0273-2289/93/3940—0727$03.20

Reduction of Nitric Oxide by Denitrifying Bacteria

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ABSTRACT

Two heterotrophic denitrifying bacteria, *Paracoccus denitrificans* and *Pseudomonas denitrificans*, have been shown to utilize nitric oxide (NO) as a terminal electron acceptor and succinate, yeast extract, and heat/alkali pretreated municipal sewage sludge as carbon and energy sources. Complete removal of NO (0.50%) from a feed gas sparged into the cultures was observed. It is suggested that reduction of NO may be a common feature of denitrifying bacteria and that a microbial process to dispose of NO_x may be economically viable.

Index Entries: Nitric oxide; NO_x; flue gas; denitrifying bacteria.

INTRODUCTION

A need exists for new technology for the disposal of concentrated NO_x streams obtained from certain regenerable, dry scrubbing processes, such as the NOXSO process, and the removal and disposal of NO_x from more dilute gas streams produced by nitric acid plants. It has previously been demonstrated that the facultative anaerobe and autotroph, *Thiobacillus denitrificans*, may be cultured anoxically in batch reactors using NO(g) as a terminal electron acceptor. Thiosulfate served as an energy source, CO_2(g) as a carbon source, and ammonium ion as a source of reduced nitrogen. The growth of *T. denitrificans* was indicated by depletion of thiosulfate and ammonium ion and the accumulation of biomass. The feed gas consisted of 0.50% NO, 5%, CO_2, and balance nitrogen. The NO concentration in the outlet gas was typically 200 ppmv (*1*).

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The ability to use NO as a terminal electron acceptor may be a common feature of denitrifying bacteria. A study is reported here of the reduction of NO by two heterotrophic bacteria, *Paracoccus denitrificans*, and *Pseudomonas denitrificans*.

MATERIALS AND METHODS

Organisms and Culture

Paracoccus denitrificans

Paracoccus denitrificans (ATCC 13543) were obtained from the American Type Culture Collection (Rockville, MD). The organism was cultured for stocks at 30°C on nutrient agar slants. Stocks were stored at 4°C until used with transfers every 30 d.

P. denitrificans was cultured anoxically on NO(g) as a terminal electron acceptor in either a succinate-supplemented mineral salts medium or the same mineral salts medium supplemented with heat and alkali-pretreated municipal sewage sludge. The basic mineral salts medium used in this study consisted of (in m*M* unless otherwise indicated): KH_2PO_4 (14.7); $NaHCO_3$ (11.9); NH_4Cl (9.3); $MgSO_4$ (6.7); and trace element solution (2.0 mL/L). The trace element solution has been described previously (*2*).

P. denitrificans was cultured anoxically on succinate as a carbon and energy source and NO(g) as a terminal electron acceptor in a Marubishi MD 300 fermenter (culture vol 2 L). In a typical batch experiment, *P. denitrificans* was grown in the mineral salts medium supplemented with 37 m*M* succinate and 50 m*M* KNO_3 at 30°C and pH 7.0 to an OD at 520 nm of about 0.6. (The medium was sparged with 30 mL/min N_2 to remove oxygen). At this time, cells were harvested aseptically by centrifugation at 5000*g* and 25°C for 10 min, and resuspended in the same medium without nitrate. A gas feed of 0.50% NO, 5% CO_2, and balance nitrogen was then initiated at 30 mL/min and sparged directly into the medium. The agitation rate was 450 rpm. The culture medium and outlet gas were sampled periodically as the culture was maintained on an NO feed for up to 8 d.

Heat/alkali pretreated sludge medium for *P. denitrificans* was prepared as follows: 100 g of wet-packed sludge (taken from the recycle line from the secondary settler of a municipal-activated sludge sewage treatment system) was suspended in 1 L of the mineral salts medium described above. The suspension was adjusted to pH 12 with 10*N* NaOH and autoclaved at 121°C for 30 min. The cooled suspension was then adjusted to pH 7.0 with 6*N* HCl and diluted to 1.5 L with additional mineral salts medium. Heat and alkali treatment solubilized a significant fraction of the biosolids that make up the sludge producing soluble carbon and energy sources for the organism (Table 1).

Table 1
Effect of Heat/Alkali Treatment
on Suspensions of Sewage Sludge[a]

	Before treatment	After treatment
MLSS (mg/L)	5800	4370
Soluble COD (mg/L)[b]	70	4400
Soluble protein (mg/L)[b]	24	550

[a]One hundred grams per liter wet-packed sludge suspended in mineral salts medium, pH adjusted to 12 with 10*N* NaOH, autoclaved at 121°C for 30 min, and then pH adjusted to 7.0 with 6*N* HCl. MLSS = mixed liquor suspended solids; COD = chemical oxygen demand.
[b]Concentration in supernatant following centrifugation at 5000 x *g* for 15 min.

When *P. denitrificans* was to be cultured on heat/alkali pretreated sewage sludge as a carbon and energy source and NO(g) as a terminal electron acceptor, the organism was first grown on succinate/nitrate as described above. Following harvesting by centrifugation at 5000*g* and 25°C, the cells were resuspended in 1.5 L of the sludge/mineral salts medium in a Marubishi MD 300 fermenter. A gas feed of 0.50% NO, 5% CO_2, and balance nitrogen was then initiated at 30 mL/min. All other operating conditions were identical to those described above for growth on succinate.

Pseudomonas denitrificans

Pseudomonas denitrificans (ATCC 13867) was also obtained from the American Type Culture Collection (Rockville, MD). The organism was cultured for stocks at 30°C on nutrient agar slants. Stocks were stored at 4°C until used and transferred every 30 d.

Ps. denitrificans was cultured anoxically on NO(g) as a terminal electron acceptor in the mineral salts medium (without nitrate) supplemented with either 3 g/L yeast extract or heat/alkali-pretreated sewage sludge. When yeast extract served as the carbon and energy source, the organism was first grown in mineral salts/yeast extract medium supplemental 50 m*M* KNO_3 (at pH 7.0 and 30°C) to an OD (520 nm) of about 0.6. Cells were then harvested aseptically as described above and resuspended in the same medium without nitrate. A gas feed of 0.50% NO, 5% CO_2, balance N_2 was then initiated at 30 mL/min. All other operating conditions were identical to those described above for growth of *P. denitrificans* on succinate/NO.

When pretreated sewage sludge served as the carbon and energy source, *Ps. denitrificans* was initially grown on yeast extract/nitrate as described above. Following harvesting by centrifugation, cells were resuspended in the sludge/mineral salts medium. A gas feed of 0.50% NO, 5%

CO_2, balance N_2 was then initiated at 30 mL/min. All other operating conditions were identical to those described above.

Analytical

Analytical methods for quantitating nitrate and ammonium ion have been described previously (2). Biomass protein was determined by the Bradford method following sonication of cell suspensions (3). The sonication procedure has been previously described (2). Bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used as a standard. Soluble chemical oxygen demand (COD) was determined by performing COD analyses on the supernatants of culture samples that had been centrifuged at 5000*g* and 4°C for 15 min. COD analyses used Hach Chemical Co. (Loveland, CO) premeasured reagent vials. Routine analysis of reactor outlet gas used Gastec Analyzer tubes (Gastec Corp., Yokohama, Japan) for NO (0–250 ppmv range). More precise determinations of NO in the outlet gas were done by gas chromatography using a Hewlett Packard 5890 gas chromatograph with a thermal conductivity detector. The column was 30 ft × 1/8-in ID stainless steel packed with 100/120 mesh Haye Sep D (Hayes Separation, Bandera, TX). The carrier gas was helium at 30 mL/min. The column oven, injector oven, and detector oven temperatures were 25, 25, and 140°C, respectively. Succinate in culture medium samples was determined by gas chromatography using a Hewlett Packard 5840 gas chromatograph with a flame ionization detector. The column was a 2 m × 1.8 mm ID (glass) packed with 80/120 Carbowax B-DA/4% Carbowax 20 M (Supelco, Bellefonte, PA). The carrier gas was helium at 24 mL/min. The column oven, injection oven, and detector temperatures were 185, 205, and 205°C, respectively.

RESULTS AND DISCUSSION

Reduction of NO by *Paracoccus denitrificans*

When NO was introduced into suspensions of *P. denitrificans* in mineral salts medium supplemented with succinate, complete removal of NO from the feed gas (30 mL/min of 0.50% NO) was observed. As NO was removed from the feed gas, there was a corresponding decrease in the succinate (Fig. 1) and ammonium ion concentrations, and an increase in the biomass protein concentration (Fig. 2). Growth of *P. denitrificans* on succinate as a carbon and energy source and NO as a terminal electron acceptor is clearly indicated. In control experiments without biomass, NO broke through rapidly at concentrations comparable to the feed gas.

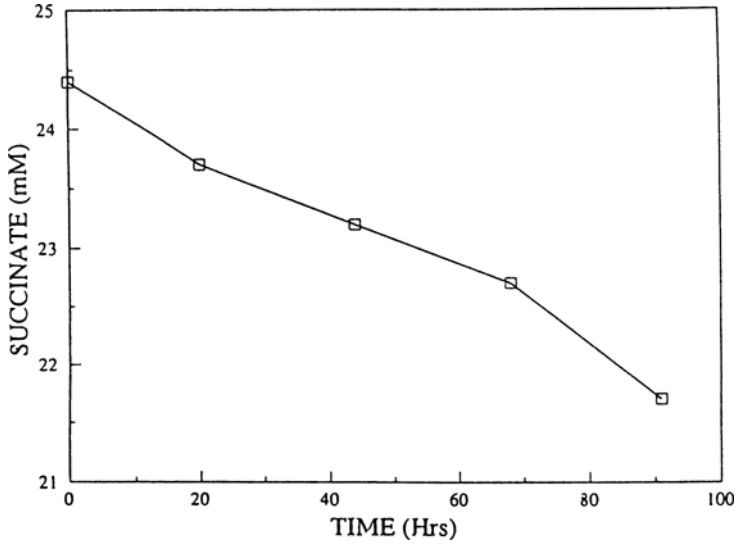

Fig. 1. Succinic acid concentration in a *P. denitrificans* culture receiving a NO feed (30 mL/min of 0.50%).

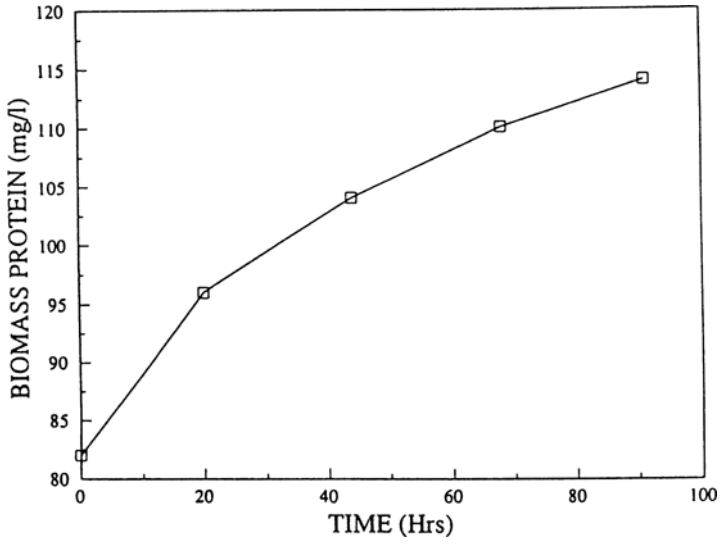

Fig. 2. Biomass protein concentration in a *P. denitrificans* culture receiving a NO feed (30 mL/min of 0.50%). Succinate was the carbon source.

During the course of the experiment illustrated by Figs. 1 and 2, the reduction of 32.3 mmol of NO gas was accompanied by the oxidation of 5.0 mmol of succinate, the utilization of 0.74 mmol of NH_4^+, and the production of 99.3 mg of biomass protein. The average NO/succinate stoichiometric ratio observed in these experiments was 6.0. The purely chemical

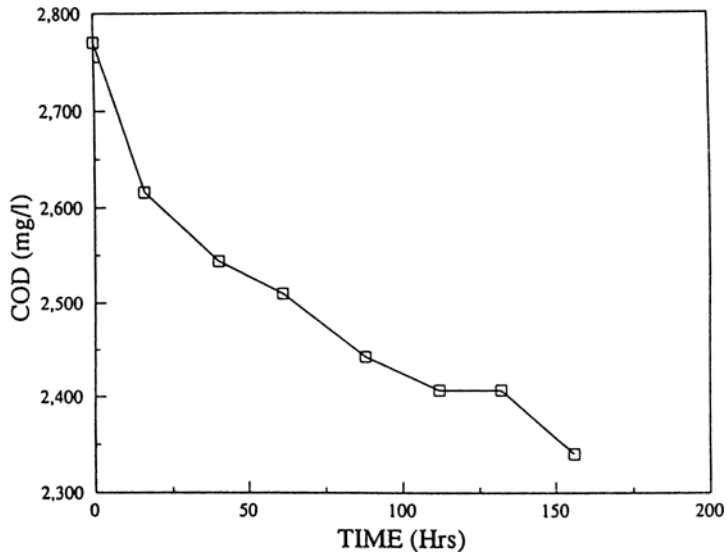

Fig. 3. Soluble COD concentration in a *P. denitrificans* culture with pretreated sewage sludge as a carbon and energy source receiving an NO feed (30 mL/min of 0.50%).

oxidation of succinate (to CO_2) would require 7.0 mol of NO/mol of succinate. The difference between the biochemical reaction and a purely chemical reaction can be attributed to diversion of reducing equivalents for biosynthesis in the microorganism.

In the specific experiment illustrated above, the initial specific NO feed rate was 4.3 mmol NO/h/g of biomass protein. However, subsequent experiments showed that specific NO feed rates of up to 23.6 mmol NO/h/g biomass protein (140 mL/min of 0.50% NO at a biomass protein concentration of 70 mg/L) could be tolerated without breakthrough of NO. At the higher NO feed rate, a proportionally higher growth rate was observed in terms of accumulation of biomass protein.

A similar series of experiments was conducted to determine whether *P. denitrificans* could use heat and alkali-pretreated sewage sludge as a carbon and energy source with NO as a terminal electron acceptor. When NO was introduced into a suspension of *P. denitrificans* in mineral salts medium supplemented with heat/alkali-pretreated sewage sludge, complete removal of NO from the feed gas (30 mL/min of 0.50% NO) was observed. As NO was removed from the feed gas, there was a corresponding decrease in the concentration of soluble COD (Fig. 3). These data indicate that *P. denitrificans* was utilizing biomolecules solubilized from the sewage sludge as sources of carbon and energy and NO as a terminal electron acceptor. The ammonium ion concentration was seen to increase as NO was removed from the feed gas. This has preliminarily been attributed to liberation of NH_4^+ during metabolism of N-containing

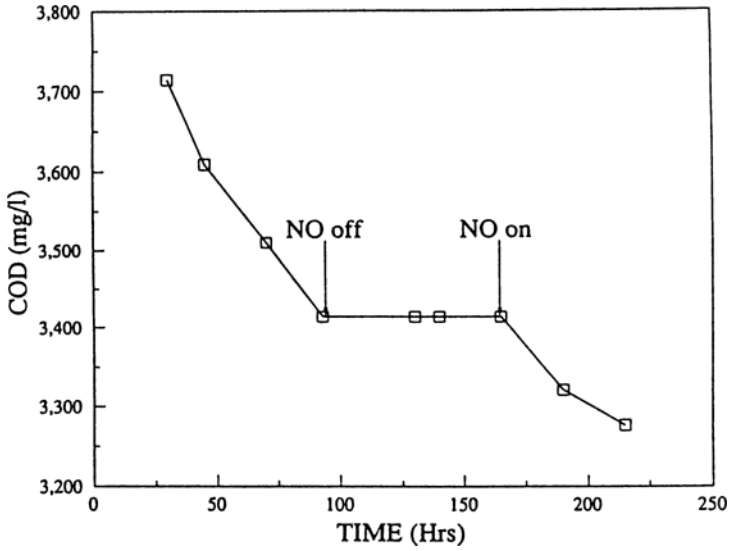

Fig. 4. Soluble COD concentration in a *P. denitrificans* culture with pretreated sewage sludge as a carbon and energy source receiving an intermittant NO feed.

compounds from the sewage sludge. In control experiments with no biomass, NO broke through rapidly at concentrations comparable to the feed gas.

In order to demonstrate clearly that the utilization of soluble COD in these cultures was directly linked to the utilization of NO as a terminal electron acceptor, two additional types of experiments were conducted. In the first type, *P. denitrificans* cultures were grown on a pretreated sewage sludge and an NO gas feed as described above. When utilization of soluble COD in the culture medium was clearly established, the NO gas feed was stopped. As seen in Fig. 4, when NO was no longer available as a terminal electron acceptor, the soluble COD concentration remained stable. When the NO feed was restarted about 72 h later, the soluble COD concentration again began to decline.

In a second type of experiment, nitrate (3 g/L KNO_3) was added to a *P. denitrificans* culture growing on pretreated sewage sludge and NO. If NO were acting as a terminal electron acceptor in these cultures, nitrate should suppress the utilization of NO. Within 72 h of the addition of KNO_3, NO was seen to break through in the outlet gas at concentrations of about 1000 ppmv. Further addition of pretreated sewage sludge did not reverse the NO breakthrough.

One additional observation is worthy of note. *P. denitrificans* cultures growing on pretreated sewage sludge were occasionally subject to NO breakthrough even when soluble COD was still available in the medium. However, addition of more pretreated sludge resulted once again in the

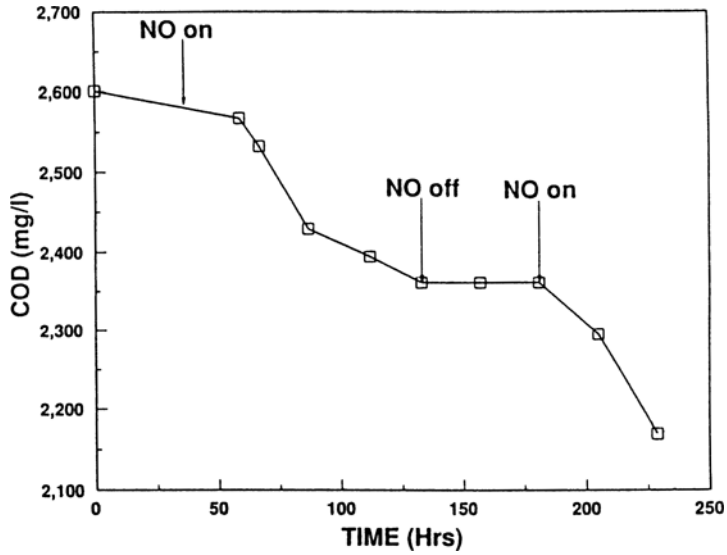

Fig. 5. Soluble COD concentration in a *Ps. denitrificans* culture receiving an NO feed (30 mL/min of 0.50%). Yeast extract was the carbon source.

complete NO removal. These observations indicate that *P. denitrificans* could use only certain components of the soluble COD as carbon and energy sources.

Reduction of NO by *Pseudomonas denitrificans*

When *Ps. denitrificans* cells were suspended in mineral salts medium supplemented with yeast extract, the suspensions initially received a feed of elemental nitrogen only. As seen in Figs. 5–7, the COD concentration, the optical density, and biomass protein concentration remained relatively constant during this time (40 h). However, when NO feed was initiated (30 mL/min of 0.50% NO), the COD concentration began to decline, and the optical density and biomass protein concentration began to increase indicating growth of the organism on components of the yeast extract as carbon and energy sources and NO as a terminal electron acceptor. Complete removal of NO from the feed gas was observed. After about 100 h of operation with the NO feed, the NO feed gas was again replaced with N_2. The results as shown in Figs. 5–7 were a cessation in growth and utilization of COD. When the NO feed was resume after about 48 h on N_2, growth resumed with further utilization of COD, and increase in optical density and biomass protein concentration. In control cultures in which NO was fed at the same rate to yeast extract/mineral salts medium without biomass, NO broke through rapidly at concentrations comparable to the feed gas.

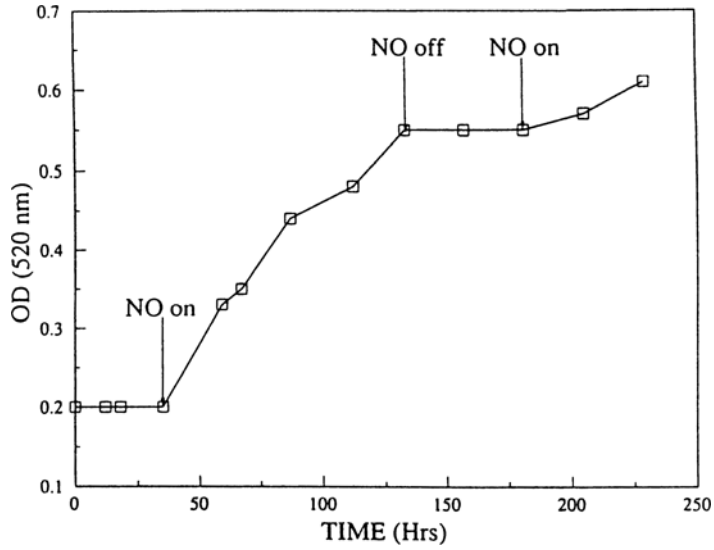

Fig. 6. Optical density in a *Ps. denitrificans* culture receiving an NO feed (30 mL/min of 0.50%). Yeast extract was the carbon source.

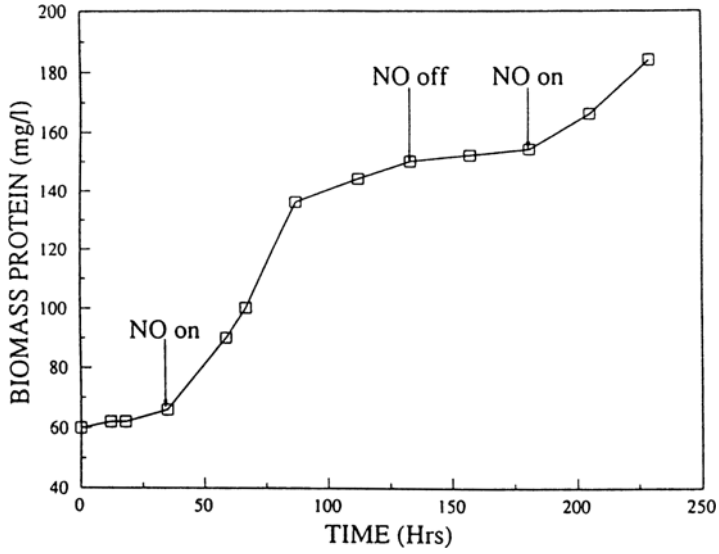

Fig. 7. Biomass protein concentration in a culture of *Ps. denitrificans* culture receiving an NO feed (30 mL/min of 0.50%). Yeast extract was the carbon source.

When NO was introduced into suspensions of *Ps. denitrificans* in mineral salts medium supplemented with heat and alkali-pretreated sewage sludge, very similar results were obtained compared to *P. denitrificans* in the same medium. Again complete removal of NO from the feed gas (30 mL/min of 0.50% NO) was observed. Figure 8 shows the soluble COD concentration in the culture medium in one of these experiments as NO is

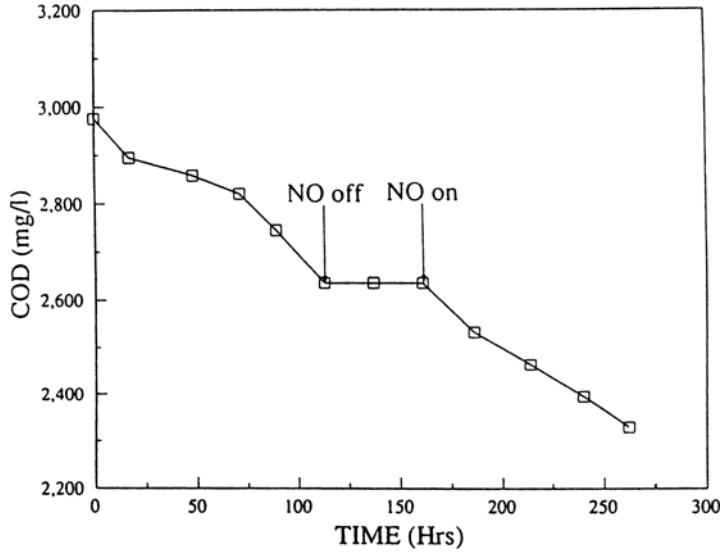

Fig. 8. Soluble COD concentration in a *Ps. denitrificans* culture with pretreated sewage sludge as a carbon and energy source receiving an NO feed (30 mL/min of 0.50%).

removed from the feed gas. The decrease in soluble COD concentration indicates growth of *Ps. denitrificans* on biomolecules derived from the sewage sludge as carbon and energy sources and NO as terminal electron acceptor. Figure 8 shows that when the NO feed was shut off at about 120 h into the experiment, the soluble COD concentration leveled off, but resumed its decline when the NO feed was restarted. The ammonium ion concentration in *Ps. denitrificans* cultures also increased as NO was removed from the feed gas. However, the ammonium ion concentration leveled off while the NO feed was shut off.

CONCLUSION

It has been demonstrated that NO will support the growth of two heterotrophic denitrifying bacteria, *Paracoccus denitrificans* and *Pseudomonas denitrificans*, as a terminal electron acceptor with succinate, yeast extract, and heat/alkali-pretreated sewage sludge as carbon and energy sources. These results, taken with those previously reported for the autotroph *Thiobacillus denitrificans*, suggests that NO reduction may be a common property of denitrifying bacteria. The use of a potentially inexpensive carbon and energy source, such as pretreated sewage sludge, to support NO reduction indicates that a microbial process to dispose of NO_x may be economically viable. Less costly pretreatment methods are currently being investigated.

ACKNOWLEDGMENT

This work was funded by the Pittsburgh Energy Technology Center of the US Department of Energy and ABB Environmental Services Inc. (Portland, ME).

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