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Biological nitrogen removal in a single aerobic reactor by association of a nitrifying ecosystem to an aerobic denitrifier, *Microvirgula aerodenitrificans*

D. Patureau *, N. Bernet, T. Bouchez, P. Dabert, J.P. Delgenes, R. Moletta

Institut National de la Recherche Agronomique, Laboratoire des Biotechnologies de l'Environnement (LBE), avenue des Etangs, 11100 Narbonne, France

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Abstract

Aerobic nitrification and anoxic denitrification are the two main steps of the biological nitrogen removal processes. However, studies have shown the ability of pure strains to consume simultaneously oxygen and nitrate. These properties of co-respiration were used to combine nitrifiers and aerobic denitrifier (*Microvirgula aerodenitrificans*) in a single aerated reactor under continuous and Sequencing Batch Reactor (SBR) cultures. The aerobic denitrifier was maintained by discontinuous addition of carbon. Under these conditions, nitrifying and denitrifying activities were observed with aerobic reduction of the N-oxides produced by autotrophs into nitrous oxide and nitrogen.

Keywords: Denitrification; Nitrification; Aerobiosis

1. Introduction

Nitrification and denitrification are steps of the nitrogen cycle in which bacteria recycle ammonia to dinitrogen. Nitrification, defined as the oxidation of reduced inorganic nitrogen compounds, is realized by autotrophic aerobic microorganisms [1]. Denitrification was generally taken to be an anoxic reaction where oxidized nitrogen compounds are reduced to gaseous nitrogen compounds [2]. Presence of oxygen was thought to inhibit activity and synthesis of denitrifying enzymes [3]. However, the inhibitory role of oxygen is now being questioned. In fact, some authors have demonstrated the ability of strains to denitrify under fully aerobic conditions: *Thiosphaera pantotropha*, *Alcaligenes faecalis* [4,5], *Pseudomonas nautica* [6], *Pseudomonas* sp. [7]. In this study, the denitrifying bacterium used was isolated from an upflow anaerobic filter inoculated with activated sludge. This strain was shown to use simultaneously oxygen and nitrate with concomitant production of dinitrogen [8]. Aerobic denitrification in this strain is accounted by a constitutive, periplasmic nitrate reductase [9], which is in agreement with the results obtained

* Corresponding author. Fax: +33-4-68-42-51-60; e-mail: patureau@ensam.inra.fr

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with *T. pantotropha*. However, in opposition to the other aerobic denitrifiers, this strain is unable to nitrify.

Existence of this kind of microorganisms allows to propose new configuration for nitrogen removal processes. In fact, conventional systems for the treatment of nitrogen-containing wastes are realized in two spatial or temporal separated steps: aerobic nitrification and anoxic denitrification. With aerobic denitrifiers, nitrification and denitrification could take place in the same aerated unit.

In this paper, we describe the behavior and the denitrifying performances of the aerobic denitrifier, *Microvirgula aerodenitrificans*, in co-culture with autotrophic nitrifiers stemmed from a piggery wastewater treatment plant. These experiments were carried out in continuous and semi-continuous culture with discontinuous addition of carbon source, using a synthetic mineral feeding medium.

2. Materials and methods

2.1. Organism and medium

Isolation and characterization of the strain *M. aerodenitrificans* (type strain SGLY2) used in this study have been described in detail elsewhere [8]. The autotrophic microflora was grown on a synthetic mineral medium [10].

2.2. Culture conditions

Discontinuous and continuous cultures of the pure strain were realized respectively in 100-ml Penicillin flasks and 2-l Biolafitte reactor. The culture and aeration conditions, medium and inoculation were described, respectively, in Refs. [8,11].

The continuous and semi-continuous (SBR) culture were realized in 2-l Biolafitte reactors [10], filled with 1.5 ml of the mineral medium. Aerobic conditions were obtained by sparging the medium with pure oxygen (quality oxygen C, Alphagas) in the continuous co-culture and with air in the SBR co-culture. The dissolved oxygen concentration was maintained around 14 mg l^{-1} and 7 mg l^{-1} respectively, implying non-limited conditions for autotrophs and heterotrophs growth. During the continuous co-culture, the feeding medium was sparged with argon to avoid nitrogen entrance which could cause errors in calculation of the nitrogen balance. The two experiments started by inoculating the 100 ml of mineral medium with 100 ml of the autotrophic microflora, by allowing the culture to grow and nitrify and by feeding the reactor to reach the working volume of 1.5 l. After stabilization of the nitrifying activity, one reactor was conducted under continuous conditions with a constant dilution rate of 0.336 d^{-1}, whereas, the other one was conducted in SBR conditions with three cycles per day and a hydraulic retention time (HRT) of 3 days. After stabilization of the culture conditions, some parameters were tested: different sources of carbon (succinate under continuous culture and acetate under SBR condition), different types of addition of carbon source with or without addition of the pure aerobic denitrifier.

Under SBR conditions, whole cell hybridization with fluorescently labeled 16 S-rRNA oligonucleotide probe was used to follow the fate of the pure aerobic denitrifier.

Analytical measurements are described in details elsewhere [9,10].

3. Results and discussion

3.1. Nitrification combined to denitrification under continuous culture

After complete stabilization of the nitrifying population, which was characterized by a stable biomass concentration, the percentage of nitrification was 90%. This implied that a part of ammonium is rejected in the effluent (9%). One percent of nitrogen was used for biomass synthesis.

The first test corresponded to continuous supply of C-succinate through the feeding medium. This resulted in a complete disequilibrium of the system with an increase of the percentage of assimilation (18%) and of residual ammonium (76%), together with a decrease of the percentage of nitrification down to 5%. This implies that, under the chosen conditions, a continuous carbon supply is favourable to the heterotrophs growth despite the nitrifying microflora. This can be explained by the difference in growth rates and by competition for ammonium between the two populations. There was no competition for oxygen because this factor was maintained at a non-limited concentration. This result, combined to those obtained in batch cultures [10], ended up to the same conclusion: to maintain a good nitrifying activity and to restrict the growth of heterotrophs, the carbon source has to be supplied discontinuously. It was thus, supplied everyday at the same time.

Before starting other tests, another steady state under strictly nitrifying conditions was reached with 80% of nitrification (90% nitrate: 10% nitrite) and a residual ammonium concentration of 39 mg l^{-1}. The different further tests corresponded to combined supplies of *M. aerodenitrificans* and succinate at various concentrations. These tests lasted 1 week, because after this period, no more aerobic denitrifier was detected in the reactor. For each test, nitrogen balances were established: one just after the combined supply and the other one at the end of the week (Table 1).

Table 1
Nitrogen balance for each combined supply of carbon source and the strain under continuous aerated mixed culture

Tests	Nitrogen balance	Percentage of denitrification	Nitrogen gas
1	12.6	7	N_2
	−2.5	–	–
2	8.1	21.7	N_2
	−1.4	–	–
3	15.5	66.6	N_2O+N_2
	−9.2	–	N_2O+N_2

Globally, just after each combined supply, there was a decrease in $N–NO_x$ concentration in the effluent, immediately followed by an increase corresponding to the disappearance of the aerobic denitrifier. These observations had to be correlated with the positive balance observed in the earlier days of each test (Table 1) and the negative or null balance observed in the latter days. A positive balance shows that assimilation, $N–NO_x$ production and ammonium drawing off did not recover supply of ammonium. This loss of nitrogen could be explained by an underestimation of the $N–NO_x$ production because of a simultaneous production–consumption by autotrophs and the pure strain, respectively. This implied that immediately after addition of the pure strain and of succinate, the aerobic denitrifier was active and that there was simultaneous consumption of the N-oxides produced by nitrification. Moreover, production of nitrogen and nitrous oxide was observed in the gas phase, correlating the other observations. A denitrifying percentage can be calculated, considering that the lost ammonium is in fact denitrified N-oxides. For instance, during the third test, 66% of the N-oxides produced by the autotrophs were reduced into nitrogen and nitrous oxide gases. However, despite increasing combined supplies of carbon source and pure strain, the denitrifying activity was lost with time. In counterpart, the discontinuous supply of carbon has implied a decrease in nitrifying activity (60%) and an increase in protein synthesis (20%).

To conclude, the higher are the quantities of aerobic denitrifiers and of carbon source, the smaller is the percentage of apparent nitrification, but higher is the percentage of denitrification. However, the lost in nitrifying activity at the end of the last supply was not only due to an increase of assimilation but also to an increase of the residual ammonium concentration. In fact, the fractionated supply of carbon limited the biomass growth, evading competition for ammonium and allowed activity of the two populations of nitrifiers and denitrifiers. However, the

main problem was to maintain all the time the heterotrophic biomass, particularly *M. aerodenitrificans*. The continuous culture allowed us to observe that the nitrifying population flocculated. These flocs could allow the sustainability of the pure strain. For this reason, we decided to cultivate the strain in Sequencing Batch Reactor.

3.2. Nitrification combined to denitrification under SBR type culture

The SBR cycle conditions were the following: feeding with synthetic mineral medium to reach 100 mg l^{-1} of $N–NH_4^+$ in the reactor, aeration with air, settling and drawing off the cell suspension of an equivalent feeding volume. There was three cycles of 8 h per day and the HRT was three days. After stabilization of the nitrifying system, addition of acetate (100 mg l^{-1} in the reactor) was realized one time every 2 days. *M. aerodenitrificans* was added only once.

During the control nitrifying culture, an accumulation of N-oxides was observed in the reactor with a concomitant complete consumption of ammonium: there was 100% of nitrification in nitrate. However, combined addition of *M. aerodenitrificans* and acetate disturbed the system. Indeed, although ammonium was completely consumed during the cycle, it was not oxidized to an equivalent quantity of N-oxides. In addition, there was a global decrease in the final concentration of N-oxides in the reactor during the period of carbon supplies. Therefore, there was one carbon supply cycle for five control cycles. This means that during the test cycles, there was less nitrate produced, whereas, during the control cycles, there was a nitrate accumulation. Fig. 1 shows one control cycle and compares it to a test cycle.

During the test cycle, nitrate began to accumulate but was rapidly consumed before the end of the cycle. Acetate concentration and in situ hybridization revealed that nitrate consumption was correlated with *M. aerodenitrificans* metabolic activity (cells luminance). Once acetate was completely consumed, cells luminance came back to a basal level and nitrate were no more consumed by the strain still present in the reactor.

These results suggest that ammonium was nitrified by the autotrophic microflora and that the nitrate produced was simultaneously reduced to nitrogen gas by the heterotrophic aerobic denitrifier. In fact, the assimilation of am-

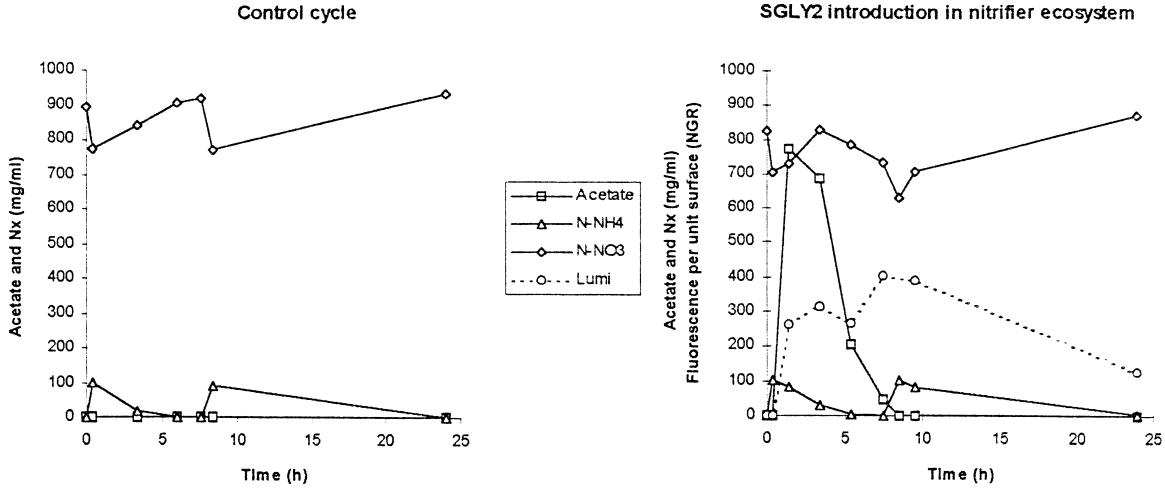

Fig. 1. Inoculation of the pure strain in a nitrifier ecosystem. On the left : nitrifier ecosystem alone. Ammonium additions were at 0 and 8 h. On the right : *M. aerodenitrificans* inoculation in the nitrifier ecosystem. Ammonium additions were as above. Acetate and strain were added after 1 h.

monium was not enough to explain the disequilibrated nitrogen balance. Here, it is interesting to underline that one addition of the strain *M. aerodenitrificans* was enough to observe this phenomenon during 20 days. This kind of reactor seems to be adapted to our ongoing project. However, at the end of the tested period, the strain seems to have disappeared. To allow sustainability of the strain, a fixed support could be used as agar beads. Another idea to maintain the aerobic strain could be the coupling with a filtration unit and re-injection of the filtered biomass in the reactor. However, these results underline that a balance has to be found between maintenance of specific biomasses concentration and regulation of activities of these present biomasses. Discontinuous supply of carbon source is one answer to the problem, SBR culture mode completes this answer, but it is not enough.

Other results lie close to those obtained with the strain *M. aerodenitrificans*. A mixed culture was realized with *T. pantotropha* and activated sludge [12]. In a continuous stirred aerated tank (dissolved oxygen concentration of 2.4 mg l^{-1}), 80% of nitrate were reduced compared to 16–28% of reduction in the control reactor without the strain. It is then possible to maintain an aerobic denitrifier in a complex heterotrophic microflora and to observe aerobic nitrate reduction. This kind of atypical behavior allows us to create attractive and revolutionary biological nitrogen removal plants.

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