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Effect of oxygen on denitrification in continuous chemostat culture with *Comamonas* sp SGLY2

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Continuous cultures of *Comamonas* sp SGLY2 were grown anaerobically prior to establishing steady states at different oxygen flow rates. At a low oxygen transfer rate, no dissolved oxygen accumulated in the medium and all nitrate was reduced to dinitrogen. Concurrently with the increase of dissolved oxygen concentration in the liquid phase, the rate of denitrification decreased. However, at a dissolved oxygen concentration near saturation (33 mg L^{-1}), a part of the electron flow always diverted to nitrate with production of dinitrogen: the aerobic denitrification rate was equivalent to 35% of that calculated under anaerobic conditions. These experiments reflected the co-utilization of oxygen and N-oxides and the production of dinitrogen up to saturated conditions, which implied synthesis and activity of the four denitrifying enzymes under various aeration conditions.

Keywords: denitrification; continuous culture; oxygen; *Comamonas* sp

Introduction

Denitrification is a part of the nitrogen cycle in which bacteria or fungi reduce nitrate to nitrogen gas with nitrite, nitric oxide and nitrous oxide as intermediates. Denitrification was generally taken to be an anaerobic process [10,18]. The presence of oxygen was thought to inhibit enzyme activities, directly or indirectly [5], or to repress enzyme synthesis: Van Spanning [26] identified an NNR-like binding site in cells of *Paracoccus denitrificans* similar to the FNR-like binding site found in cells of *Escherichia coli* responsible for anaerobic expression of genes. The inhibitory role of oxygen is now being questioned, however. Early reports on aerobic denitrification had been queried because of inadequate controls of dissolved oxygen concentration and possible existence of anaerobic microenvironments in the experimental systems [4,25,27]. More recently, some authors have clearly shown the possibility of aerobic denitrification in *Thiosphaera pantotropha*, *Alcaligenes faecalis* [20,22], *Pseudomonas nautica* [3] and *Pseudomonas* sp [24] whereas others have described the complete inhibition of denitrifying enzymes in *Paracoccus denitrificans* [6,9] and *Pseudomonas aeruginosa* [7] or partial inhibition of the enzymatic system in *Pseudomonas stutzeri* [11].

The denitrifying bacterium used in this study, *Comamonas* sp SGLY2, was isolated in our laboratory from an upflow anaerobic filter. In batch assays, it denitrifies in the presence of high oxygen gas levels and can use the two electron acceptors simultaneously [16]. We have also previously reported that addition of a protein synthesis inhibitor, nitrate and ammonium (to prevent nitrate assimilation) in aerobic cultures inoculated with cells that had never been submitted to nitrate, resulted in immediate nitrate consumption. This could be explained by the existence of a constitutive nitrate reductase. Absence of the protein synthesis inhibitor in the same culture conditions resulted in an increase of the nitrate and nitrite reduction rate. It seemed that synthesis and activity of the two primary denitrifying enzymes could occur under high dissolved oxygen levels [17]. The different levels of inhibition of nitrate reductase activity by respiratory inhibitors and detergent, according to the aerobic and anaerobic cultures, suggest the existence of a double nitrate reductase enzymatic system: one 'aerobic' enzyme insensitive to membrane damage caused by triton and less sensitive to azide, and one 'anaerobic' partially inhibited by 0.02% of triton and completely inhibited at 0.1 mM of azide [17]. These experiments, carried out in batch culture, could be criticised because of the lack of direct measurements of dissolved oxygen concentration. In this paper, we describe how increasing concentrations of oxygen in the gaseous phase of continuous cultures, fed with a synthetic medium, affect the rate of nitrate-oxygen consumption and the nature of the intermediates.

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Materials and methods

Organisms and medium

Isolation and characterisation of *Comamonas* sp SGLY2 have been described in detail elsewhere [16]. Cells were grown in a synthetic medium that contained: phosphate buffer 0.01 M pH 7.0; $MgSO_4$ 0.39 g L^{-1}; yeast extract 250 mg L^{-1}; 1 ml L^{-1} of trace element solution; $(NH_4)_2SO_4$ as nitrogen source (NH_4-N = 58 mg L^{-1}); KNO_3 as electron acceptor (NO_3-N = 257 mg L^{-1}). Ethanol (C_2H_5OH-C = 360 mg L^{-1}) was filter-sterilized and added to the cooled, autoclaved medium. Cells were grown aerobically in a 2-L Erlenmeyer flask filled with 200 ml of the medium described above without nitrate. At the end of the exponential phase, the aerobic cells were harvested by centrifugation and washed twice with sterile phosphate buffer

(0.01 M, pH 7.0) and concentrated ten times in the same solution. This concentrated cell suspension was used to inoculate a 2-L Biolafitte (Paris, France) reactor filled with 1.5 L of the medium described above where the carbon/nitrate-nitrogen ratio was constant and fixed at 1.4.

Continuous culture conditions

The medium was stirred at a constant rate of 700 rpm. Temperature was regulated at 35°C. pH was measured using an Ingold pH electrode, connected to an Ingold transmitter 2300 and was maintained at 7 by addition of sterile 2N hydrochloric acid or 5N sodium hydroxide. The dissolved oxygen levels were measured by a polarographic electrode. Anaerobic conditions were obtained by sparging the medium with oxygen/nitrogen-free argon (quality argon N56, Alphagas, Toulouse Labège, France) immediately after autoclaving it and by continuously sparging the feeding medium. Aerobic conditions were obtained by sparging the medium with a known quantity of pure oxygen (quality oxygen C, Alphagas) using a peristaltic pump, while the feeding medium was sparged with argon to avoid nitrogen entrance which could cause errors in the calculation of the nitrogen balance.

The experiment started by inoculating the 1.5 L of medium with 10 ml of aerobically grown cell suspension and by allowing the culture to grow anaerobically to the late exponential phase before starting the continuous culture. A dilution rate of 0.033 h^{-1} was used throughout the study. Anaerobic conditions were maintained for one month to achieve stable culture. Increasing oxygen flows were then applied: 35 and 75 ml h^{-1}. Each condition was applied for 1 month. Gas production and other parameters were measured every 2 days.

The Oxygen Transfer Rate (OTR, mg h^{-1}) was calculated from the formula: $OTR = Q_i n_i - Q_e n_e - Q'_i n'_i + Q'_e n'_e$ where Q_i and Q_e are the gas flow rates (ml h^{-1}) of the influent and effluent gas respectively, n_i the oxygen concentration (mg ml^{-1}) in the influent gas and n_e the oxygen concentration in the effluent gas, Q'_i and Q'_e are the liquid flow rates (ml h^{-1}) of the influent and effluent respectively, n'_i and n'_e the dissolved oxygen concentrations of the influent and effluent liquid respectively. Equations 1 and 2 were used to calculate the stoichiometric ratios: the quantities of carbon necessary to reduce 1 mg of oxygen or 1 mg of nitrate are respectively 0.25 (C_2H_5OH-C/O_2) and 0.71 (C_2H_5OH-C/NO_3/N).

$$CH_3CH_2OH + 3O_2 \rightarrow 2CO_2 + 3H_2O \quad (1)$$

$$5CH_3CH_2OH + 12NO_3^- \rightarrow 6N_2 + 12OH^- + 9H_2O + 10CO_2 \quad (2)$$

The Oxygen Uptake Rate (OUR in mg h^{-1}) was calculated from the formula: $(q_{tot} - q_{NO3})/0.25$ where q_{tot} (mg h^{-1}) is the overall quantity of reduced ethanol and q_{NO3} the quantity of carbon consumed to reduce nitrate calculated from the previous ratio. It is considered that the part of carbon used for biomass synthesis is insignificant. In our configuration where the dissolved oxygen concentration in the liquid influent is zero, the following equation can be written:

r'''_{O_2} = oxygen variation rate (mg O_2 h^{-1} L^{-1} of reactor)

$$= kla\ (c^* - c_L) - Q_{O2}X - (Q'_e/V)\ c_e$$

where

- V = working volume
- c^* = dissolved oxygen concentration in balance with the partial pressure of oxygen in the gaseous phase (or oxygen solubility);
- c_L = dissolved oxygen concentration in the medium;
- c_e = dissolved oxygen concentration in the liquid effluent;
- $Q_{O2}X$ = oxygen consumption rate;
- kla = volumetric mass transfer coefficient.

The nitrogen balance is obtained from the difference of concentrations between the effluent and the influent for nitrate, nitrite, ammonium and proteins in the liquid phase, and dinitrogen, nitric and nitrous oxide in the gaseous phase. The percentage of nitrate reduction is calculated from the formula: $([NO_3 - N]_i - [NO_3 - N]_e)/[NO_3 - N]_i * 100$.

Analytical measurements

The liquid samples were centrifuged at 17500 × *g* and 4°C for 15 min. The supernatant phases were diluted as required for the different analyses.

Ammonium was determined using a Büchi 320 apparatus (Bioblock, Strasbourg, France) according to the method recommended by Rodier [23].

Nitrate and nitrite were measured by an ion exchange chromatography system using conductivity detection (DIONEX-100). Separation and elution of the anions were carried out on a IonPacAS4A Analytical Column (Dionex, Toulouse, France) using a carbonate-bicarbonate eluant and a sulfuric acid regenerant. Protein concentrations were determined by the method of Lowry *et al* [14] using bovine serum albumin as a standard. Ethanol was measured by gas chromatography with nitrogen as carrier gas using a flame ionisation detector.

Gas composition was analysed by gas chromatography with a Shimadzu GC-8A apparatus (Touzant et Matignon, Paris, France) with argon as a carrier gas, using a katharometer detector. Carbon dioxide and nitrous oxide were separated on a Haye Sep Q column (Touzant et Matignon; 80–100 mesh, 2.0 m × 3.15 mm). Oxygen and nitrogen were separated on a molecular sieve column 5A (20–100 mesh, 2.0 m × 3.15 mm). The injector and detector temperatures were 100°C. The column temperature was 35°C. Nitric oxide was measured on a Shimadzu GC-14A with helium as a carrier gas, using a katharometer detector. The molecular sieve 5A (80–100 mesh, 2.0 m × 3.15 mm) column was maintained at 220°C, the injector at 150°C and the detector at 105°C.

Results

Effect of increasing oxygen gas flow rates on denitrifying enzyme activity

Using chemostat culture at a fixed dilution rate of 0.033 h^{-1} with a feeding medium containing 257 mg L^{-1} NO_3-N with

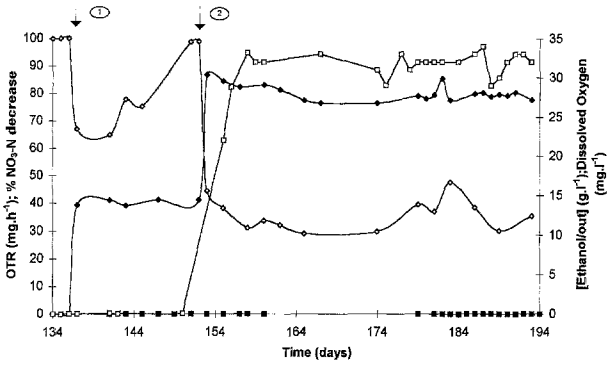

Figure 1 Changes in the percentage of denitrification (% NO_3-N decrease) with increasing oxygen flow rate in continuous culture of *Comamonas* sp. Arrows indicate the time at which oxygen was supplied (① 35 ml h^{-1}, ② 75 ml h^{-1}). ◆ Oxygen Transfer Rate (OTR) in mg h^{-1}. ◇ % decrease of nitrate. ■ Remaining ethanol (mg L^{-1}). □ Dissolved oxygen concentration (mg L^{-1}).

a carbon (ethanol) to nitrogen (nitrate) ratio of 1.4 (twice as much as the stoichiometric ratio), a series of steady states were established at different oxygen gas flow rates. The different phases of oxygen supply are successively described for the reactor on Figure 1. During anaerobic growth in continuous culture, all the nitrate was reduced to dinitrogen. At the commencement of the oxygen supply (35 ml h^{-1}), nitrate reduction and nitrogen production sharply decreased and no accumulation of other denitrifying intermediates was observed: 65–75% of nitrate was reduced to nitrogen over a period of 8 days. After 15 days, the percentage of nitrate reduction reached 100% with a simultaneous consumption of the small quantity of transferred oxygen. In fact, the oxygen concentrations in the liquid as well as in the gaseous phases were zero. Table 1 summarizes the parameters calculated for each step of oxygen supply. At 35 ml h^{-1}, the overall quantity of carbon supplied was consumed: 47% was oxidised by nitrate and 53% by oxygen. The earlier 8 days reflected a phase of acclimatisation of the biomass. It seemed that the electron flow preferably diverted to oxygen, but with a concomitant flow to the complete denitrifying enzymatic system: the quantity of carbon was sufficient for nitrate and oxygen reduction. In this case, oxygen supply was the limiting factor.

The second oxygen flow rate applied to the reactor (75 ml h^{-1}) was accompanied by a decrease in the percentage of nitrate consumption to 35% and by accumulation of oxygen in the aqueous phase equivalent to 33 mg L^{-1}. This value corresponds to the maximum quantity of dissolved oxygen that can be transferred with pure oxygen at one atmosphere of pressure and at 35°C. In these conditions, the transfer of oxygen was not a limiting factor. Ethanol was completely oxidised: 19% was consumed to reduce nitrate and 81% to reduce oxygen. The maximum Oxygen Transfer Rate was 96 mg h^{-1} and the maximum Oxygen Uptake Rate for a total consumption of ethanol was 72 mg h^{-1}. In fact, the OUR was 58.6 mg h^{-1}. However, the high concentration of oxygen in the aqueous phase showed that the strain could consume more than what it really consumed. The consumption of oxygen was limited by the simultaneous reduction of nitrate. These results are inconsistent with the common view of diversion of electron flow solely to oxygen, when the two acceptors are present in the medium. In counterpart, they underline the ability of the strain to co-respire the two acceptors and the ability of the aerobic nitrate reductase to be active even at 33 mg O_2 L^{-1}. Moreover, the major product of denitrification at steady state was dinitrogen (Table 2): 85–57% of the nitrate used was reduced to nitrogen, 1–3% was transformed to nitrite, 9–18% to nitrous oxide and 0–4% to nitric oxide.

The concentrations of the aqueous forms of nitrous-nitric oxide can be determined with the Bunsen coefficient. However, their estimation gives low values that can be negligible in the nitrogen balance. This balance is slightly desequilibrated, with more nitrogen in the influent than in the effluent (1–5% of errors). This could be explained by errors in the analytical measurements. It shows too that there is an equivalence between ammonium consumption and protein synthesis. This result overcomes the hypothesis of existence of nitrate assimilation. Therefore, it confirms the fact that the four denitrifying enzymes of the strain were active and synthesized under non-limiting aerobic conditions.

Effect of increasing concentration of ethanol on oxygen-nitrate respiring system

After a steady state was established in the reactor at an oxygen flow rate of 75 ml h^{-1}, ethanol was added to the reaction volume to observe the consequences on the denitrification rate and the OUR. The results are summarized in Figure 2. At time zero, 2.5 ml of pure ethanol were added. Concurrently to the increase of OUR which was correlated to the decrease in dissolved oxygen concentration and in oxygen gas concentration, nitrates were consumed with concomitant nitrogen gas production. However, the nitrate consumption rate was lower than the oxygen consumption rate: during the first 4 hours, 4.4% of the added carbon allowed consumption of 7% more nitrate, whereas the remaining quantity was used to consume all transferred oxygen. Afterwards, denitrification was favoured by the dissolved oxygen concentration decrease. Thus, it implies that the two enzymatic systems could function simultaneously and that the electron flow diverted both to oxygen and to nitrate whatever the conditions.

Table 1 Oxygen Transfer Rate, Oxygen Uptake Rate, nitrate and carbon consumption at each aerated steady state in the reactor

O_2 flow rate ml h^{-1}	OUR mg h^{-1}	DO mg L^{-1}	C/O_2 mg h^{-1}	C/NO_3-N mg h^{-1}	C total mg h^{-1}	NO_3-N total mg h^{-1}	% NO_3-N decrease
35	41.8	0	10	8.87	18.87	12.5	100
75	58.6	33	14.6	3.33	18	4.77	35

DO = dissolved oxygen concentration, C/O_2 = quantity of carbon consumed to reduce oxygen, C/NO_3-N = quantity of carbon consumed to reduce nitrate.

Table 2 Nitrogen balance at two points (1 and 2) during the last steady state for the reactor (75 ml h^{-1})

		Influent at point		Effluent at point	
		1	2	1	2
Liquid phase	NO_3-N	12.8	13.48	7.74	9.016
	NO_2-N	0.095	0	0.088	0.12
	NH_4-N	2.51	2.8	1.19	1.3
	Protein-N	0	0	1.53	1.53
Gaseous phase	N_2-N	0	0	4.34	2.54
	N_2O-N	0	0	0.46	0.83
	NO-N	0	0	0	0.161
Total		15.4	16.2	15.34	15.49

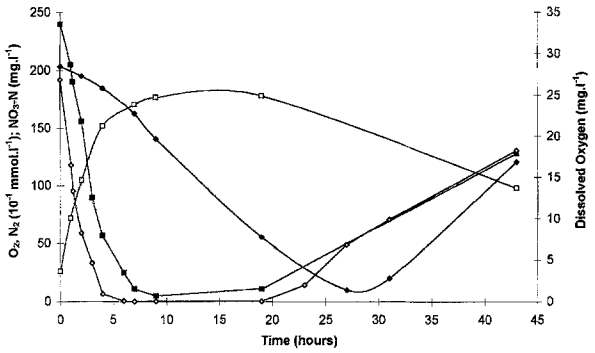

Figure 2 Effect of addition of ethanol (2.5 ml) at steady state on nitrate reductase activity (oxygen flow rate = 75 ml h^{-1}). ◇ Dissolved oxygen concentration (mg L^{-1}). ■ Oxygen in gaseous phase (10^{-1} mmol L^{-1}). □ Dinitrogen in gaseous phase (10^{-1} mmol L^{-1}). ◆ Concentration of NO_3-N (mg L^{-1}) in the outflow.

Discussion

Continuous culture of the *Comamonas* sp SGLY2 allowed a better characterization of the denitrifying enzymatic system of the strain and its behaviour towards oxygen, because of a constant measurement of dissolved oxygen concentration. At a low oxygen flow rate, the apparent absence of dissolved oxygen in the culture medium suggests that the cells consumed the electron acceptor. At the same time, however, nitrate was also reduced to dinitrogen. In these conditions, oxygen was the limiting factor implying consumption of nitrate to saturate the respiratory chain. These results confirmed the facts observed in batch assay [16]: oxygen and nitrate are consumed simultaneously. This co-utilization of the two electron acceptors contrasted with the observations of John [9] and Ferguson [6] in *Paracoccus denitrificans* NCIB 8944, where oxygen inhibited nitrate reduction in intact cells. Other experiments carried out in our laboratory with aerobically grown nitrate non-adapted cells of *Pseudomonas aeruginosa* (ATCC 10145), *Alcaligenes eutrophus* (ATCC 17699) and *Pseudomonas stutzeri* (ATCC 14405) gave the same results: under partial aerobic conditions, nitrate consumption started only after complete disappearance of oxygen. Lloyd *et al* [13] using the same strain, *Paracoccus denitrificans* NCIB 8944, reached a contrary conclusion: at oxygen concentrations greater than the atmospheric saturation value, NOx production was rapid. In addition, Hochstein *et al* [8] suggested that under conditions of nitrate limitation, anaerobically grown nitrate-adapted cells of *P. halodenitrificans* were able to reduce nitrate over a broad range of oxygen concentrations with a concomitant accumulation of nitrite. Bonin and Gilewicz [3] also reported that *Pseudomonas nautica* used nitrate and oxygen at the same time. *Thiosphaera pantotropha* and *Alcaligenes faecalis* have also been found to use the two acceptors simultaneously resulting in a higher growth rate than if either of them was used alone [21]. It could be thus concluded that aerobic reduction of nitrate was the general case. However, these experiments were carried out with anaerobically grown nitrate-adapted cells, in batch assays for incubation periods not exceeding 5 h (except for *T. pantotropha* [19]). The nitrate reductase activity measured under these conditions resulted from the activity of preformed enzymes. As suggested by Ferguson [6], the constitutive electron transport chain for oxygen might have a relatively low capacity in anaerobically grown cells, thus a switch to aerobic conditions could not result in complete transfer of electrons to oxygen. In contrast, with the continuous culture of strain SGLY2, implying a long experimental time, the consumption of the two electron acceptors was observed up to saturation of the medium with pure oxygen.

When *Comamonas* sp SGLY2 was grown under conditions of carbon limitation, denitrification took place over a wide range of dissolved oxygen concentrations. In non-limiting aeration conditions, where 100% of the carbon could be used to reduce all transferred oxygen, a part of the electron flow (19%) diverted to nitrate. The rate of nitrate reduction decreased with increasing levels of oxygen flow rate: 74.5 nmol NO_3 min^{-1} mg $protein^{-1}$ under anaerobic conditions to 26.1 nmol NO_3 min^{-1} mg $protein^{-1}$ under aerobic conditions. However the main product of the reaction was dinitrogen (85–57%). This production of dinitrogen in non-limiting aeration conditions involved activity of the four denitrifying enzymes, although it was believed for a long time that nitrous oxide reductase was an oxygen-labile enzyme. The same kind of behaviour was noticed with *P. halodenitrificans* [8]: with increasing levels of dissolved oxygen, a progressive reduction of dinitrogen production was observed with accumulation of nitrous oxide at first and then nitrite. However, at 0.1 mg L^{-1} of dissolved oxygen, all nitrate was transformed to nitrite whereas at the same concentration, *Comamonas* sp SGLY2 reduced 75%

of nitrate to dinitrogen, and at 1.7 mg L^{-1}, a culture of *P. halodenitrificans* did not reduce significant amounts of nitrate. In the same way, *T. pantotropha* had a smaller denitrification rate under fully aerobic conditions equivalent to 10% of that obtained under anaerobic conditions [12].

Aerobic denitrification in *T. pantotropha* was accounted for by a periplasmic nitrate reductase enzyme, which is constitutive and less sensitive to azide compared to the membrane-bound nitrate reductase, similar to other reductases found in classical oxygen-sensitive denitrifiers [1,2]. Localisation of the enzyme in the periplasmic fraction evaded the inhibitory effect of oxygen on the antiport nitrate-nitrite system. Subsequently, Moir *et al* [15] showed that the cd_1-type nitrite reductase was not expressed under aerobic conditions. The status of aerobic denitrifier of *T. pantotropha* was then not obvious.

At this point, the behaviour of *Comamonas* sp SGLY2 differed from that of *T. pantotropha* because synthesis as well as activity of all enzymes could occur under fully aerobic conditions as shown here. Other experiments carried out in batch culture, with different inhibitors of the respiratory system and of the protein synthesis, led to the conclusion that there is a double nitrate reductase system with an aerobic constitutive enzyme [17]. It seems that the denitrifying system of the strain SGLY2 is particulate and complex. Oxygen may not have a direct inhibitory effect on activity and synthesis of the enzymes, but may directly compete for the electron flow. In the reactor, under non-limiting aeration conditions (33 mg L^{-1}), the electron flow diverted to oxygen and nitrate; it looks like a kind of limitation of consumption of the most energetic electron acceptor to prevent the saturation of the respiratory chain.

However, further investigations on the regulatory system of synthesis of enzymes, involving purification of the enzymes would be necessary for a better understanding of the particular behaviour of this aerobic denitrifier. From an ecological point of view, existence of this kind of aerobic denitrifier is interesting. It may explain nitrogen losses in agronomic systems and have to be considered in agricultural practices especially for the mode of application of nitrogen fertilizers. From the point of view of waste water treatment plants, a combination of nitrifiers and aerobic denitrifiers may result in conversion of ammoniacal pollution to nitrogen in a single aerated unit.

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