

High cell density cultivation of the chemolithoautotrophic bacterium *Nitrosomonas europaea*

Benedek Papp¹ · Tibor Török² · Erzsébet Sándor³ · Erzsébet Fekete¹ · Michel Flipphi¹ · Levente Karaffa¹

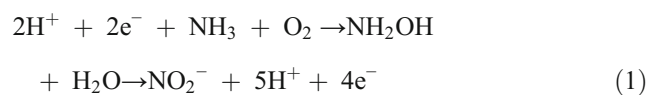
Received: 16 February 2015 / Accepted: 2 September 2015 / Published online: 10 September 2015
© Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i. 2015

Abstract *Nitrosomonas europaea* is a chemolithoautotrophic nitrifier, a gram-negative bacterium that can obtain all energy required for growth from the oxidation of ammonia to nitrite, and this may be beneficial for various biotechnological and environmental applications. However, compared to other bacteria, growth of ammonia oxidizing bacteria is very slow. A prerequisite to produce high cell density *N. europaea* cultures is to minimize the concentrations of inhibitory metabolic by-products. During growth on ammonia nitrite accumulates, as a consequence, *N. europaea* cannot grow to high cell concentrations under conventional batch conditions. Here, we show that single-vessel dialysis membrane bioreactors can be used to obtain substantially increased *N. europaea* biomasses and substantially reduced nitrite levels in media initially containing high amounts of the substrate. Dialysis membrane bioreactor fermentations were run in batch as well as in continuous mode. Growth was monitored with cell concentration determinations, by assessing dry cell mass and by monitoring ammonium consumption as well as nitrite formation. In addition, metabolic activity was probed with in vivo acridine orange

staining. Under continuous substrate feed, the maximal cell concentration (2.79×10^{12} /L) and maximal dry cell mass (0.895 g/L) achieved more than doubled the highest values reported for *N. europaea* cultivations to date.

Introduction

Nitrifying bacteria such as *Nitrosomonas europaea* can use ammonia as their sole source of energy. The carbon required for growth and anabolism is obtained by carbon dioxide fixation (see for a recent review: Monteiro et al. 2014). Nitrification in *N. europaea* involves two conversions catalyzed by ammonia monooxygenase and hydroxylamine oxidoreductase, respectively (conveniently reviewed by Arp et al. 2002).



The two electrons released [1] serve as the reducing equivalents driving growth.

Ammonia oxidizers are difficult to grow in the laboratory. Growth of *N. europaea* is slow, with specific growth rates in the range of 0.4–1/day, with generation times reportedly between 8 and 24 h (Sato et al. 1985). In addition, several factors (e.g., light or salts comprising sulfate or multivalent metal cations) may have a negative influence on biomass formation (Hyman and Arp 1992; Yan et al. 2010; Park and Ely 2008). Nevertheless, chemolithoautotrophic nitrifying bacteria are essential components of activated sludge used in domestic or industrial wastewater treatment systems (Terada et al. 2013). Their ammonia monooxygenase is also capable of oxidizing many non-physiological substrates, like halogenated

Electronic supplementary material The online version of this article (doi:10.1007/s12223-015-0425-8) contains supplementary material, which is available to authorized users.

✉ Levente Karaffa
karaffa.levente@science.unideb.hu

- ¹ Department of Biochemical Engineering, Faculty of Science and Technology, University of Debrecen, H-4032, Egyetem tér 1, Debrecen, Hungary
- ² Safety and Environmental Department, TEVA Pharmaceutical Ltd., H-4032, Pallagi u. 13, Debrecen, Hungary
- ³ Institute of Food Science, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, H-4032, Böszörményi út 138, Debrecen, Hungary

hydrocarbons and certain aromatic compounds, suggesting a much sought after potential in a variety of biotechnological applications (for a review, see Arp and Stein 2003). However, to be practicable at the industrial scale, one needs to produce and operate viable, high cell density cultures.

In simple batch cultures (e.g., serum bottles, Erlenmeyer flasks), the metabolism of *N. europaea* constantly decreases the pH (see Eq. (1)) and consequently the amount of free ammonia (NH_3) available in the medium (beyond its consumption). While this effect can be counterbalanced by applying external pH control, the principal inhibitory metabolite, nitrite, continues to accumulate, inhibiting growth and limiting cell concentration (Tan et al. 2008). Fed-batch and continuous cultures of *N. europaea*, with or without biomass recycling, were also reported (Tappe et al. 1999; Güven and Schmidt 2009; Yingling and Zhengfang 2013). The highest cell concentrations reported to date—using systems retaining the biomass—were about 400 mg/L dry cell mass (DCW) (Tappe et al. 1996; Chapman et al. 2006).

Ultrafiltration and microfiltration are frequently used to remove low molecular mass, inhibitory compounds from the growth medium, but the necessary hydraulic flux often results in membrane fouling, particularly at higher cell concentrations (Fuchs et al. 2002). In contrast, single-vessel dialysis fermentors are very efficient in continuously removing low molecular mass by-products during cultivations, while biomass can be supplied concurrently with substrate (Märkl et al. 1990). Dialysis mass transfer does not require hydraulic flow across the membrane; hence, fouling will not occur swiftly. Using a dialysis membrane reactor, *Escherichia coli* could be grown to cell concentrations as high as 174 g/L DCW (Märkl et al. 1993). Prokaryotes with typically low biomass yields in fermentations, such as the hyperthermophile, anaerobic Archaea *Pyrococcus furiosus*, the thermoacidophile *Sulfolobus shibatae*, and the halophile isolate *Marinococcus* M52, could also be grown to substantially higher cell concentrations using this cultivation strategy (Krahe et al. 1996). Furthermore, the system was suitable to obtain high cell density mammalian cell cultures (Kurosawa et al. 1991; Pörtner et al. 1992).

In this work, we demonstrate the conduciveness of the dialysis membrane reactor technology in obtaining high cell density *N. europaea* cultures, compared to more conventional fermentation systems.

Materials and methods

Microorganism and cultivation conditions

N. europaea Winogradsky (ATCC 19718) was obtained from Dr. Daniel Arp (Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR, USA). Strain maintenance and basic cultivations (including medium preparation

and the monitoring of growth) were done as described by the “nitrification network protocol” (NNP) (<http://nitrificationnetwork.org/Nerecipe.php>). Care was taken to protect all ongoing cultures (maintenance, seed as well as bioreactors) from light; bioreactors were shielded with prefabricated covers. LB agar-based count plates were employed to regularly verify that *N. europaea* cultures were free of contaminating heterotrophic bacteria.

Minimal media for shake flask cultivations and fermentations were formulated as described in the NNP, except that the initial soluble ammonium ion concentrations (with sulfate as the anion) were set at 800 mg/L (~45 mmol/L). The medium additionally contained 29.3 mmol/L KH_2PO_4 , 3.71 mmol/L Na_2CO_3 , 3.93 mmol/L NaH_2PO_4 , 0.73 mmol/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.19 mmol/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 mmol/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.49 mmol/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Shake flask cultures (100-mL medium in 500-mL Erlenmeyer flasks) grown for 4 days at 120 revolutions per min (rpm) and 30 °C in a rotary shaker were used as inoculum (seed) cultures. Such seed cultures typically yielded an in vitro absorbance of 0.09–0.12 at $\lambda=600$ nm (henceforth referred to as A_{600}) by the time they were used as inoculum. Bioreactors were inoculated with 10 % (v/v) seed culture, and the initial A_{600} was (thus) at 0.01 ± 0.002 .

Batch fermentations were carried out in 2-L glass bioreactors (Sartorius) with a working volume of 1.5 L and equipped with a six-blade Rushton disc turbine impeller ($d_i=0.053$ m, $W_i=0.0106$ m). Operating conditions were pH 7.8, 30 °C, and 0.5 volumes of air per volume of liquid per minute (vvm). The pH was controlled with the automatic addition of 4 mol/L HCl or 4 mol/L Na_2CO_3 solutions. Dissolved oxygen (DO) levels were maintained at 20 % saturation and were controlled by means of the agitation (=stirring) rate. To minimize medium loss, the waste gas was cooled in a reflux condenser connected to an external cooling bath (4 °C) before exiting the system.

For cultivations with facilitated medium exchange, a single-vessel dialysis membrane reactor (Bioengineering), with 1.5 L total/1 L working and 5.5 L total/4.5 L working volumes for the inner and outer chambers, respectively, was used (Krahe et al. 1996; Sándor et al. 1999). The outside wall of the bioreactor was made of heavy duty, transparent polyamide with a thickness of 80 μm . A transparent, cylindrical cuprophane (regenerated cellulose) membrane (thickness 20 ± 2 μm , cutoff 10 Da, permeability coefficient for water at 20 °C 1.5×10^3 mL/m²/h/bar, as specified by the manufacturer) was placed inside the polyamide reactor, forming a closed off, inner chamber. Each chamber was independently stirred with its own six-blade Rushton disc turbine impeller ($d_i=0.040$ m, $W_i=0.008$ m). Temperature, pH, DO, aeration conditions, and means to minimize medium loss means were identical to those described above for the glass batch bioreactor. During in situ sterilization, the elastic polyamide foil was supported externally by two prefabricated, cylindrical metal shells; these were

also used as covers to shield away light during fermentations. Cells grew in the inner chamber, surrounded by the outer chamber filled with (the same) growth medium. Sampling occurred simultaneously from both the inner and outer chamber. Because of their deleterious effect on *N. europaea*, the use of organic solvents to prevent contamination during inoculation and sampling was omitted, and instead, the (metal) fittings, tubes, and ports were briefly flamed in situ with a Bunsen burner prior to each use. For graphic schemes that illustrate the layout, design, and function of the dialysis membrane fermentor employed in this study, we refer to the literature contained in Supplementary File 1.

Continuous operations in the dialysis reactor were performed by feeding fresh growth medium into the outer chamber of the reactor while concurrently draining spent medium out at the same rate, thereby keeping the culture volume constant. Substrate feeding started after approximately 80 % of the initial ammonium/ammonia had been converted.

Analytical methods

The combined medium concentration of ammonia plus ammonium ions (ammonia/ammonium-nitrogen, henceforth referred to as “Ammono-N”), the concentration of nitrite ions (“nitrite-N”) as well as that of the phosphate ions in solution were estimated with a colorimeter (Orion AQUAfast IV AQ4000, Thermo Scientific) equipped with purpose-specific cuvettes. Due to the relatively high Mg^{2+} concentrations in the growth medium (as defined at NNP, see above), a slightly amended procedure recommended by the manufacturer was used.

Total cell number was determined by direct cell count using a Bürker hemocytometer. Cell number data reported in the “Results” section are the average of one count of each of the four big squares of the gridded area (standard deviation [SD], 4.9 %). *N. europaea* cell mass was also defined as dry cell mass (DCW), determined from 10 mL culture aliquots, and acidified to pH 4 with 2 mol/L acetic acid after sampling (to solubilize any struvite crystals, see below). Biomass was subsequently harvested on a preweighted filter paper of 0.2- μ m average pore size (Millipore) by suction filtration and washed with sterile water on the filter. Then, the filter was dried at 80 °C until constant mass weight. Dry weight data are the average of two parallel measurements, which never deviated more than 21 %. In addition, cell concentrations were monitored by means of culture’s light absorbance at 600 nm (A_{600}) using a spectrophotometer (Amersham), where sterilized cell-free culture medium served as a blank.

Specific growth rates (μ , given as the reciprocal of time, e.g., 1/h or 1/day) were calculated from the increased cell count over the time elapsed between two subsequent samplings (i.e., sampling time points); the highest of the thus obtained values was taken as the maximal specific growth rate

of the culture. Generation (=doubling) time (T_d) was defined as $\ln 2/\mu$ (Pirt 1975).

The metabolic status of the cells was probed using acridine orange (AO) (Freudenberg et al. 1996), the staining performed as described earlier (Sándor et al. 2000). Samples were studied under an epifluorescent microscope (Zeiss AxioImager) equipped with AxioCam MRc5 camera at a magnification of 500 times.

The presence of struvite (magnesium ammonium phosphate hexahydrate $NH_4MgPO_4 \cdot 6H_2O$) crystals in the medium was verified by chemical analyses. The struvite particles were collected by centrifugation (20,000 rpm, 10 min) and resolubilized in deionized water. All three constituents of struvite were found present in this solution at equimolar concentrations. Phosphate and ammonium ions were quantified as described above, while magnesium (II) ions were determined by the “Titan yellow” method (Heaton 1960).

Reproducibility

All the analytical data presented are the means of three independent experiments (i.e., fermentations). Data were analyzed and visualized with SigmaPlot software (Jandel Scientific), and for each procedure, a SD was determined. In the case of fermentations with different agitation rates, the significance of changes in maximal cell concentrations and specific growth rates, relative to the values obtained at the lowest agitation rate (200 rpm), was assessed using Student’s *t* test, with probability (*p*) values given in the “Results” section.

Results and discussion

Verification of the experimental system

Kinetic analysis of *N. europaea* submerged cultures is prone to inaccuracies, mainly due to low biomass yields (see, e.g., Grady and Lim 1980; Farges et al. 2012). Therefore, we first verified the analytical tools at our disposal. Farges et al. (2012) put to evidence the existence of a strong linear correlation between culture absorbance (A_{600}) and *N. europaea* cell concentration (=cell count/mL):

$$\text{Cell concentration} = 1.010 \times 10^9 \times A_{600} \pm 0.022 \times 10^9 \quad (2)$$

The correlation between cell concentration and A_{600} data in our study was essentially identical to Eq. (2) for a wide range of cell concentrations (data not shown). We used the same *N. europaea* strain as Farges et al. (2012) and an essentially identical minimal growth medium. Notwithstanding, given the unpredictable growth patterns of this species under different environmental conditions (e.g., Grady and Williams 1975;

Prinčič et al. 1998), this correlation may not be as accurate for other *N. europaea* strains and/or different growth regimes.

We correlated our culture absorbance (A_{600}) data to dry cell mass (Fig. 1). A high correlation coefficient ($R^2=0.976$) was obtained for the full range of biomass yields that could be achieved, although SDs for DCW determinations (<21 %; see “Material and methods” section) appeared consistently higher for *N. europaea* than what we usually observe for (eukaryotic) microbial cultures with bigger cell size and/or with mycelial morphology (for comparison, see, e.g., Jónás et al. 2014: <14 %). In practical terms, the trilateral correlations for *N. europaea* meant that the initial turbidity of the fermentations (i.e., for medium plus inoculum at time point zero), $A_{600}\sim 0.01$, corresponded to a cell concentration of approximately 10^7 /mL and a 2.72 mg/L DCW, respectively. Assuming a wet/dry cell mass ratio of 5 (Krahe et al. 1996), our values were proportional to the 0.1–0.2 g/L wet mass at $A_{600}\sim 0.1$ given at the NNP website. A conversion factor within the same order of magnitude (6.3×10^{12} cells for 1 g of DCW) was obtained by Farges et al. (2012).

DO levels were controlled with the agitation rate. A series of fermentations at different agitation rates were first performed to assess the sheer rate effect generated by the impeller on the growth kinetics of *N. europaea* cultures. Sheer rate is a linear function of the impeller speed (Sanchez Pérez et al. 2006). Without investigating the underlying (biological) mechanisms, we found that agitation rates between 200 and 800 rpm did not influence the maximal cell concentration ($p<0.1$ %) or the maximal specific growth rate ($p<0.05$ %). Agitation rates between 800 and 1300 rpm decreased progressively the growth rate ($p<0.1$ %), while stirring at higher than 1300 rpm resulted in progressive reduction of both parameters ($p<0.1$ %; Fig. 2). The maximal sheer rate at 1300 rpm—

calculated after Bowen (1986)—was 3.740/s. Agitation rates in the glass bioreactor (batch cultures) did not exceed 350 rpm while DO was kept at 20 % saturation. In agreement with Yu and Chandran (2010), DO levels elevated up to 90 % saturation although increased stirring rates in the glass reactor did not influence growth kinetics. By contrast, in the dialysis membrane reactor, the highest cell density was achieved at an agitation rate of 800–820 rpm (data not shown), i.e., at those speeds where the maximal growth rate started to progressively decline in the batch fermentation reactor.

In the early stages of cultivations (up until 12–14 h), crystals identified as struvite were formed (Supplementary Fig. S1). Based on the concentration of magnesium (II) ions (the limiting component of struvite in the growth medium at 0.75 mmol/L), the solubility of struvite in water (Bhuiyan et al. 2007), and the calculated difference between calibrated and measured Ammo-N concentrations, maximal amount of struvite formed was estimated at 800 mg/L, out of which roughly 200 mg/L remained dissolved. The rest likely precipitated and thus could potentially interfere with DCW determinations. Samples were therefore acidified to pH 4 to solubilize struvite prior to DCW measurements. Indeed, at this acidic pH, medium aliquots were visually devoid of struvite crystals (i.e., none were seen upon microscopic inspection).

To resume our assessment of the available analytical tools, while SDs for DCW determinations—particularly at lower cell concentrations—were relatively high, they unlikely affected the general experimental strategy we wished to employ in continuation. We likewise expect that the sheer rate elicited by mechanical agitation or the struvite crystal formation in the growth medium would not cause aberrations under our established experimental conditions. We thus considered the experimental system appropriate for the purposes of this study.

Conventional batch fermentation of *N. europaea*

Initial soluble ammonium ion concentrations in the growth medium were set at 800 mg/L (~45 mmol/L), roughly corresponding to the average NH_4^+ concentration in the primary (untreated) wastewater generated in the Debrecen plant of the TEVA Pharmaceutical Ltd. The pK_a value of ammonia (i.e., the pH at which the concentration of ammonia equals that of ammonium) is 9.25. Consequently, at medium pH 7.8—consistently used throughout this work—the ratio of ammonium ions to molecular ammonia is about 25:1, i.e., the relative ammonia concentration (<2 mmol/L) in the medium was low (Schmidt et al. 2004). The pH of the *N. europaea* cytoplasm is about 6.8–7.2 (Hollocher et al. 1982; Kumar and Nicholas 1983). To meet the K_M value (i.e., the concentration at which the reaction rate is half the maximum (V_{\max}) at infinite substrate concentration under Michaelis-Menten kinetics) for ammonia oxidation (estimated at 20 $\mu\text{mol/L}$; Wood 1986),

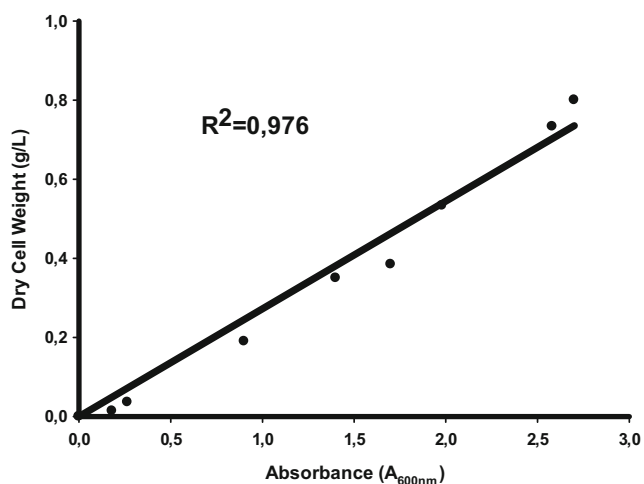
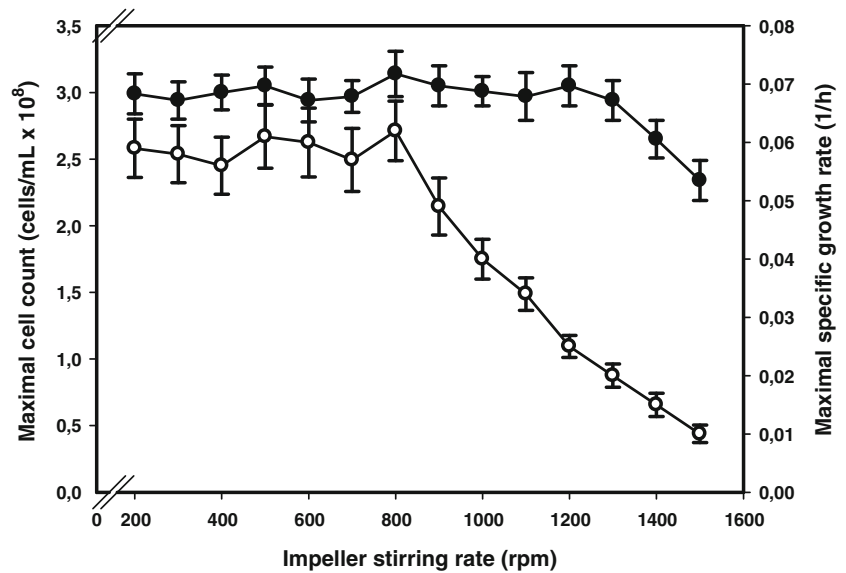


Fig. 1 Correlation between dry cell mass (DCW) and turbidity of *N. europaea* cultures measured at $\lambda=600$ nm (A_{600}). Samples with an absorbance of over 0.4 were diluted with sterilized medium. Turbidity data were adjusted with the dilution factor

Fig. 2 Maximal cell concentration (filled symbols) and maximal specific growth rate (empty symbols) as a function of the impeller stirring rate in *N. europaea* cultures grown batchwise in a conventional bioreactor



the intracellular ammonium concentration should range between 2 and 5 mmol/L. Since cell membranes are highly permeable for ammonia by means of diffusion (backed up by an efficient active transport of the ammonium ion; Schmidt et al. 2004), the initial ammonium concentration was likely high enough to drive fast ammonia oxidation and ditto growth of *N. europaea*. By its own standards, *N. europaea* indeed grew relatively fast in our bench-scale batch fermentors (Table 1): The values we obtained resemble the maximal cell count of $1.5\text{--}2 \times 10^8/\text{mL}$ reported by Yu and Chandran (2010). In the growth profile of the culture acceleration, exponential and stationary phases could be distinguished; the latter phase appears to be reached rather abruptly, without a clear phase of progressive deceleration (Fig. 3). Cultures grew till approximately 80 h and attained a maximal cell count of $2 \times 10^8/\text{mL}$ (~ 54 mg/L DCW), corresponding to a 20 times increase compared to the zero time point.

Time profile of the residual Ammo-N in the medium mirrored that of growth, i.e., the initial amount of roughly 45 mmol/L was almost depleted in 80 h (Fig. 3). Assuming that proteins represent around 50 % of the dry mass of a microorganism (Stephanopoulos et al. 1998),

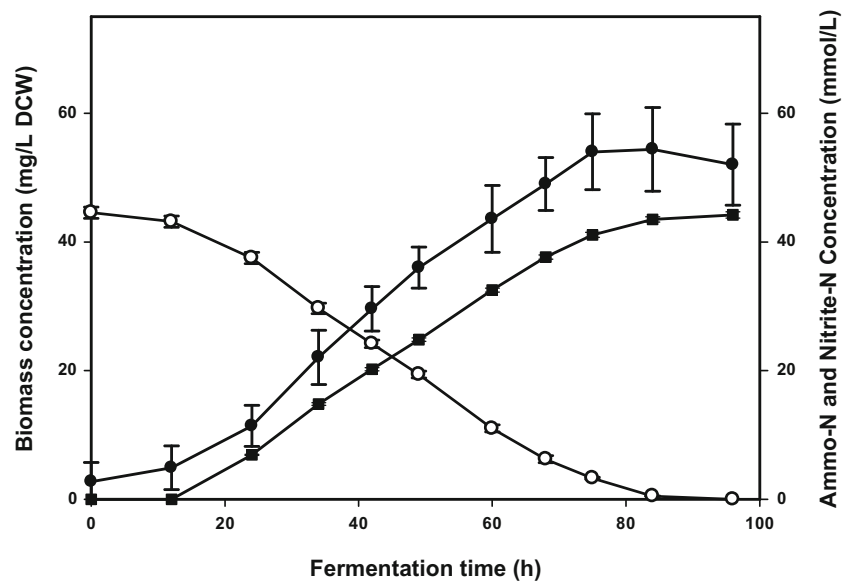
the highest ammonium uptake rate—calculated from the residual ammonium concentration data—was estimated at 1.5 ± 0.3 mmol/g DCW/h at the mid-exponential phase (Fig. 3). This rate is of the same order as previously reported by Schmidt et al. (2004): 0.075–0.079 mmol/g protein/min (i.e., $\sim 2.25\text{--}2.37$ mmol/g DCW/h). As expected, nitrite-N was formed stoichiometrically from Ammo-N consumption, reaching a final concentration of about 44 mmol/L (Fig. 3).

Viability of cells is of considerable concern when one deals with slow-growing cultures/organisms. AO staining enables to probe cellular metabolic activity in a visual, quantitative, reproducible, and fast way (McFeters et al. 1991). AO interacts differently with double- and single-stranded polynucleotides, fluorescing green and red, respectively (McMaster and Carmichael 1977). In brief, actively growing cells containing high messenger RNA (mRNA) levels appear orange-red, while cells with low mRNA levels stain green. AO staining reflected the growth profile of the cultures, i.e., cells displayed an overwhelmingly redish fluorescence in the exponential growth phase and a yellow-greenish one in the later, stationary stage (Supplementary Fig. S2).

Table 1 Key kinetic parameters of *N. europaea* cultures using different cultivation methods

Growth parameters of cultures	Method of cultivation		
	Conventional batch	Dialysis, batch mode	Dialysis, continuous mode (5 L/h flux)
Maximal cell concentration (mg/L)	54±5.8	258.4±21.9	895±75.6
Maximal cell count (cells×10 ⁸ /mL)	2±0.04	9.4±0.06	32.4±0.81
Maximal specific growth rate (1/h)	0.067±0.006	0.094±0.009	0.102±0.01
Shortest doubling time (h)	10.36±1.02	7.35±0.87	6.79±0.75

Fig. 3 Time profile of growth (filled circle) as well as residual Ammo-N (empty circle) and nitrite-N (square) concentrations in a conventional batch fermentation of *N. europaea*



Dialysis fermentations of *N. europaea*

To improve growth yield, *N. europaea* was cultivated in the dialysis reactor using cuprophane membrane through which only components with a molecular mass smaller than 10,000 can pass. Permeability depends on the molecular mass; thus, all principal components of the growth medium including ammonium and nitrite moved practically unhindered between the two chambers. As a consequence, while bacterial growth was restricted to the inner chamber, it was supported by the combined nutrients in the internal and external chambers. Moreover, the principal inhibitory metabolite nitrite was continuously diluted down in the inner chamber to an extent that depended on the prevalent concentration gradient over the membrane.

In qualitative terms, time profiles for cell growth, ammonium consumption, and nitrite formation were similar to those attained in the conventional glass fermentor (data not shown). AO staining time profile (visualizing cellular metabolic activity) was likewise essentially identical. Quantitatively, however, all growth parameters were significantly higher in dialysis fermentation (Table 1). Maximal cell density was almost 100-fold increased compared to the zero time point (i.e., the inoculum) and was collaborated by the increased ammonium uptake rate, estimated at 2.8 ± 0.6 mmol/g DCW/h at the most rapid phase of growth (between 60 and 80 h of the fermentation).

Nitrite is toxic to *N. europaea* (for a review: Stein and Arp 2003), with reported thresholds varying from 12 and >30 mmol/L (Painter 1970; Yu and Chandran 2010). Accordingly, the peak nitrite concentration in our batch fermentations (~ 44 mmol/L; see above) could well be high enough to limit further growth if more ammonium would be supplied to the system.

To assess this hypothesis, a dialysis fermentation strategy involving continuous dilution of the outer chamber with fresh medium was applied, with medium fluxes at flow rates from 1 through 5 L/h. Under such conditions, the nitrite concentrations remained below 5 mmol/L (in both chambers) throughout this set of fermentations (data not shown). The exponential phase of growth was proportionally prolonged with increasing flux, the rate at which the dialysate in the outer chamber was exchanged, also when visualized by AO staining (not shown). As a consequence, maximal specific growth rates did not significantly ($p < 0.1\%$) increase with the maximal cell concentration (Table 1). On the contrary, the maximal cell concentration was proportional to the flow rate applied (Fig. 4). At the highest dialysate flow rate tested, 5 L/h, the mean biomass yield was close to 900 mg/L. This represents a >16 -fold increase relative to conventional batch fermentations (Table 1) and doubles the highest values reported in literature for

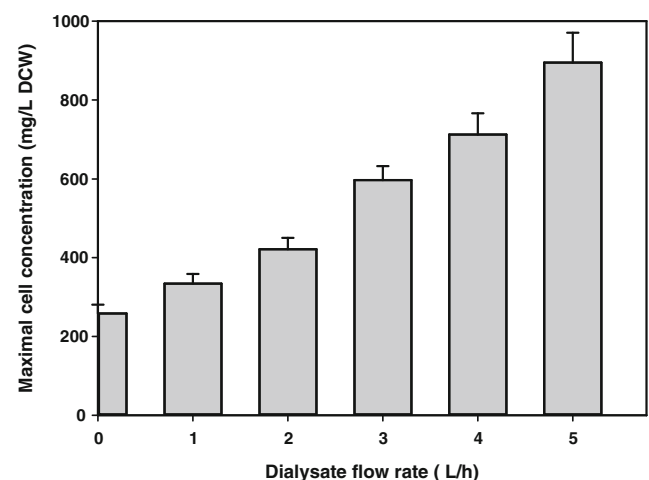


Fig. 4 Effect of the rate at which the dialysate in the outer chamber was exchanged on the maximal cell concentration of *N. europaea*

N. europaea (Tappe et al. 1996; Chapman et al. 2006). This substantial increase may either be due to the removal of nitrite from the biomass and/or to the continuous supply of (fresh) ammonium for energy, growth, and anabolism. Further experiments with altered ammonium input would be needed to distinguish the two potential effects.

Due to its geometry and construction, the type of dialysis membrane bioreactor used in this study is available at laboratory scale only. However, technical-scale dialysis applications employing external membrane modules with minimal residence time and the provision of oxygen are readily commercially available (Fuchs et al. 2002). Using *E. coli*, these fermentation systems proved to be as efficient as their lab-scale counterparts in obtaining high cell concentrations. It remains to be seen if such scaled-up dialysis bioreactor could also be employed for the production of viable *N. europaea* cultures with high biomass yield.

Conclusions

In this work, we have demonstrated a methodological improvement of the cultivation of the slow-growing nitrifying bacterium *N. europaea* that resulted in significantly increased final cell concentrations. In their natural environment, soil-borne nitrifying bacteria have to cope with low nutrient concentrations. By contrast, industrial wastewaters typically contain high amounts of ammonium, and its conversion to nitrite may limit the growth of the very microorganisms performing it. Our work showed that *N. europaea* can nevertheless be grown to relatively high cell concentrations (up to 900 mg/L DCW) in the presence of ammonium at concentrations comparable of that in industrial wastewater by employing continuous fermentation technology coupled with medium dialysis.

Acknowledgments The research was supported by the EU and cofinanced by the European Social Fund under the project ENVIKUT (TÁMOP-4.2.2.A-11/1/KONV-2012-0043) and by the Hungarian Scientific Research Fund (OTKA K1006600 and NN116519). The authors are grateful to Zoltán Németh, Zoltán Fekete, Antal Kökényesi, Csilla Noémi Lipták, and István Kolláth for their help. We thank József Kozma (Gedeon Richter Plc, Budapest, Hungary) for critically reading the manuscript.

References

- Arp DJ, Stein LY (2003) Metabolism of inorganic N compounds by ammonia-oxidizing bacteria. *Crit Rev Biochem Mol Biol* 38:471–495
- Arp DJ, Sayavedra-Soto LA, Hommes NG (2002) Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. *Arch Microbiol* 178:250–255
- Bhuiyan MI, Mavinic DS, Beckie RD (2007) A solubility and thermodynamic study of struvite. *Environ Technol* 28:1015–1026
- Bowen R (1986) Unraveling the mysteries of shear-sensitive mixing systems. *Chem Eng* 9:55–63
- Chapman BD, Schleicher M, Beuger A, Gostomski P, Thiele JH (2006) Improved methods for the cultivation of the chemolithoautotrophic bacterium *Nitrosomonas europaea*. *J Microbiol Methods* 65:96–106
- Farges B, Poughon L, Roriz D, Creuly C, Dussap C-G, Lasseur C (2012) Axenic cultures of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* in autotrophic conditions: a new protocol for kinetic studies. *Appl Biochem Biotechnol* 167:1076–1091
- Freundenberg S, Fasold K-I, Müller SR, Siedenberg D, Kretzmer G, Schügerl K, Giuseppin M (1996) Fluorescent microscopic investigation of *Aspergillus awamori* growing on synthetic and complex media and producing xylanase. *J Biotechnol* 46:265–273
- Fuchs C, Köster D, Wiebusch S, Mahr K, Eisbrenner G, Märkl H (2002) Scale-up of dialysis fermentation for high cell density cultivation of *Escherichia coli*. *J Biotechnol* 93:243–251
- Grady CPL Jr, Williams DR (1975) Effects of influent substrate concentration on the kinetics of natural microbial population in continuous culture. *Water Res* 9:171–180
- Grady CPL Jr, Lim HC (1980) Biological wastewater treatment, theory and applications. Marcel Dekker, New York, pp 291–299
- Güven D, Schmidt I (2009) Specific activity and viability of *Nitrosomonas europaea* during discontinuous and continuous fermentation. *Proc Biochem* 44:516–520
- Heaton FW (1960) Determination of magnesium by the Titan yellow and ammonium phosphate methods. *J Clin Pathol* 13:358–360
- Hollocher TC, Kumar S, Nicholas DJD (1982) Respiration-dependent proton translocation in *Nitrosomonas europaea* and its apparent absence in *Nitrobacter agilis* during inorganic oxidations. *J Bacteriol* 149:1013–1020
- Hyman MR, Arp DJ (1992) $^{14}\text{C}_2\text{H}_2$ - and $^{14}\text{CO}_2$ -labeling studies of the *de novo* synthesis of polypeptides by *Nitrosomonas europaea* during recovery from acetylene and light inactivation of ammonia monooxygenase. *J Biol Chem* 267:1534–1545
- Jónás Á, Fekete E, Flippi M, Sándor E, Jäger S, Molnár ÁP, Szentirmai A, Karaffa L (2014) Extra- and intracellular lactose catabolism in *Penicillium chrysogenum*: phylogenetic and expression analysis of the putative permease and hydrolase genes. *J Antibiot* 67:489–497
- Krahe M, Antranikian G, Märkl H (1996) Fermentation of extremophilic microorganisms. *FEMS Microbiol Rev* 18:271–285
- Kumar S, Nicholas DJD (1983) Proton electrochemical gradients in washed cells of *Nitrosomonas europaea* and *Nitrobacter agilis*. *J Bacteriol* 154:65–71
- Kurosawa H, Märkl H, Niebuhr-Redder C, Matsumura M (1991) Dialysis bioreactor with radial-flow fixed bed for animal cell culture. *J Ferment Bioeng* 1:41–45
- Märkl H, Lechner M, Götz F (1990) A new dialysis fermentor for the production of high concentrations of extracellular enzymes. *J Ferment Bioeng* 69:244–249
- Märkl H, Zenneck C, Dubach AC, Ogbonna JC (1993) Cultivation of *Escherichia coli* to high cell densities in a dialysis reactor. *Appl Microbiol Biotechnol* 39:48–52
- McPeters A, Singh S, Byun PR, Callis S, Williams (1991) Acridine orange staining reaction as an index of physiological activity in *Escherichia coli*. *J Microbiol Methods* 13:87–97
- McMaster GK, Carmichael GG (1977) Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc Natl Acad Sci U S A* 74:4835–4838
- Monteiro M, Seneça J, Magalhães C (2014) The history of aerobic ammonia oxidizers: from the first discoveries to today. *J Microbiol* 52: 537–547
- Painter HA (1970) A review of literature on inorganic nitrogen metabolism in microorganisms. *Water Res* 4:393–450

- Park S, Ely RL (2008) Candidate stress genes of *Nitrosomonas europaea* for monitoring inhibition of nitrification by heavy metals. *Appl Environ Microbiol* 74:5475–5482
- Pirt SJ (1975) Principles of microbe and cell cultivation. Blackwell Scientific Publications, Oxford, UK, pp 156–170
- Pörtner R, Bohmann A, Märkl H (1992) Membrane dialysis bioreactor with integrated radial-flow fixed bed—a new concept for continuous cultivation of animal cells. In: Murakami H, Shirahata S, Tachibana, H (eds) *Animal Cell Technology: Basic and Applied Aspects*, Vol.4. Proceedings of the Fourth Annual Meeting of the Japanese Association for Animal Cell Technology, Fukuoka, Japan, 13–15 November 1991. Kluwer Academic Publisher, pp 217–223
- Prinčič A, Mahne I, Megušar F, Paul EA, Tiedje JM (1998) Effects of pH and oxygen and ammonium concentrations on the community structure of nitrifying bacteria from wastewater. *Appl Environ Microbiol* 64:3584–3590
- Sanchez Pérez JA, Rodríguez Porcel EM, Casas López JL, Fernández Sevilla JM, Chisti Y (2006) Shear rate in stirred tank and bubble column bioreactors. *Chem Eng J* 124:1–5
- Sándor E, Karaffa L, Paul GC, Pócsi I, Thomas CR, Szentirmai A (2000) Assessment of the metabolic activity of *Acremonium chrysogenum* using acridine orange. *Biotechnol Lett* 22:693–697
- Sándor E, Szentirmai A, Biró S, Karaffa L (1999) Specific cephalosporin C production of *Acremonium chrysogenum* is independent of the culture density. *Biotechnol Technol* 13:443–445
- Sato C, Schnoor JL, McDonald DB, Huey J (1985) Test medium for the growth of *Nitrosomonas europaea*. *Appl Environ Microbiol* 49:1101–1107
- Schmidt I, Look C, Bock E, Jetten MSM (2004) Ammonium and hydroxylamine uptake and accumulation in *Nitrosomonas*. *Microbiology* 150:1405–1412
- Stein LY, Arp DJ (2003) Loss of ammonia monooxygenase activity in *Nitrosomonas europaea* upon exposure to nitrite. *Appl Environ Microbiol* 64:4098–4102
- Stephanopoulos G, Aristidou A, Nielsen J (1998) *Metabolic engineering: principles and methodologies*. Academic Press, San Diego, CA, USA
- Tan NCG, Kampschreur MJ, Wanders W, van der Pol WLJ, van de Vossenberg J, Kleerebezem R, van Loosdrecht MCM, Jetten MSM (2008) Physiological and phylogenetic study of an ammonium-oxidizing culture at high nitrite concentrations. *Syst Appl Microbiol* 31:114–125
- Tappe W, Laverman A, Bohland M, Braster M, Rittershaus S, Groeneweg J, van Verseveld HW (1999) Maintenance energy demand and starvation recovery dynamics of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* cultivated in a retentostat with complete biomass retention. *Appl Environ Microbiol* 65:2471–2477
- Tappe W, Tomaszewski C, Rittershaus S, Groeneweg J (1996) Cultivation of nitrifying bacteria in the retentostat, a simple fermenter with internal biomass retention. *FEMS Microbiol Ecol* 19:47–52
- Terada A, Sugawara S, Yamamoto T, Zhou S, Koba K, Hosomi M (2013) Physiological characteristics of predominant ammonia-oxidizing bacteria enriched from bioreactors with different influent supply regimes. *Biochem Eng J* 79:153–161
- Wood PM (1986) Nitrification as a bacterial energy source. In: Prosser JI (ed) *Nitrification*. IRL Press, Oxford, UK, pp 39–62
- Yan J, Jetten M, Rang J, Hu Y (2010) Comparison of the effects of different salts on aerobic ammonia oxidizers for treating ammonium-rich organic wastewater by free and sodium alginate immobilized biomass system. *Chemosphere* 81:669–673
- Yingling B, Zhengfang Y (2013) Application of an integrated statistical design for optimization of culture condition for ammonium removal by *Nitrosomonas europaea*. *PLoS ONE* 8:e60322
- Yu R, Chandran K (2010) Strategies of *Nitrosomonas europaea* 19718 to counter low dissolved oxygen and high nitrite concentrations. *BMC Microbiol* 10:70