Modeling of Biochemical Nitrate Reduction in Constant Electric Field

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Experiments on the bioelectrochemical stimulation of enzyme reduction of nitrate to nitrite in a potentiostatic regime at different catode potentials were carried out. It was established that the stimulation effect of the constant electric field on nitrate reduction is also relevant for cell-free enzyme preparation, i.e. the effect is related to the constitutive enzymes nitrate-reductase and nitrite-reductase, contained in the cell membranes.

Mathematical modeling of these experimental data as well as data for the same process accomplished by living immobilized cells was carried out. The purpose of the modeling was to select the most suitable kinetic model and then estimate the kinetic parameters and their dependence on the cathode potential.

The mathematical models were based on the Michaelis-Menten kinetics taking into account inhibition by nitrate and nitrite. This modeling helped to conclude that the stimulation consists of two effects: enhanced maximum rate of nitrate enzyme reduction and faster nitrite reduction to eliminate nitrite inhibition on the overall process. It was found that the maximum reaction rates of nitrate and nitrite reduction depend on the cathode potential with maxima at + 0.01 V vs. the saturated hydrogen electrode.

Key words:
Nitrate reduction, enzyme process, cell-fee crude extract, bacterial cells, bioelectrochemical stimulation

Introduction

Pollution of natural waters, like rivers, lakes and ground water by nitrate is one of the main environmental problems nowadays. The origin of nitrate in those waters is different: *in situ* oxidation of ammonia resulting from biodegradation of nitrogen containing organic compounds or excessive use of fertilizers.[1] Although nitrate does not in itself pose a human or animal health threat, it could be converted to nitrite in the gastro-intestinal tract or to nitrous organic compounds through reduction and thus cause severe environmental and health problems.[2,3] It is established that nitrate reduction to molecular nitrogen follows the scheme:[2]

$$NO_3^- \rightarrow NO_2^- \rightarrow NO\ (g) \rightarrow N_2O\ (g) \rightarrow N_2\ (g) \quad (1)$$

The main problem in water treatment to nitrate is the enormous amount of water to be treated and the excessive amount of energy to be applied for this purpose in case of traditional methods. That is why new techniques have been developed and tested, like electro-chemical reduction of nitrate in the presence of bacteria.[1,4–8] On the other hand, the practical application of such a process requires appropriate organization in order to reduce the bioreactor size and attain repeated use of the microbial cells in a continuous culture. The best way to do this is to use immobilized denitrifying bacteria, as reported in.[9–11]

Two different regimes of bio-electrochemical nitrate reduction have been studied: galvanostatic[5–7] and potentiostatic.[8] There are different explanations for this effect. Some authors claim that the enhanced bio-electrochemical reduction of nitrate in galvanostatic regime is due to the hydrogen release on the cathode.[5–7]

It was shown in a previous paper[8] that the potentiostatic regime of nitrate reduction is associated with very low electric currents and current efficiencies and could not be related to purely electro-chemical process or reduction by released hydrogen. It was shown there, that the electric field leads to shortening the lag-phase in microbial growth and thus enhances the overall nitrate reduction process.

There are different effects of weak electric fields on living cells based on facilitated movement of ions across the cell membranes through trans-membrane proteins[12,13] on changes in hydration, orientation and in conformation of molecules in the cell membranes,[14] provoked shift in redox potentials, resulting in electron transfer processes,[15] genetic effects,[16] stimulation of microbial growth,[17] etc.

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The first step to clarify the effect of bio-electrochemical stimulation of nitrate reduction is to study the denitrification capacity of crude cell free enzyme preparations with and without application of constant electric field in the broth. The obtained results could answer the question whether the constant electric field stimulates the constitutive enzymes after disintegration or it consists in stimulation of certain life activity of the intact cells. Comparison with data for the process accomplished with immobilized bacteria was done as well. Appropriate kinetic models were tested and the kinetic constants were evaluated by mathematical modeling of the experimental results. These quantitative results lead to conclusions about the sensitivity of the denitrification process on the cathode potential.

Experimental

Materials and methods

Strain and cultivation media

The strain *Pseudomonas denitrificans* (NBIMCC 1625) supplied by the Bulgarian National Bank of Industrial Microorganisms and Cell Cultures was inoculated in a medium containing: peptone, 10 g L^{-1}; yeast extract, 1 g L^{-1}; NaCl, 10 g L^{-1}. The inoculum was developed for 24 h at 30 °C in a rotary shaker at a very low agitation speed (50 rpm) to avoid undesired aeration. Bacterial cells were immobilized on support of co-polymer of acrylonitrile and acrylamide by the method described in.[18] The concentration of the immobilized cells on the solid support was estimated from the cell mass balance before and after immobilization, as about 25 grams per liter of particle volume, or 0.008 g cm^{-2} on the support surface.

For the continuous process, the feeding medium contained phosphate buffer (pH ~ 7) with controlled addition of KNO_3 corresponding to different initial nitrate concentrations within 0.05 and 0.300 g m^{-3}.

Crude cell-free extracts

The cell-free extracts were prepared by ultrasonic disintegration of cells of the cultivated strain. Disintegration was accomplished by UD-20 disintegrator (produced in Poland) at 14 kHz for 30 min. The culture was kept in an ice-cold bath during disintegration to avoid thermal denaturation. Afterwards the resulting suspension was filtered through cellulose acetate filters with 0.2 μm pore size. The filtrate and the non-filtered suspension were tested for denitrification activity separately.

Denitrification activity of the enzyme preparations was tested on nitrate solutions at initial concentrations within 10 and 50 mg L^{-1} at 30 °C and pH 7 in a stirred cell. Samples were taken in the first 45 minutes after crude enzyme addition. The enzyme activity was calculated from the amount of reduced nitrate related to the total protein content.

Analyses

Biomass concentration

The biomass release from the particles with immobilized cells was monitored off-line photometrically following the optical density of the broth at λ = 660 nm by means of a UV/Vis spectrophotometer (Unicam/Heλios β, Cambridge, UK).

Nitrate concentrations

Nitrate was determined by the method of Cataldo *et al.*,[19] based on nitration of salicylic acid in strong acid solutions. The method consists of the following.

Reagents: 2 N NaOH and salycilic acid solution in sulfuric acid (5 g in 100 mL conc. sulfuric acid). A fresh solution is prepared weekly and stored in a brown bottle.

Procedure: an aliquot (e.g. 0.25 mL) of sample or standards are transferred into a 50 mL Erlenmeyer flask. An amount of 0.8 mL of 5 % (w/v) salicylic acid in conc. H_2SO_4 is added at thorough mixing. After 20 minutes at room temperature, 19 mL of 2 N NaOH are added to raise the pH above 12. The sample is cooled to room temperature and the light absorbance is measured at 410 nm against distilled water. The nitrate concentrations were calculated from a calibration curve. The standard error of this analysis was ± 1 %.

Nitrite concentrations

Nitrite was determined photometrically by a standard method, based on Griess reaction, involving the formation of purple-colored azo dye upon treatment of a NO_2^--containing sample with sulfanilic acid and naphthyl-1-amine in the presence of acid.[20] The analysis standard error was ± 4 %.

Protein content

Protein content in the crude cell-free extracts was determined according to the method of Bradford.[21] It was about 0.14 mg protein mL^{-1}.

Experimental set-up

Experiments with immobilized microbial cells were carried out under moderate stirring conditions. A stirred tank bioreactor of 500 mL volume was

used with a continuous feed corresponding to hydraulic retention times within 30 minutes to 5 hours. The particles with the attached microbial cells were put in the reactor prior to use. Samples from the outlet flow were taken and analyzed for biomass, nitrate and nitrite as described above. The transient processes with respect of the outlet nitrate concentration took 18 hours or more. Steady states were assumed when samples taken within four hours showed constant concentrations within the analysis accuracy. When cell release was detected, the process was stopped.

The area of the stainless steel cathode dipped in the broth was 13 cm^2. The cathode potential was kept constant within –0.06 and +0.02 V/S.H.E. by potentiostat with a reference saturated calomel electrode. These potential values were chosen to match the optimum range established in.[8] The electric circuit was closed through an agar salt bridge with an anode outside the fermentor. The ohmic resistance of the fermentor was about 35 kΩ. The measured electric current did not exceed 0.01 mA.

Two types of experiments with cell-free extract were carried out: without and with electric field application. When electric field was applied, the cathode potential was maintained within –0.06 and 0.14 V/S.H.E. The experiments with constant electric field were carried out in a stirred tank bioreactor as it was with immobilized microbial cells. When no field was applied, they have been carried out in a thermostated stirred cell of 50 mL volume.

The enzyme process was started with addition of the cell-free extract to the initial nitrate-containing buffer solution (pH ~ 7) at a volumetric ratio cell-free extract: reaction solution = 1 : 50. The initial nitrate concentrations were between 10 and 200 g m^{-3}.

During these experiments, different initial amounts of sodium nitrite were added to the initial nitrate solutions to test the inhibition effect of nitrite with pre-set concentrations 0, 5, 10, 20 and 40 g m^{-3} NO_2^-. No growth factors, like yeast extract, corn steep liquor, etc., or any other carbon sources were added in order to avoid growth of possibly not destroyed cells. Samples were taken regularly and analyzed for nitrate and nitrite. The lack of growth in these cases was established by monitoring of optical density of the reaction mixture.

All experiments were carried out at 30 °C.

Three separate and parallel cell-free preparations were tested. There was no significant difference between the kinetic results according to the statistical analysis.

Mathematical modeling

The mathematical modeling had two goals in this study. The first one was to select suitable enzyme kinetics of nitrate reduction with further nitrite degradation by the crude enzyme and the immobilized cells. For this purpose the experimental data obtained at low initial nitrate concentrations were handled by different kinetic models.

The second goal was to use the selected kinetics to evaluate the kinetic constants of the nitrate reduction at different cathode potential from the experimental data obtained at constant electric field and to estimate the studied effect quantitatively.

Nitrate degradation by immobilized cells in continuous stirred tank reactor

The mathematical modeling of the denitrification process by immobilized cells was made with the data for continuous process at steady state at different inlet nitrate concentrations S_{inlet}. The following simple balance equations for the steady state were used:

$$D(S_{inlet} - S) = f(S)$$

or (2)

$$D = \frac{1}{S_{inlet} - S} \cdot f(S)$$

where the function f(S) contains the kinetics of the reaction. At very high dilution rates D (the inverse values of hydraulic retention times), there is practically no conversion and the outlet substrate concentration S tends toward inlet one S_{inlet}. Here we can consider that the immobilized cells do not grow and the microbial nitrate reduction is a purely enzyme process. The function f(S) may take different forms. The simplest one is the Michaelis-Menten kinetic equation. We tested the substrate inhibition described by two different models: the one of Andrews:[22]

$$-\frac{dS}{dt} = f(S) = V_m \frac{S}{K'_m + S + S^2/K_i}, \quad (3a)$$

and of Aiba *et al.*:[23]

$$-\frac{dS}{dt} = f(S) = V_m \frac{S}{K'_m + S} \exp(-S/K_i). \quad (3b)$$

When $K_i \to \infty$, both equations give the classical Michaelis-Menten kinetics. It is usually expected that accumulation of nitrite may cause additional product inhibition, particularly at high initial nitrate concentrations.[24] However, our experimental data showed that, under the conditions of continu-

ous stirred tank reactor, there was no such accumulation and this inhibition effect could be neglected for the studied case.

The model equations (2, 3) were solved by the TUTSIM simulator[25] coupled with an optimization procedure according to the Nelder-Mead method. The model parameters were estimated by fitting the experimentally determined outlet steady-state concentrations for different dilution rates with those computed by the models minimizing the sum of the squares:

$$\text{Sum} = \sum_j (S_j^{\text{calc}} - S_j^{\text{exp}})^2 \tag{4}$$

The experimental data were handled by the two different kinetic models, i.e. eqs. (3a, 3b). The more suitable model with the corresponding function f(S) was selected by the Fisher-test comparing the minimized sums of the least squares (4) calculated by the two models at a confidence level of 99 %.

The confidence intervals of the evaluated kinetic parameters were established according to the Student test at a confidence level of 99 %.

Nitrate degradation by crude cell-free extract under batch conditions

The numerical procedure described in previous section was applied for the kinetic data evaluation for nitrate degradation with cell-free extracts as biocatalyst.

Two kinetic models for product inhibition were tested: the Levenspiel's one[26] and that of competitive product inhibition. The Levenspiel's model assumes there is a critical product concentration beyond which no process takes place. Nitrite ions degrade further according to the scheme in eq. (1) with maximum reaction rate V_P and Michaelis constant K_{mP}. For the case of Levenspiel's model the balance equations for these consecutive enzyme reactions are:

$$-\frac{dS}{dt} = f(S)\left(1-\frac{P}{P_{cr}}\right), \quad \text{for } P \le P_{cr}, \quad \text{or}$$

$$\frac{dS}{dt} = 0, \quad \text{if } P > P_{cr}; \tag{5}$$

$$\frac{dP}{dt} = -\frac{M_{NO_2^-}}{M_{NO_3^-}}\frac{dS}{dt} - V_P\frac{P}{K_{mP}+P}(1-P/P_{cr})$$

In the case of competitive inhibition by the product the system (5) for the nitrate and nitrite reduction rate takes the form:

$$-\frac{dS}{dt} = V_m\frac{S}{K'_m\left(1+\frac{P}{K_{iP}}\right)+S}\exp(-S/K_i)$$

$$\frac{dP}{dt} = -\frac{M_{NO_2^-}}{M_{NO_3^-}}\frac{dS}{dt} - V_P\frac{P}{K_{mP}+P}(1-P/P_{cr}) \tag{6}$$

The substrate (i.e. nitrate) inhibition was taken into account by the function f(S) taken from each of eqs. (3a, 3b). At $K_{iP} \to \infty$ the first equation in (6) turns into the Aiba's model, eq. (3b). In all cases the corresponding initial conditions are:

$$t = 0,\ S = S_0,\ P = P_0 \tag{7}$$

Note, that the initial nitrite concentration P_0 was not zero in all experiments.

The kinetic parameters in eqs. (5) or (6) were sought using the sum of squares, taking into account the substrate (i.e. nitrate) as well as the product (i.e. nitrite) concentrations:

$$\text{Sum} = \sum_j [(S_j^{\text{calc}} - S_j^{\text{exp}})^2 + (P_j^{\text{calc}} - P_j^{\text{exp}})^2] \tag{8}$$

The sums in eq. (8) were calculated from six to eight experimental points comprising the product concentrations.

The test of validity of the model (5) and the parameter evaluation was done by the Fisher-test as described in previous section. For this purpose, the kinetic data at low initial nitrate concentrations (i.e. up to 50 g m^{-3}) were used.

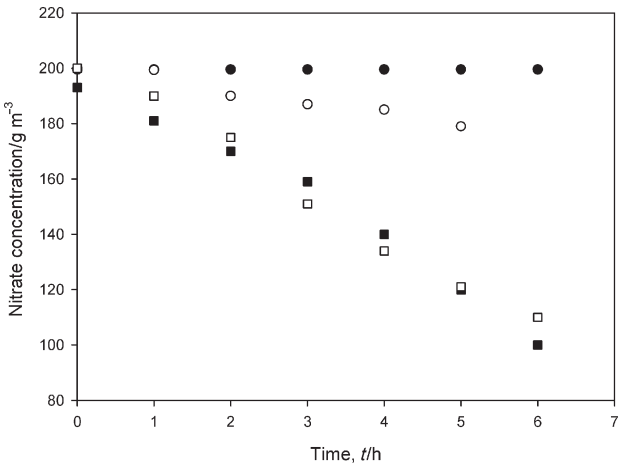
Then the effect of the constant electric field on the denitrification process and the dependence of the kinetic parameters in the selected model on the cathode potential were quantitatively estimated. It was accomplished by minimization of the sum of least squares, see eq. (8).

Results and discussion

Denitrification by crude cell-free extracts

First, the filtrate after disintegration and filtration was tested. It was established that there was no denitrification activity of the filtrate, neither with nor without electric field application. Significant denitrification activity was detected in the remaining suspension. This result follows the fact, that the enzyme nitrate-reductase is not released by the cells but it is bound to the cells membranes and it is retained there after cell disintegration. This fact was pointed out for other strains of the genus *Pseudomonas* in the literature.[27–30]

The experimental data carried out in the presence of cell-free extract with and without preliminary filtration showed denitrification activity, considerably enhanced by the electric field, cf. Fig. 1.



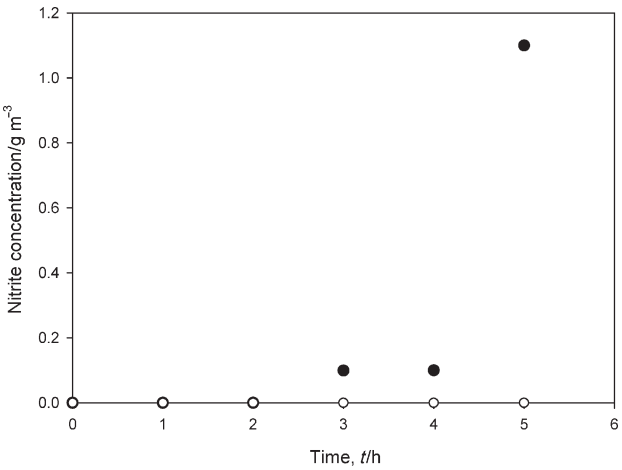
F i g . 1 – *Denitrification by crude cell-free extract at initial concentration 200 g m^{-3} at different cathode potentials. (●) – filtrate, no el. field; (○) – crude cell-free extract; no el. field; (□) – E = 0.06 V; (■) – E = 0.095 V.*

Additional results on the effect of enhanced reduction of nitrite ions by crude cell-free extract at electric field application are shown in Fig. 2a and Fig. 2b. Although very low, nitrite concentrations were measurable when no electric field was applied, cf. Fig. 2a. Nitrite ions could not be detected in the broth for any of the experiments for nitrate reduction in case of electric field application. We could assume that the process is additionally enhanced by the electric field due to the faster nitrite reduction and removal from the broth, and therefore its inhibition effect is avoided.

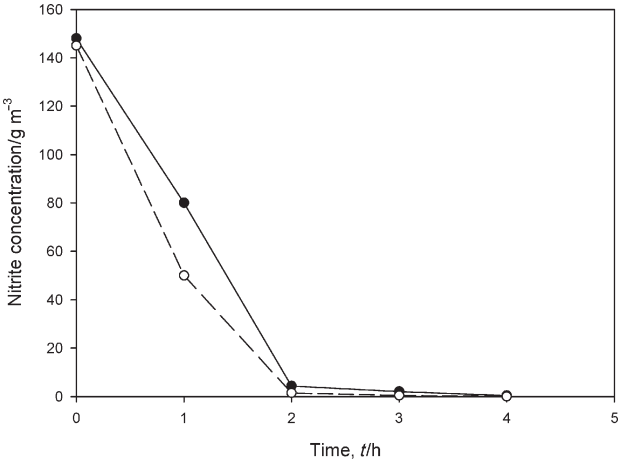
The effect of electric field in case of nitrite reduction as a single substrate at a higher concentration is shown in Fig. 2b.

The kinetic experiments for denitirification by crude cell-free extract with initial nitrite addition showed that nitrate or nitrite inhibition takes place at relatively higher concentrations. However, the process started to slow down considerably at initial nitrite concentrations of 20 g m^{-3}. Neither nitrate, nor nitrite degradation was observed at initial concentrations of nitrite higher than 20 g m^{-3}.

Illustration of these results is shown in Figs. 3a and 3b. Whereas nitrate reduction runs at a considerable rate, nitrite reduction stops at an initial nitrite concentration of 20 g m^{-3}. The experimental data for lower nitrate concentrations with different initial nitrite concentrations handling by the TUTSIM simulator with the model (6) also showed



F i g . 2 a – *Nitrite concentrations for experiments with cell free extract without electric field application (●) and at cathode potential of –0.06 V/S.H.E (○). Initial nitrate concentration – 200 g m^{-3}.*



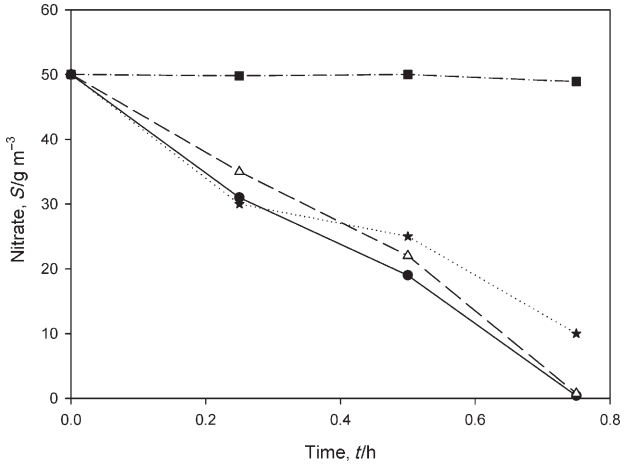
F i g . 2 b – *Nitrite concentrations from experiments with cell free extract without electric field application (●) and at cathode potential of –0.06 V/S.H.E (○). Initial nitrite concentration – 150 g m^{-3}.*

that the critical product concentration beyond which no nitrite degradation takes place was P_{cr} = 20 g m^{-3}.

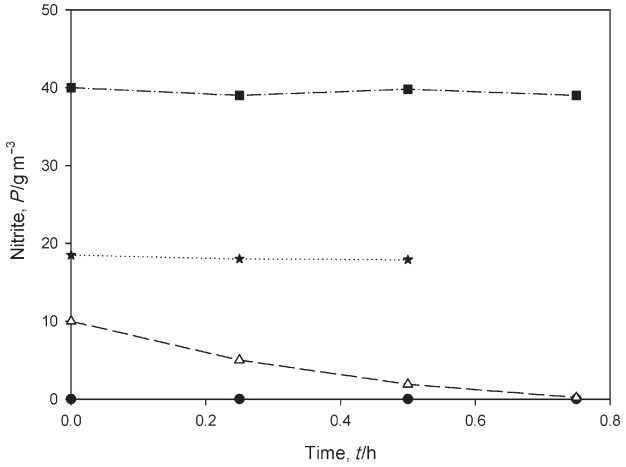
This fact prompted us to assume a Levenspiel's kinetics for the second consecutive reaction, i.e. nitrite reduction.

Modeling of denitrification by crude cell-free extract in constant electric field

The first results from the mathematical modeling were that the kinetic model for substrate inhibition proposed by Aiba *et al.,* eq. (3b) coupled with the competitive product inhibition, see eqs. (6), gave better results compared to the models of Andrews (eq. 3a) and Levenspiel, eq. (5). This statement was confirmed by the *F*-test at a confidence



F i g . 3 a – *Time profiles for nitrate reduction by cell free extract without electric field application at different initial nitrite concentrations. Initial nitrate concentration – 50 g m^{-3}. Solid line, (●) – NO_2^- = 0; dashed line, (△) – NO_2^- = 10 g m^{-3}; dotted line, (*) – NO_2^- = 18.5 g m^{-3}; broken line, (■) – NO_2^- = 40 g m^{-3}.*



F i g . 3 b – *Time profiles for nitrite reduction by cell free extract without electric field application at different initial nitrite concentrations. Initial nitrate concentration – 50 g m^{-3}. Solid line, (●) – NO_2^- = 0; dashed line, (△) – NO_2^- = 10 g m^{-3}; dotted line, (*) – NO_2^- = 18.5 g m^{-3}; broken line, (■) – NO_2^- = 40 g m^{-3}.*

level of 99 %. The values of the kinetic constants for the reference experiments (without electric field application) are given below:

$$V_m = 3.2 \pm 0.1 \quad \text{mmol dm}^{-3}\ \text{h}^{-1};$$
$$K'_m = 0.04 \pm 0.002 \quad \text{mmol dm}^{-3};$$
$$K_i = 1.34 \pm 0.01 \quad \text{mmol dm}^{-3}; \qquad (9)$$
$$K_{mp} = 0.22 \pm 0.01 \quad \text{mmol dm}^{-3}\ \text{h}^{-1};$$
$$K_{iP} = 0.15 \pm 0.03 \quad \text{mmol dm}^{-3}$$

The apparent Michaelis constant K'_m = 0.04 mmol dm^{-3} is lower than the one found by Garbayo *et al.*[31] for nitrate reduction by *Chlamydomonas reinhardti* (0.21 mmol dm^{-3}) but closer to the one, reported by Afshar *et al.*[32] for nitrate reductase isolated from cell membranes of *Pyrobaculum aerophilum*, i.e. 0.057 mmol dm^{-3}.

The Aiba's model presented in eq. (6) was used further for parameter estimation for the constant electric field stimulated processes. The values of the estimated kinetic parameters are shown in Table 1. In all cases V_m at constant electric field was higher than the reference value when no electric field was applied. There is a neat maximum at cathode potential of 0.01 V *vs*. the saturated hydrogen electrode.

Similar maxima were found of the Michaelis constant K'_m and the maximum nitrite reduction rate V_p. However, the sensitivity of the evaluation procedure was not high enough at higher initial nitrate concentration. Therefore, we cannot claim that there is such a clearly pronounced dependence as it is for the maximum nitrate reduction rate.

The substrate inhibition constants K_i, although dispersed, are quite high to consider a moderate substrate inhibition.

T a b l e 1 – *Comparison of kinetic parameters for crude cell-free extract evaluated by the Aiba model, combined with competitive product inhibition, see eq. (6) for experiments in constant electric field. Initial nitrate concentration S_0 = 100 g m^{-3}. Confidence intervals are in accordance with the Student test at confidence level of 99 %.*

Cathode potential, V/S.H.E.	V_m mmol dm^{-3} h^{-1}	K'_m mmol dm^{-3}	V_p mmol dm^{-3} h^{-1}	K_i mmol dm^{-3}
No electric field	3.2 ± 0.1	0.040 ± 0.002	0.050 ± 0.02	1.34
–0.11	4.2 ± 0.1	0.064 ± 0.003	0.050 ± 0.01	1.11
–0.060	4.4 ± 0.2	0.08 ± 0.01	0.050 ± 0.01	1.12
–0.010	5.7 ± 0.1	0.49 ± 0.03	0.050 ± 0.01	1.12
0.010	8.6 ± 0.1	0.050 ± 0.004	7.1 ± 0.1	1.85
0.060	7.0 ± 0.8	0.110 ± 0.02	0.02 ± 0.02	5.4
0.095	6.1 ± 0.3	0.040 ± 0.007	0.12 ± 0.02	5.4
0.140	5.7 ± 1.0	0.060 ± 0.01	0.10 ± 0.01	5.4

Modeling of denitrification by immobilized cells in constant electric field

The values of the estimated parameters of the kinetics (1) for immobilized cells at different cathode potentials are shown in Table 2. The modeling has shown that moderate substrate inhibition described by the model of Aiba *et al.*, cf. eq. (2b) is present. The maximum rate V_{max} for immobilized bacterial cells remains almost constant for different inlet nitrate concentrations: V_{max} = 56.4 ± 5 g m^{-3} h^{-1} or 0.91 mmol dm^{-3} h^{-1}. Of course, the maximum reaction rate depends on the concentration of cells attached to the solid support and on the related nitrate uptake coefficient for nitrate reduction.

Table 2 – *Kinetic parameters estimated from the experimental data with immobilized cells in CSTR for S_0 = 100 g m^{-3} by the Aiba model, eq. (2b)*

Cathode potential, V/S.H.E.	V_{max} mmol dm^{-3} h^{-1}	K'_m mmol dm^{-3}	K_i mmol dm^{-3}
No electric field	0.91 ± 0.09	0.02 ± 0.01	3.5
–0.06	1.3 ± 0.2	0.05 ± 0.02	1.5
–0.015	1.19 ± 0.02	0.07 ± 0.01	2.0
0.0095	1.43 ± 0.02	0.08 ± 0.01	1.7
0.02	1.06 ± 0.01	0.97 ± 0.03	3.2

As may be seen in this case, the positive effect of the constant electric field was present as well. However, it was not as strongly pronounced for the maximum reaction rate as it was for the crude cell-free extract, see Table 1 and 2. Moreover, there was an increase in the Michaelis constant compared to the reference experiment.

Comparison of the experimental values and the model ones for continuous process with electric field application are shown in Fig. 4. For reference, a theoretical curve with Michaelis-Menten kinetics without substrate inhibition was plotted also. It is evident that the positive effect of electric field can compensate the substrate inhibition with the attained higher maximum reaction rate and enable higher dilution rates at equal outlet substrate concentrations *S*.

Discussion

The presented data with the mathematical modeling showed that there was an obvious effect of the constant electric field on the denitrification either by immobilized microbial cells of *Pseudomonas denitrificans*, or by cell-free preparation from disintegrated microbial culture. Comparison of the data for cell-free extract and for microbial cells showed that the bioelectrochemical stimulation was more pronounced for the enzyme process than for the one with intact or immobilized cells. Furthermore, the electric field stimulated further reduction of the produced nitrite ions, thus reducing their inhibition effect on the overall process.

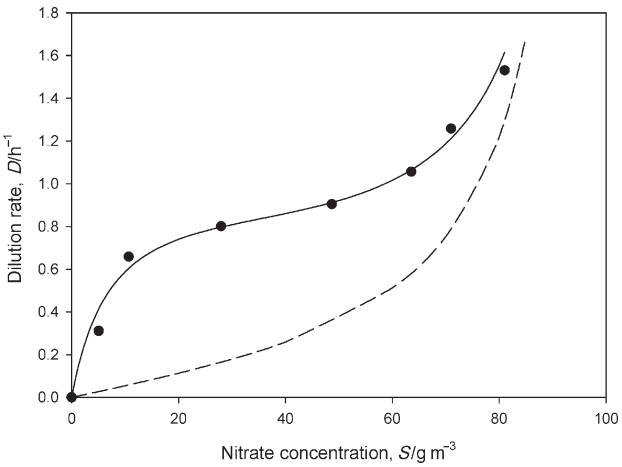


Fig. 4 – *Comparison of the experimental values (●) and the model ones (solid line) for continuous process with immobilized cells with electric field application. Cathode potential: 0.0095 V/S.H.E. Inlet nitrate concentration – 100 g m^{-3}. Parameter values are in Table 2. Dashed line – Michaelis-Menten kinetics without substrate inhibition ($K_i \rightarrow \infty$) with V_{max} = 0.91 mmol dm^{-3} h^{-1} and K'_m = 0.02 mmol dm^{-3}.*

One possible explanation of this effect is the nitrate and nitrite reduction by the released hydrogen due to electrode process. However, the current efficiency is about three orders of magnitude less than the expected stoichiometric one for purely electro-chemical process of hydrogen production. It is reasonable to assume direct electron transfer on the cathode since the maximum effect is observed at 0.01 V/S.H.E., which coincides with the standard redox potential of the couple nitrate/nitrite for the electrode reaction:

$$NO_3^- + H_2O + 2e^- \rightarrow NO_2^- + 2OH^- \qquad (10)$$

However, the observed current efficiency was three orders of magnitude lower than the expected stoichiometric one. Next, control experiments of direct electro-chemical nitrate reduction in absence of enzyme or microbial cells showed considerably (twice as less) lower denitrification rates.[8] Moreover, the estimated maximum nitrate reduction rate was considerably lower at more negative cathode potentials (see Table 1).

Another explanation could be sought in the electrochemical cofactor regeneration. Similar results have been reported recently[4] and long ago.[33,34]

Some data indicate NAD^+/NADH – dependent nitrate-reductase EC 1.7.1.1 with a redox optimum of –0.05 V/S.H.E.[35] However, the very low current efficiencies observed in our experiments are against this explanation, because we should expect stoichiometric values as above.

We can state only that the enzymes capable of converting nitrate to nitrite and further are very sensitive to the constant electric field, and that there is an obvious stimulating effect on the denitrification process even when no living cells are in the reacting solution. More precise targeted experiments coupled with additional measurements are required for clarification of the observed effect from the viewpoint of biophysics and enzymology.

Conclusions

Two main conclusions can be drawn from the experimental data and the mathematical modeling:

1. The effect of nitrate enzyme reduction stimulated by constant electric field in a potentiostatic regime is inherent to the constitutive enzymes nitrate reductases and nitrite reductase of the strain *Pseudomonas denitrificans* which are membrane and periplasmic bound.

2. It is shown by the mathematical modeling that there is a cathode potential E = +0.01 V/S.H.E where the electric field has the strongest impact on the enzyme nitrate and nitrite reduction process. There is no reasonable explanation of this effect in terms of direct electro-chemical reduction, hydrogen release or cofactor regeneration, because of the extremely low current efficiencies compared to the stoichiometric ones.

Further efforts for clarification of the observed effect will be concentrated on the study of isolated and purified enzymes under well defined conditions.

List of symbols

D – dilution rate (reciprocal to the hydraulic retention time), h^{-1}

K_i – inhibition constant, g m^{-3}, mmol dm^{-3}

K'_m – Michaelis constant, g m^{-3}, mmol dm^{-3}

K_{mP} – Michaelis constant for nitrite degradation, eq. (4), kg m^{-3}, mmol dm^{-3}

M – equivalent mass of nitrate or nitrite ions, g

P – product (nitrite) concentration, g m^{-3}, mmol dm^{-3}

S – substrate (nitrate) concentration, g m^{-3}, mmol dm^{-3}

t – time, h

V_m – maximum reaction rate for nitrate reduction, g m^{-3} h^{-1}, mmol dm^{-3} h^{-1}

V_p – maximum reaction rate for nitrite reduction, g m^{-3} h^{-1}, mmol dm^{-3} h^{-1}

Subscripts

0 – initial concentrations

cr – critical product concentration, beyond which no process is possible

i – inhibition constant

inlet – inlet substrate concentration

max – maximum values

p – values related to the product

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