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56 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.

57	Keywords separated by ' - '	
58	Foot note information	The online version of this article (doi:10.1007/s12223-015-0425-8) contains supplementary material, which is available to authorized users.

Electronic supplementary material

ESM 1
(PDF 26 kb)

Fig. S1
Microscopic image of struvite crystals formed in the early stages of *N. europaea* fermentations. For technical details, see Materials and methods section. (PDF 43 kb)

Fig. S2
Microscopic images of *N. europaea* cells stained with Acridine Orange at 20 (image 1.), 40 (2.), 70 (3.) and 80 h (4.) of the conventional batch fermentation presented in Fig. 3. For technical details, see Materials and methods section. (PDF 85 kb)

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

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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.

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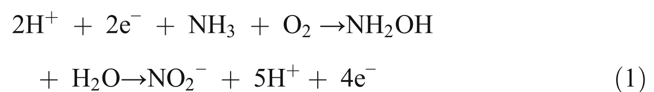
Introduction

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).



$$2H^+ + 2e^- + NH_3 + O_2 \rightarrow NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (1)$$

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

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 $MgSO_4 \cdot 7H_2O$, 0.19 mmol/L $CaCl_2 \cdot 2H_2O$, 0.01 mmol/L $FeSO_4 \cdot 7H_2O$, and 0.49 mmol/L $CuSO_4 \cdot 5H_2O$. 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 λ=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/m^2/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

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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 “Ammo-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-μm 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 ln2/μ (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;

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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}~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}~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.

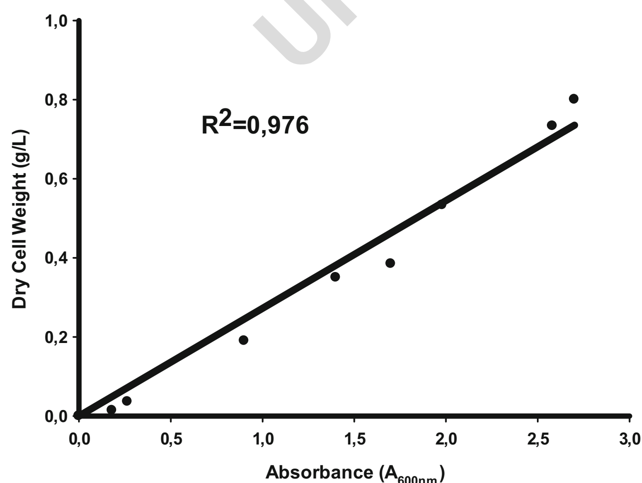

Fig. 1 Correlation between dry cell mass (DCW) and turbidity of *N. europaea* cultures measured at λ=600 nm (A_{600}). Samples with an absorbance of over 0.4 were diluted with sterilized medium. Turbidity data were adjusted with the dilution factor

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 μmol/L; Wood 1986),

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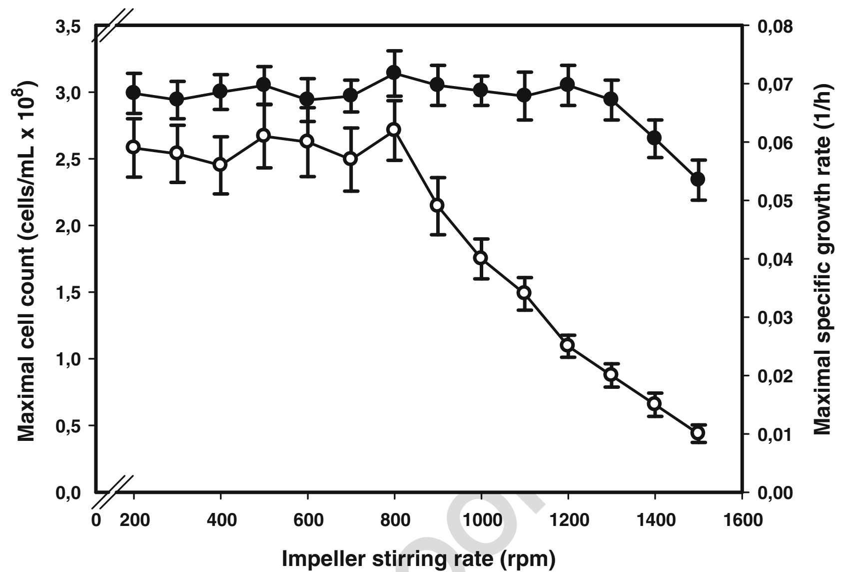

Fig. 2 Maximal cell concentration (*filled symbols*) and maximal specific growth rate (*empty symbols*) as a function of the impeller stirring rate in *N. europaea* cultures grown batchwise in a conventional bioreactor

344 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 1.5–2×10^8/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 2×10^8/mL (~54 mg/L DCW), corresponding to a 20 times increase compared to the zero time point. 360

361 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., ~2.25–2.37 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). 374

375 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). 387

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

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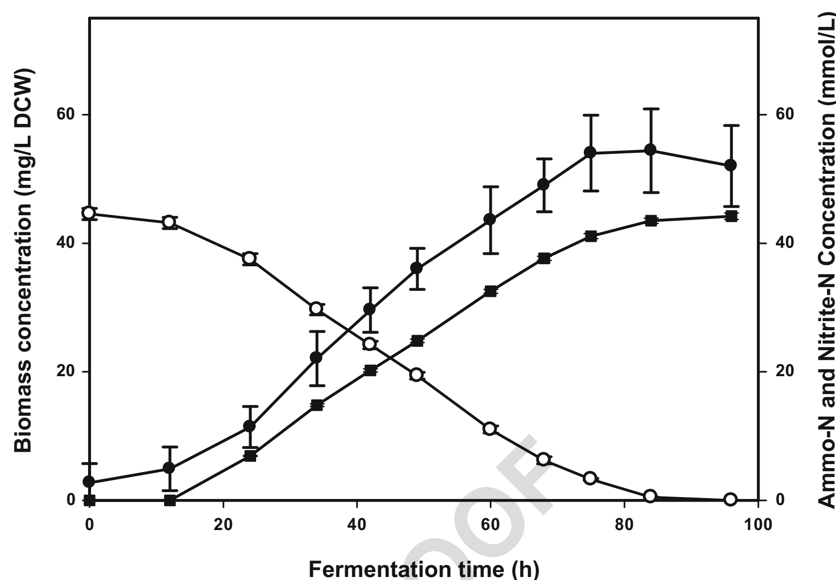

Fig. 3 Time profile of growth (*filled circle*) as well as residual Ammo-N (*empty circle*) and nitrite-N (*square*) concentrations in a conventional batch fermentation of *N. europaea*

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388 **Dialysis fermentations of *N. europaea***

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

402 In qualitative terms, time profiles for cell growth, ammoni-
403 um consumption, and nitrite formation were similar to those
404 attained in the conventional glass fermentor (data not shown).
405 AO staining time profile (visualizing cellular metabolic activ-
406 ity) was likewise essentially identical. Quantitatively, howev-
407 er, all growth parameters were significantly higher in dialysis
408 fermentation (Table 1). Maximal cell density was almost 100-
409 fold increased compared to the zero time point (i.e., the inoc-
410 ulum) and was collaborated by the increased ammonium up-
411 take rate, estimated at 2.8±0.6 mmol/g DCW/h at the most
412 rapid phase of growth (between 60 and 80 h of the
413 fermentation).

414 Nitrite is toxic to *N. europaea* (for a review: Stein and Arp
415 2003), with reported thresholds varying from 12 and
416 >30 mmol/L (Painter 1970; Yu and Chandran 2010).
417 Accordingly, the peak nitrite concentration in our batch fer-
418 mentations (~44 mmol/L; see above) could well be high
419 enough to limit further growth if more ammonium would be
420 supplied to the system.

421 To assess this hypothesis, a dialysis fermentation strategy
422 involving continuous dilution of the outer chamber with fresh
423 medium was applied, with medium fluxes at flow rates from 1
424 through 5 L/h. Under such conditions, the nitrite concentra-
425 tions remained below 5 mmol/L (in both chambers) through-
426 out this set of fermentations (data not shown). The exponential
427 phase of growth was proportionally prolonged with increasing
428 flux, the rate at which the dialysate in the outer chamber was
429 exchanged, also when visualized by AO staining (not shown).
430 As a consequence, maximal specific growth rates did not sig-
431 nificantly ($p<0.1$ %) increase with the maximal cell concen-
432 tration (Table 1). On the contrary, the maximal cell concentra-
433 tion was proportional to the flow rate applied (Fig. 4). At the
434 highest dialysate flow rate tested, 5 L/h, the mean biomass
435 yield was close to 900 mg/L. This represents a >16-fold in-
436 crease relative to conventional batch fermentations (Table 1)
437 and doubles the highest values reported in literature for

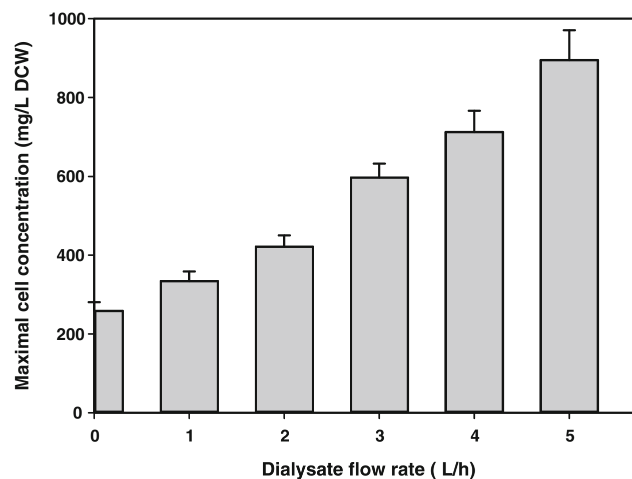

Fig. 4 Effect of the rate at which the dialysate in the outer chamber was exchanged on the maximal cell concentration of *N. europaea*

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.

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