was made. That is, the rate of accumulation of nitrite within the column, $d(VC)/dt$, was equated with the rate at which nitrite flowed into the column ($F_{in}C_{in}$) less the rate at which nitrite flowed out from the column ($F_{out}C_{out}$) less the rate at which nitrite was oxidized in the column $f(t)$, which represents a function of

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TABLE 1. Kinetics of *Nitrobacter* floating-bed columns

Test variable	F (ml/h)	Initial time course (h)	Rate (k) calculations[a]		Retention time (h)		
			mg of NO_2^--N/h[b]	mg of NO_2^--N/h per mg of protein[c]	t_r'	t_r	
						I[d]	II[d]
$S_2O_3^{2-}$	17.9 ± 1.1	6	9.91	0.244	15.7	17.4	14.4
$S_4O_6^{2-}$	16.8 ± 0.9	9	7.99	0.210	16.8	17.8	16.8
pH	18.9 ± 1.1	6	8.04	0.198	14.9		
MoO_4^{2-}	18.5 ± 1.1	6	7.77	0.233	15.2		
Ni^{2+}	17.7 ± 0.9	9	7.37	0.193	15.9		
Al^{3+}	18.2 ± 1.2	6	7.76	0.232	15.5		

[a] All k values are calculated from the time course before the introduction of the test variable.
[b] Mean = 8.14 ± 0.90.
[c] Mean = 0.218 ± 0.021.
[d] I, Values derived from the increasing effluent concentrations after introduction of the marker; II, values derived from the decreasing effluent concentrations after discontinued feeding of the marker.

several variables including nitrite concentration, the number of cells in the column, and the activity of the cells. The construction of this balance is based on the assumption that the contents of the column were perfectly mixed, or equivalently, that conditions were the same at all points within the column. If it is further assumed that the column volume (V) and flow rates into and from the column were constant ($F_{in} = F_{out} = F$), the nitrite balance becomes $V(dC/dt) = FC_{in} - FC_{out} - f(t)$, where V is the total liquid volume (282 ml); F is the flow rate (in milliliters per hour); C is the concentration of nitrite in the column, which is also that of nitrite in the effluent, C_{out} (NO_2^--N, in milligrams per liter); and $f(t)$ is the oxidation rate (NO_2^--N, in milligrams per hour). Solution of this differential equation in terms of C, the nitrite concentration in the column, is presently impossible since the form of the function $f(t)$ is unknown. However, since it is desired to estimate only the average nitrite oxidation rate between two points in time (0 and t), the function $f(t)$ can be replaced by the constant average rate k, which simplifies the balance, $V(dC/dt) = F(C_{in} - C) - k$. Solution of this first-order equation in terms of C may be accomplished by several techniques. Use of $\exp(Ft/V)$ as an integrating factor (13) or application of Laplace transforms (5) are two such methods. Ultimately, the derived solution is $C(t) = (C_{in} - k/F)[1 - e^{-(F/V)t}] + C(0)e^{-(F/V)t}$, or the average nitrite oxidation rate becomes $k = -\{FC(t) + FC_{in}[e^{-(F/V)t} - 1] - FC(0)e^{-(F/V)t}\}/[1 - e^{-(F/V)t}]$.

The average rate of nitrite oxidation in each column was calculated by using the above equation (Table 1). The average activity in the first 6 to 9 h was approximately 0.22 ± 0.02 mg of NO_2^--N/h per mg of protein. This activity assumes no change in biomass during that period which seems consistent with previous observations that instantaneous growth rates (μ) of *Nitrobacter* spp. are in the range of 0.015 to 0.056/h (3). This particular strain grows in batch culture at μ = 0.04/h, but its μ in continuous flow systems has not been determined. The oxidation rate was equal to the flow rate during the steady state (4.39 ± 0.17 mg of NO_2^--N/h, or 0.11 ± 0.01 mg of NO_2^--N/h per mg of protein). The comparison of the initial and steady-state rates indicates that the columns were operated at flow rates which were below the maximum nitrite oxidation capacity of the immobilized *N. agilis*.

Sulfur compounds and effectiveness of mixing. Nitrite oxidation was not influenced by either $S_2O_3^{2-}$ or $S_4O_6^{2-}$ at concentrations of up to 20 mM (Fig. 1 and 2). The residual effluent concentration of $S_2O_3^{2-}$ and $S_4O_6^{2-}$ indicated that *N. agilis* did not oxidize these test compounds.

Because of the chemical and biological stability of $S_2O_3^{2-}$ and $S_4O_6^{2-}$ in the column, the effluent concentrations of

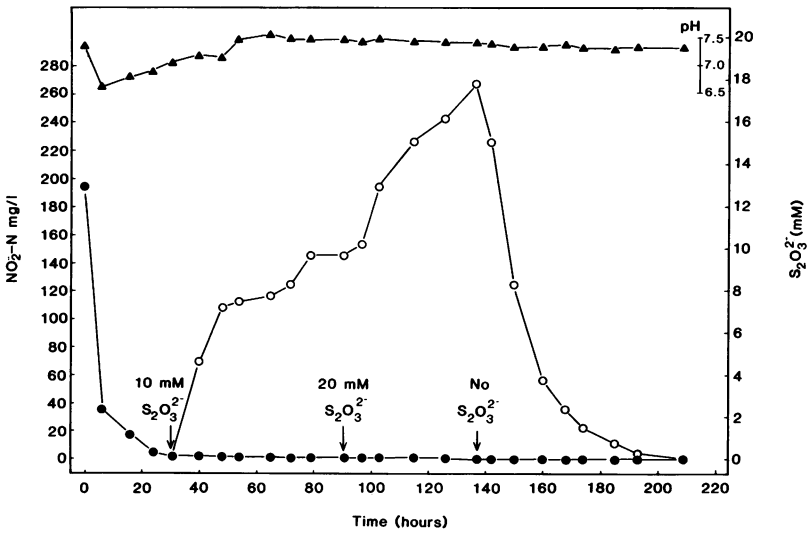

FIG. 1. Effect of thiosulfate on nitrite oxidation by immobilized *N. agilis*. Symbols: ●, effluent NO_2^- concentration; ○, effluent $S_2O_3^{2-}$ concentration; ▲, effluent pH.

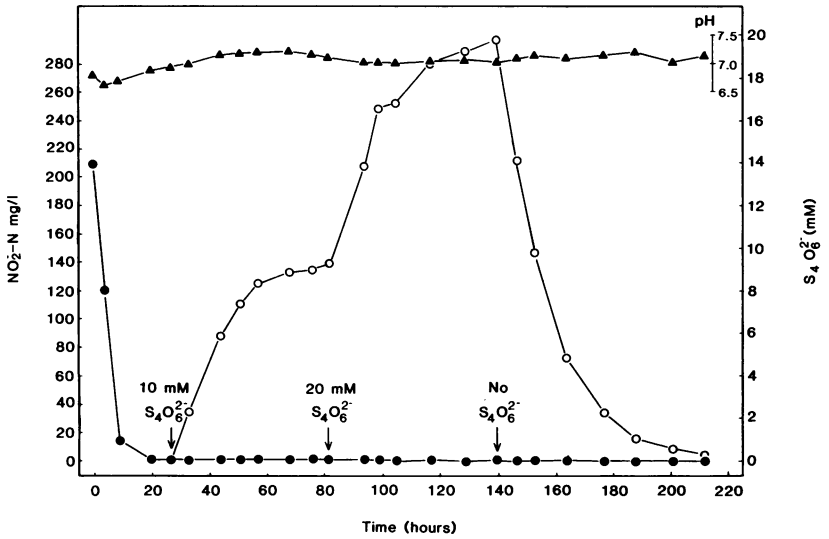

FIG. 2. Effect of tetrathionate on nitrite oxidation by immobilized *N. agilis*. Symbols: ●, effluent NO_2^- concentration; ○, effluent $S_4O_6^{2-}$ concentration; ▲, effluent pH.

these two compounds were used to estimate the observed retention time ($t_r + V/F$). Again, assuming perfect mixing conditions, constant volume, $F_{in} = F_{out}$, $C = C_{out}$, C_{in} constant, and no reaction of either $S_2O_3^{2-}$ or $S_4O_6^{2-}$, an equation similar to that of nitrite balance may be written (where C now represents the concentration of the markers) $V(dC/dt) = F(C_{in} - C)$. [Note the absence of the $f(t)$ owing to the condition that *N. agilis* does not oxidize the markers.] As before, solution of this first-order differential equation may be accomplished by use of an integrating factor $\exp(Ft/V)$ (13), application of Laplace transforms, or other methods. The result is $C(t) = C_{in}[1 - e^{-t/(V/F)}]$. Therefore, the observed t_r can be obtained by fitting measured marker concentrations to the above equation.

On the other hand, the theoretical retention time t_r' is a value directly calculated from the working volume (V) divided by the flow rate (F). Under perfect mixing conditions, $t_r = t_r'$. The t_r values derived from the thiosulfate and tetrathionate experiments (Table 1) were within 92 to 111% of the respective t_r' values. These values indicated that the mixing was acceptable, in agreement with the observation that 3 to 4 retention times were required for the effluent concentration (C) to reach the influent concentration (C_{in}) of the marker.

Acidity. The effect of pH on immobilized *N. agilis* is shown in Fig. 3. Influent media of pH 6.0, 4.5, and 4.0 were used to adjust the pH in the floating-bed column. No inhibition was apparent when the effluent pH decreased to 5.5, and the oxidation activity slightly declined as the effluent pH reached 4.8. The nitrite utilization ceased completely below this pH and was not restored upon increasing the pH to 7 (Fig. 3).

Metal ions. Nitrite oxidation was not affected by 1 mM MoO_4^{2-}, and only about 2% inhibition was observed after the introduction of 10 mM MoO_4^{2-}. An inhibitory effect on nitrite oxidation was apparent when immobilized *N. agilis*

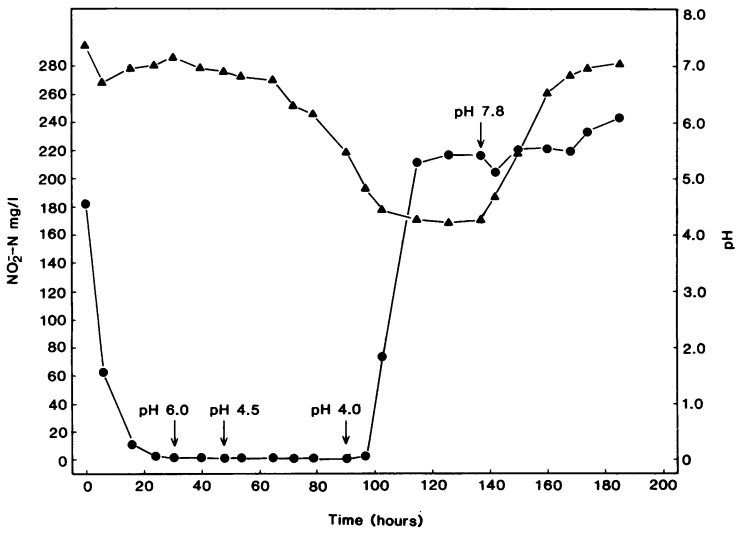

FIG. 3. Effect of acidity on nitrite oxidation by immobilized *N. agilis*. Symbols: ●, effluent NO_2^- concentration; ▲, effluent pH.

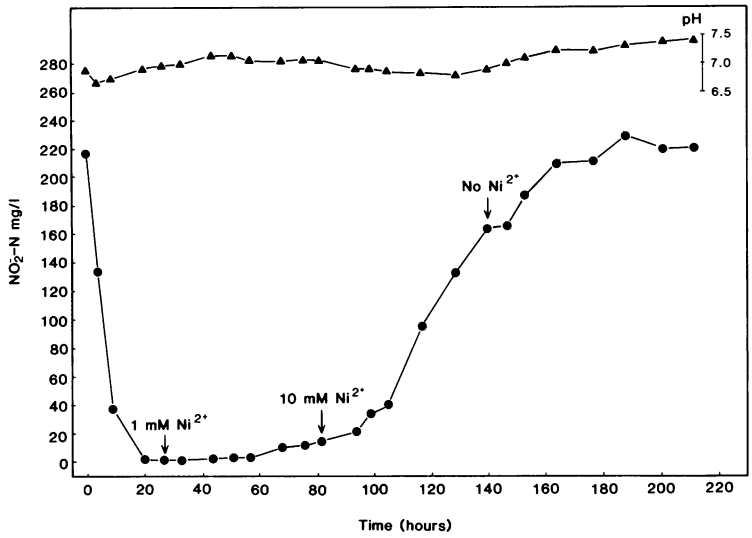

FIG. 4. Effect of nickel ions on nitrite oxidation by immobilized *N. agilis*. Symbols are as in the legend to Fig. 3.

cells were exposed to Ni^{2+}. Inhibition remained slight (6%) after 54.5 h (3.2 × t_r') of the introduction of 1 mM Ni^{2+} (Fig. 4). About 70% inhibition was apparent when Ni^{2+} was increased to 10 mM at 3.5 × t_r', and the Ni^{2+}-exposed cells did not recover in nickel-free medium (Fig. 4). Although not determined, the lack of recovery may be related to the retention of nickel in the bead matrix or its slow exchange with freely mixing ions. There were no significant pH changes in the effluents as a result of the addition of either MoO_4^{2-} or Ni^{2+}.

Figure 5 shows the effect of Al^{3+} on the immobilized *N. agilis*. Inhibition was not detected in the first 11 h of contact time with 1 mM Al^{3+}. Subsequently, the effluent pH declined as the Al^{3+} concentration increased. The inhibition by Al^{3+} was evident after 11 h of nitrite oxidation at effluent pH < 6.5. In the acidity test, no inhibitory effect was observed at this pH regimen (Fig. 3). Inhibition of 67% was determined after 59 h of contact (3.6 × t_r') of bacteria with Al^{3+}, at which time the effluent pH had declined to 4.4. The effluent pH further decreased to 3.6 after the introduction of 10 mM Al^{3+} (Fig. 5), and the parallel effluent nitrite concentrations indicated continuing inhibition of the biological activity. At this time, the influent nitrite concentration was only approximately one-half of the one initially added, suggesting that aluminum-catalyzed nitrite oxidation took place in the sterile fresh medium reservoir. (With the colorimetric nitrite assay, interference by aluminum at concentrations of up to 17 mM resulted in a slight overestimation [up to 15%] of standard nitrite concentrations.) After discontinuation of the aluminum feed, the biological nitrite oxidation activity was not resumed, and the effluent nitrite concentration continued to increase toward the concentration present in the fresh influent medium.

Conclusions. The results demonstrate that *N. agilis* can be successfully immobilized by entrapment and used in continuous flow-through column systems. Complete biological nitrite oxidation occurred with the use of this system for at least 210 h. Problems due to bead packing aeration retarded the oxidation but could be resolved by resorting to floating-bed design and direct aeration of the column. Nitrite oxida-

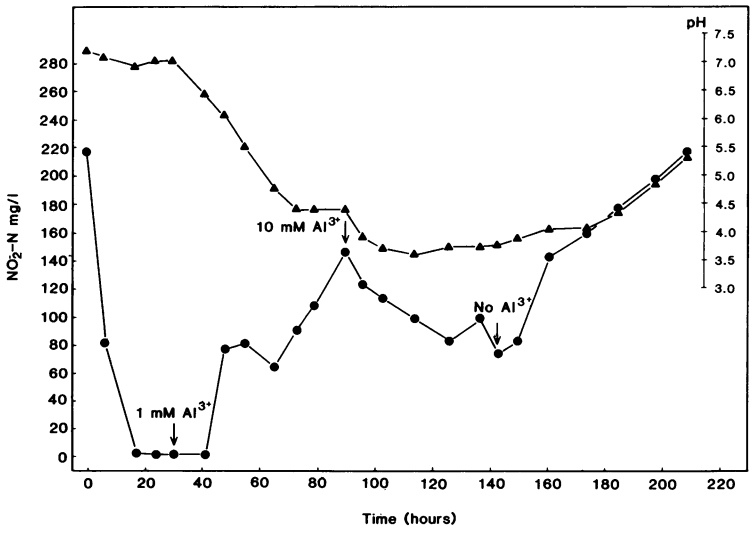

FIG. 5. Effect of aluminum ions on nitrite oxidation of immobilized *N. agilis*. Symbols are as in the legend to Fig. 3.

tion by *N. agilis* was insensitive to thiosulfate, tetrathionate, and molybdate, whereas it was adversely influenced by nickel and aluminum ions as well as pH < 4.5.

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