Simultaneously Occurring Nitrification and Denitrification under Oxygen Gradient by Polyelectrolyte Complex-Coimmobilized Nitrosomonas europaea *and* Paracoccus denitrificans *Cells*

Etsuo Kokufuta, Masato Shimohashi, and Isei Nakamura
Institute of Applied Biochemistry, University of Tsukuba, Sakura-mura, Niihari-gun, Ibaraki 305, Japan

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Nitrification followed by subsequent denitrification is the easiest and most economical method currently available for removal of nitrogen from wastewaters. However, both nitrification and denitrification could not occur optimally in the same locale at the same time, since the oxidation–reduction potential acts in opposite ways in these biochemical reactions.[1] In principle, this requires two different operations for removing nitrogen from wastewaters; i.e., 1) the oxidation of ammonia to nitrate or nitrite under aerobic conditions by nitrifying bacteria, and 2) the reduction of nitrite or nitrate to gaseous products (primarily molecular nitrogen) under anaerobic conditions by denitrifying bacteria. It is thus desirable to develop a novel method of permitting the concurrence of nitrification and denitrification in the same system.

In this communication, we wish to report that the coimmobilization of *Nitrosomonas europaea* cells with *Paracoccus denitrificans* cells using a polyelectrolyte complex (PEC) makes it possible for nitrification and denitrification to occur simultaneously in the same system under aerobic conditions, because of the presence of aerobic and anaerobic regions within the PEC support.

MATERIALS AND METHODS

Nitrosomonas europaea ATCC 25978 and *Paracoccus denitrificans* IFO 12442 were used for the present investigation. Both microorganisms were maintained and grown as already described for *N. europaea* and *P. denitrificans*.[2,3] Potassium poly(vinyl alcohol) sulfate (KPVS)[2–4] and poly(diallyl-dimethylammonium chloride)[3] (PDDA) were the same as those used in our previous studies.[2–4]

N. europaea cells (6.1 mg dry wt), *P. denitrificans* cells (330 mg dry wt), or mixed nitrifying (6.1 mg dry wt) and denitrifying (177 mg dry wt) cells were suspended into 100 mL phosphate buffer (0.1*M*; pH 6.5), and immobilized by the following procedure: An excess of PDDA solution (30 mmol/L in terms of monomer units; 50 mL) was first mixed with 100 mL of the cell suspension to aggregate cells, then KPVS solution (30 mmol/L in terms of monomer units; 50 mL) was added to form PEC with excess PDDA and to entrap the aggregated cells. The resulting yellowish PEC–precipitate containing cells was separated by decantation, washed with a large amount of 0.1*M* phosphate buffer, and stored at 3°C before use.

To evaluate the activity of singly immobilized cells (SIC) or coimmobilized cells (CIC), batch reaction kinetics was studied by following the concentration changes of nitrate, nitrite, and ammonia during incubation. The analyses of these ions were made colorimetrically, as described previously.[2,3] The incubation was carried out with the following liquid mediums: medium A (pH 6.75, for SIC of *P. denitrificans*): KNO_3, 0.14 g/L (or $NaNO_3$, 0.098 g/L); potassium aspartate, 0.57 g/L; $(NH_4)_2SO_4$, 5.3 g/L; medium B (pH 8.0, for SIC of *N. europaea*): $(NH_4)_2SO_4$, 2.0 g/L; $NaHCO_3$, 0.5 g/L; Na_2HPO_4, 13.5 g/L; KH_2PO_4, 0.7 g/L; $MgSO_4 \cdot 7H_2O$, 0.1 g/L; $CaCl_2 \cdot 2H_2O$, 3 mg/L; $FeSO_4 \cdot 7H_2O$, 0.03 g/L; medium C (pH 8.0; for CIC of *N. europaea* and *P. denitrificans*): 0.25% (v/v) ethanol plus the same amounts of all the components of medium B. Potassium aspartate in medium A and ethanol in medium C were required as a donor of hydrogen to permit the dissimilatory reduction of nitrate or nitrite. These were chosen out of several organic compounds by carrying out preliminary experiments. The cultivations of SIC and CIC were done with a water-jacketed cylindrical glass vessel (200 mL) containing 100 mL medium into which a mixed gas (21% O_2 and 79% N_2) was bubbled at a flow rate of 0.3 L/min. In the case of the cultivation with medium C, in order to avoid the volatilization of ethanol, its concentration was checked at 8 h intervals by liquid chromatography and, if necessary, adjusted to the initial value by an additional amount of ethanol.

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RESULTS AND DISCUSSION

In a cell suspension of *N. europaea, P. denitrificans*, or a mixture of both bacteria, the complexation between PDDA and KPVS was complete within 2–3 min, and a yellowish formless precipitate of the PEC-entrapped cells was formed. Residual cells were not observed in the supernatant solution decanted from the precipitated PEC-entrapped cells, while a small residue of KPVS was detected by the technique of colloid titration.[5] This indicates that cells are almost or entirely immobilized in PEC. Electron microscopic analyses for SIC and CIC were carried out in the same manner as described previously,[2,4] showing that the aggregated cells were entrapped or surrounded by an amorphous phase of PEC.

Figure 1 depicts the results of aerobic incubation of the free cells and SIC of *P. denitrificans*. It is found that not only nitrate but also nitrite is little consumed by the free cells. However, SIC exhibits a marked decrease in the nitrogen concentration caused by the consumption of nitrite or nitrate. In the SIC system, the formation of nitrite during the nitrate consumption and the evolution of N_2 gas during the nitrite consumption were observed by colorimetric and gas chromatographic methods, respectively. It is therefore proved that, even in an externally aerobic environment, SIC of *P. denitrificans* has an ability to permit the dissimilatory reduction of nitrate or nitrite. This could be interpreted as follows: Because of both the diffusional restriction on the transport of O_2 dissolved in the medium and the absorption of O_2 by the cells entrapped, a concentration gradient of O_2 is generated within the PEC support. The rates of nitrate and nitrite consumption under aerobic conditions were found to be ca. 8% (for NO_3^-) and 10% (for NO_2^-) of those under anaerobic conditions, as estimated by comparing the results obtained here and reported previously.[3]

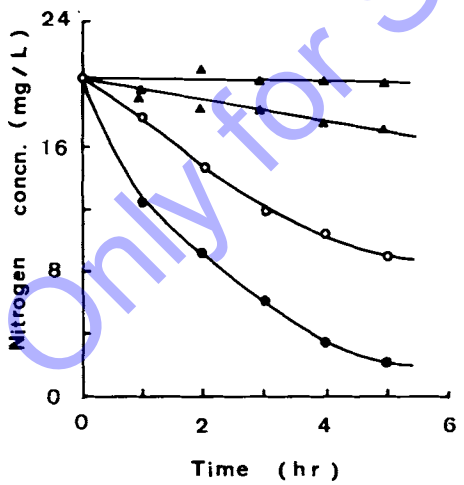

Figure 1. Changes in nitrogen concentrations during aerobic cultures of (△, ▲) free cells and (○, ●) SIC of *P. denitrificans*: (▲, ●) NO_2^-–N and (△, ○) NO_3^-–N. Free cells (330 mg dry wt) and SIC (720 mg dry wt; composed of 54.2% PEC and 45.8% cells) were incubated at 21°C using 100 mL of medium A with a carbon (as aspartate) to nitrogen (as nitrate or nitrite) ratio of 7.2:1 by weight.

Taking these results into account, we prepared CIC of *P. denitrificans* plus *N. europaea* and compared it with SIC of *N. europaea*. Both CIC and SIC were aerobically incubated under the same conditions, except that medium C employed for CIC contained a slight amount of ethanol as a hydrogen donor. In Figure 2, the time courses of ammonia consumption and nitrite formation by SIC were compared with those by CIC. There is a remarkable difference between SIC and CIC; that is, SIC quantitatively converts ammonia to nitrite, while CIC consumes ammonia without forming nitrite. From these results, it is reasonable to conclude that nitrite, formed from ammonia *via* the oxidation by *N. europaea* cells, is immediately decomposed to gaseous terminal products *via* the dissimilatory reduction by the denitrifying cells coexisting in CIC. However, the ability of *Nitrosomonas* to produce nitrous oxide from nitrite (or hydroxylamine), which has been reported by

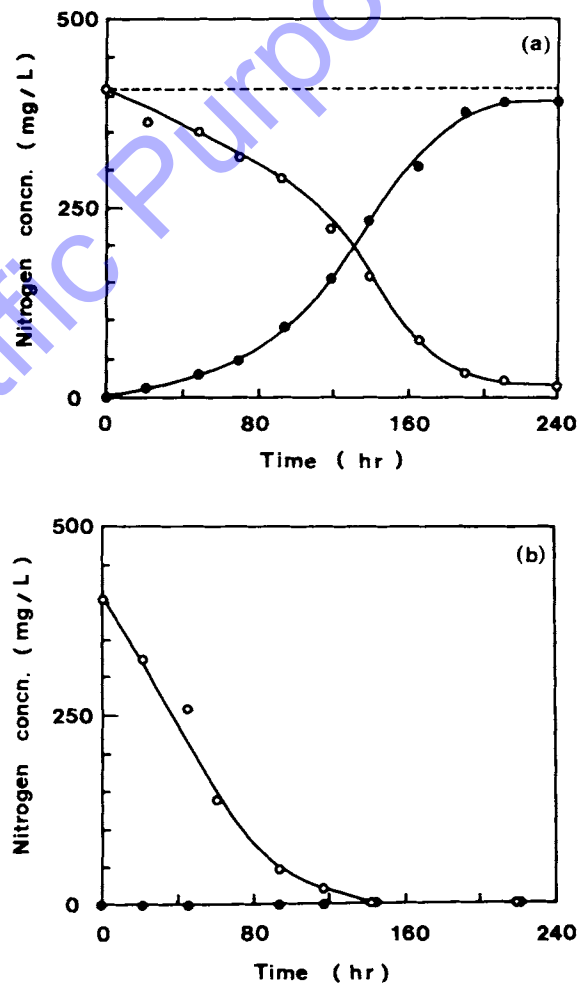

Figure 2. Changes in nitrogen concentrations during aerobic cultures of (a) SIC of *N. europaea* and (b) CIC of *N. europaea* with *P. denitrificans*: (○) NH_4^+–N and (●) NO_2^-–N. The SIC (396 mg dry wt, composed of 98.4% PEC and 1.6% cells) was incubated at 30°C on 100 mL of medium B, while the incubation of CIC (565 mg dry wt, composed of 67.6% PEC plus 31.3% denitrifying cells plus 1.1% nitrifying cells) was made at 30°C on medium C (100 mL) with a carbon (as ethanol) to nitrogen (as ammonium sulfate) ratio of 2.4:1 by weight. Dashed line in part (a) indicates a complete conversion of ammonium to nitrite.

some authors,[6–8] must be considered, since it may cause the decomposition of nitrite to gaseous nitrogen without recourse to the denitrifying cells. To clarify whether the decomposition of nitrite in the CIC system is related to *N. europaea* or *P. denitrificans*, an aerobic incubation of CIC was carried out using medium B which was free from hydrogen donor required for the dissimilatory nitrite reduction by denitrifying bacteria. The results obtained were found to be in close agreement with those of SIC in Figure 2. Thus, it is clearly indicated that nitrification (ammonia oxidation) and denitrification (nitrite reduction) occur simultaneously in the same system containing CIC under aerobic conditions.

Detailed comparison of CIC with SIC in Figure 2 showed the following important characteristics: 1) the initial rate of ammonia oxidation by CIC is ca. 3.4 times that by SIC, as given on the basis of 1 g *N. europaea* cells (dry weight) in SIC or CIC; 2) a complete ammonia oxidation takes place in CIC but not in SIC. In addition, a stability test of CIC showed that the initial activity remained unaltered over at least four runs (total 24 days), when each run was repeated on freshly prepared medium C after a complete removal of nitrogen. These principal advantages could be related to the ability of CIC to decompose nitrite, because accumulation of nitrite inhibited the ammonia-oxidizing activity of the previous preparation of PEC-entrapped *N. europaea*[2] and the growth of *Nitrosomonas* in a suspension culture.[9]

Soil is a redox system characterized by bearing aerobic zones in close proximity to anaerobic zones. This situation has an important implication for the "natural concurrence" of nitrification and denitrification.[10] The observed "artificial occurrence" of nitrification and denitrification could be, in analogy with soil, explained by assuming the coexistence of aerobic and anaerobic regions within the body of PEC. Generating a concentration gradient of O_2 within PEC seems to be the major cause for the coexistence of both regions.

In conclusion, the idea that the coimmobilization of *N. europaea* cells with *P. denitrificans* cells by using PEC enables the simultaneous occurrence of nitrification and denitrification, as proposed in this communication, would be useful in simplifying or modifying the present processes of nitrogen removal in wastewater treatment.

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