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56	Abstract	<p><i>Nitrosomonas europaea</i> 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 <i>N. europaea</i> cultures is to minimize the concentrations of inhibitory metabolic by-products. During growth on ammonia nitrite accumulates, as a consequence, <i>N. europaea</i> 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 <i>N. europaea</i> 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</p>	

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 ( $2.79 \times 10^{12}/L$ ) 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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57 Keywords separated  
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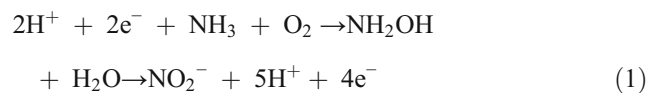
58 Foot note  
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## Electronic supplementary material

**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)

4 **High cell density cultivation of the chemolithoautotrophic**  
5 **bacterium *Nitrosomonas europaea***6 **Benedek Papp<sup>1</sup> · Tibor Török<sup>2</sup> · Erzsébet Sándor<sup>3</sup> · Erzsébet Fekete<sup>1</sup> · Michel Flippi<sup>1</sup> ·**  
7 **Levente Karaffa<sup>1</sup>**  
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10 © Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i. 201511 **Abstract** *Nitrosomonas europaea* is a chemolithoautotrophic  
12 nitrifier, a gram-negative bacterium that can obtain all energy  
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14 and this may be beneficial for various biotechnological and  
15 environmental applications. However, compared to other bac-  
16 teria, growth of ammonia oxidizing bacteria is very slow. A  
17 prerequisite to produce high cell density *N. europaea* cultures  
18 is to minimize the concentrations of inhibitory metabolic by-  
19 products. During growth on ammonia nitrite accumulates, as a  
20 consequence, *N. europaea* cannot grow to high cell concen-  
21 trations under conventional batch conditions. Here, we show  
22 that single-vessel dialysis membrane bioreactors can be used  
23 to obtain substantially increased *N. europaea* biomasses and  
24 substantially reduced nitrite levels in media initially contain-  
25 ing high amounts of the substrate. Dialysis membrane biore-  
26 actor fermentations were run in batch as well as in continuous  
27 mode. Growth was monitored with cell concentration deter-  
28 minations, by assessing dry cell mass and by monitoring am-  
29 monium consumption as well as nitrite formation. In addition,  
30 metabolic activity was probed with in vivo acridine orangestaining. Under continuous substrate feed, the maximal cell 31  
concentration ( $2.79 \times 10^{12}/L$ ) and maximal dry cell mass 32  
(0.895 g/L) achieved more than doubled the highest values 33  
reported for *N. europaea* cultivations to date. 3435 **Introduction** 36Nitrifying bacteria such as *Nitrosomonas europaea* can use 37  
ammonia as their sole source of energy. The carbon required 38  
for growth and anabolism is obtained by carbon dioxide fixa- 39  
tion (see for a recent review: Monteiro et al. 2014). 40  
Nitrification in *N. europaea* involves two conversions cata- 41  
lyzed by ammonia monooxygenase and hydroxylamine oxi- 42  
doreductase, respectively (conveniently reviewed by Arp et al. 43  
2002). 44The two electrons released [1] serve as the reducing equiv- 47  
alents driving growth. 48Ammonia oxidizers are difficult to grow in the laboratory. 49  
Growth of *N. europaea* is slow, with specific growth rates in 50  
the range of 0.4–1/day, with generation times reportedly be- 51  
tween 8 and 24 h (Sato et al. 1985). In addition, several factors 52  
(e.g., light or salts comprising sulfate or multivalent metal 53  
cations) may have a negative influence on biomass formation 54  
(Hyman and Arp 1992; Yan et al. 2010; Park and Ely 2008). 55  
Nevertheless, chemolithoautotrophic nitrifying bacteria are 56  
essential components of activated sludge used in domestic or 57  
industrial wastewater treatment systems (Terada et al. 2013). 58  
Their ammonia monooxygenase is also capable of oxidizing 59  
many non-physiological substrates, like halogenated 60  
61

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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<sub>3</sub>) 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<sub>2</sub>PO<sub>4</sub>, 3.71 mmol/L Na<sub>2</sub>CO<sub>3</sub>, 3.93 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 0.73 mmol/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.19 mmol/L CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.01 mmol/L FeSO<sub>4</sub>·7H<sub>2</sub>O, and 0.49 mmol/L CuSO<sub>4</sub>·5H<sub>2</sub>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 λ=600 nm (henceforth referred to as A<sub>600</sub>) by the time they were used as inoculum. Bioreactors were inoculated with 10 % (v/v) seed culture, and the initial A<sub>600</sub> 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<sub>2</sub>CO<sub>3</sub> 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<sup>3</sup> mL/m<sup>2</sup>/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

164 also used as covers to shield away light during fermentations.  
 165 Cells grew in the inner chamber, surrounded by the outer  
 166 chamber filled with (the same) growth medium. Sampling  
 167 occurred simultaneously from both the inner and outer cham-  
 168 ber. Because of their deleterious effect on *N. europaea*, the use  
 169 of organic solvents to prevent contamination during inocula-  
 170 tion and sampling was omitted, and instead, the (metal) fit-  
 171 tings, tubes, and ports were briefly flamed in situ with a  
 172 Bunsen burner prior to each use. For graphic schemes that  
 173 illustrate the layout, design, and function of the dialysis mem-  
 174 brane fermentor employed in this study, we refer to the liter-  
 175 ature contained in Supplementary File 1.

176 Continuous operations in the dialysis reactor were per-  
 177 formed by feeding fresh growth medium into the outer cham-  
 178 ber of the reactor while concurrently draining spent medium  
 179 out at the same rate, thereby keeping the culture volume con-  
 180 stant. Substrate feeding started after approximately 80 % of  
 181 the initial ammonium/ammonia had been converted.

## 182 Analytical methods

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

192 Total cell number was determined by direct cell count using  
 193 a Bürker hemocytometer. Cell number data reported in the  
 194 “Results” section are the average of one count of each of the  
 195 four big squares of the gridded area (standard deviation [SD],  
 196 4.9 %). *N. europaea* cell mass was also defined as dry cell  
 197 mass (DCW), determined from 10 mL culture aliquots, and  
 198 acidified to pH 4 with 2 mol/L acetic acid after sampling (to  
 199 solubilize any struvite crystals, see below). Biomass was sub-  
 200 sequently harvested on a preweighted filter paper of 0.2- $\mu$ m  
 201 average pore size (Millipore) by suction filtration and washed  
 202 with sterile water on the filter. Then, the filter was dried at  
 203 80 °C until constant mass weight. Dry weight data are the  
 204 average of two parallel measurements, which never deviated  
 205 more than 21 %. In addition, cell concentrations were moni-  
 206 tored by means of culture’s light absorbance at 600 nm ( $A_{600}$ )  
 207 using a spectrophotometer (Amersham), where sterilized cell-  
 208 free culture medium served as a blank.

209 Specific growth rates ( $\mu$ , given as the reciprocal of time,  
 210 e.g., 1/h or 1/day) were calculated from the increased cell  
 211 count over the time elapsed between two subsequent sam-  
 212 plings (i.e., sampling time points); the highest of the thus  
 213 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).

216 The metabolic status of the cells was probed using acridine  
 217 orange (AO) (Freundenberg et al. 1996), the staining performed  
 218 as described earlier (Sándor et al. 2000). Samples were studied  
 219 under an epifluorescent microscope (Zeiss AxioImager)  
 220 equipped with AxioCam MRc5 camera at a magnification of  
 221 500 times.

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

## 231 Reproducibility

232 All the analytical data presented are the means of three inde-  
 233 pendent experiments (i.e., fermentations). Data were analyzed  
 234 and visualized with SigmaPlot software (Jandel Scientific),  
 235 and for each procedure, a SD was determined. In the case of  
 236 fermentations with different agitation rates, the significance of  
 237 changes in maximal cell concentrations and specific growth  
 238 rates, relative to the values obtained at the lowest agitation rate  
 239 (200 rpm), was assessed using Student’s *t* test, with probabil-  
 240 ity (*p*) values given in the “Results” section.

## 241 Results and discussion

### 242 Verification of the experimental system

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

$$250 \text{ Cell concentration} = 1.010 \times 10^9 \times A_{600} \pm 0.022 \times 10^9 \quad (2)$$

251 The correlation between cell concentration and  $A_{600}$  data in  
 252 our study was essentially identical to Eq. (2) for a wide range  
 253 of cell concentrations (data not shown). We used the same  
 254 *N. europaea* strain as Farges et al. (2012) and an essentially  
 255 identical minimal growth medium. Notwithstanding, given  
 256 the unpredictable growth patterns of this species under differ-  
 257 ent environmental conditions (e.g., Grady and Williams 1975;  
 258 259

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

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

280 DO levels were controlled with the agitation rate. A series  
281 of fermentations at different agitation rates were first per-  
282 formed to assess the sheer rate effect generated by the impeller  
283 on the growth kinetics of *N. europaea* cultures. Sheer rate is a  
284 linear function of the impeller speed (Sanchez Pérez et al.  
285 2006). Without investigating the underlying (biological)  
286 mechanisms, we found that agitation rates between 200 and  
287 800 rpm did not influence the maximal cell concentration  
288 ( $p<0.1$  %) or the maximal specific growth rate ( $p<0.05$  %).  
289 Agitation rates between 800 and 1300 rpm decreased progres-  
290 sively the growth rate ( $p<0.1$  %), while stirring at higher than  
291 1300 rpm resulted in progressive reduction of both parameters  
292 ( $p<0.1$  %; Fig. 2). The maximal sheer rate at 1300 rpm—

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

303 In the early stages of cultivations (up until 12–14 h), crys-  
304 tals identified as struvite were formed (Supplementary  
305 Fig. S1). Based on the concentration of magnesium (II) ions  
306 (the limiting component of struvite in the growth medium at  
307 0.75 mmol/L), the solubility of struvite in water (Bhuiyan  
308 et al. 2007), and the calculated difference between calibrated  
309 and measured Ammo-N concentrations, maximal amount of  
310 struvite formed was estimated at 800 mg/L, out of which  
311 roughly 200 mg/L remained dissolved. The rest likely precipi-  
312 tated and thus could potentially interfere with DCW determi-  
313 nations. Samples were therefore acidified to pH 4 to solubilize  
314 struvite prior to DCW measurements. Indeed, at this acidic  
315 pH, medium aliquots were visually devoid of struvite crystals  
316 (i.e., none were seen upon microscopic inspection).

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

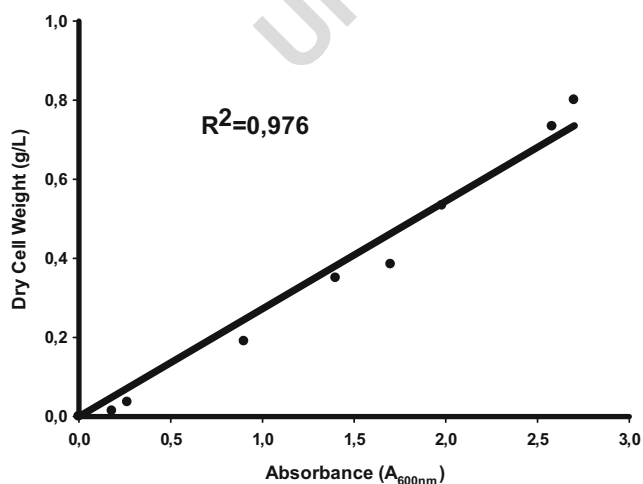
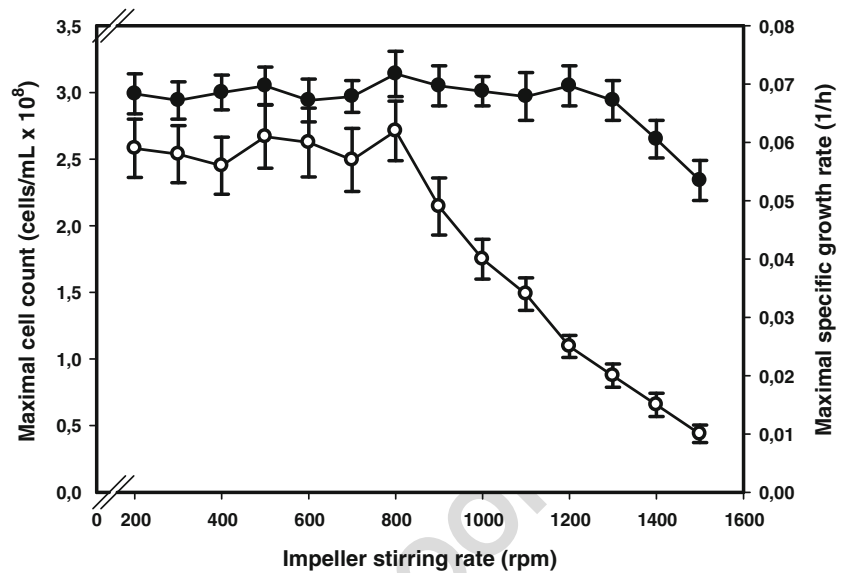


Fig. 1 Correlation between dry cell mass (DCW) and turbidity of *N. europaea* cultures measured at  $\lambda=600$  nm ( $A_{600}$ ). Samples with an absorbance of over 0.4 were diluted with sterilized medium. Turbidity data were adjusted with the dilution factor

### Conventional batch fermentation of *N. europaea* 327

328 Initial soluble ammonium ion concentrations in the growth  
329 medium were set at 800 mg/L (~45 mmol/L), roughly corre-  
330 sponding to the average  $NH_4^+$  concentration in the primary  
331 (untreated) wastewater generated in the Debrecen plant of the  
332 TEVA Pharmaceutical Ltd. The  $pK_a$  value of ammonia (i.e.,  
333 the pH at which the concentration of ammonia equals that of  
334 ammonium) is 9.25. Consequently, at medium pH 7.8—con-  
335 sistentlly used throughout this work—the ratio of ammonium  
336 ions to molecular ammonia is about 25:1, i.e., the relative  
337 ammonia concentration (<2 mmol/L) in the medium was  
338 low (Schmidt et al. 2004). The pH of the *N. europaea* cyto-  
339 plasm is about 6.8–7.2 (Hollocher et al. 1982; Kumar and  
340 Nicholas 1983). To meet the  $K_M$  value (i.e., the concentration  
341 at which the reaction rate is half the maximum ( $V_{max}$ ) at infi-  
342 nite substrate concentration under Michaelis-Menten kinetics)  
343 for ammonia oxidation (estimated at 20  $\mu$ mol/L; Wood 1986),

**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  $1.5\text{--}2 \times 10^8/\text{mL}$  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  $2 \times 10^8/\text{mL}$  ( $\sim 54$  mg/L DCW), corresponding to a 20 times increase compared to the zero time point.

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),

366 the highest ammonium uptake rate—calculated from the residual ammonium concentration data—was estimated at  $1.5 \pm 0.3$  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 Ammonium consumption, reaching a final concentration of about 44 mmol/L (Fig. 3).

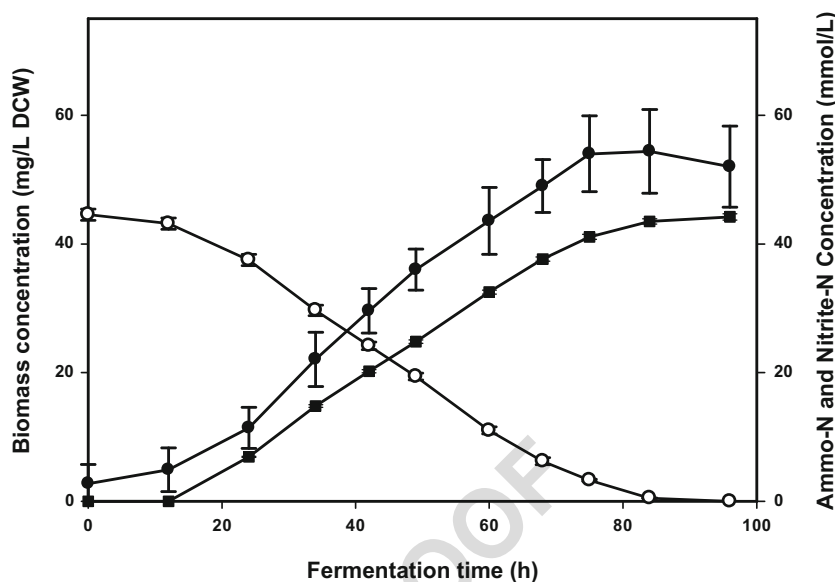
375 Viability of cells is of considerable concern when one deals with slow-growing cultures/organisms. AO staining enables to probe cellular metabolic activity in a visual, quantitative, reproducible, and fast way (McFeters et al. 1991). AO interacts differently with double- and single-stranded polynucleotides, 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).

t1.1 **Table 1** Key kinetic parameters of *N. europaea* cultures using different cultivation methods

t1.2	Growth parameters of cultures	Method of cultivation		
		Conventional batch	Dialysis, batch mode	Dialysis, continuous mode (5 L/h flux)
t1.4	Maximal cell concentration (mg/L)	54±5.8	258.4±21.9	895±75.6
t1.5	Maximal cell count (cells×10 <sup>8</sup> /mL)	2±0.04	9.4±0.06	32.4±0.81
t1.6	Maximal specific growth rate (1/h)	0.067±0.006	0.094±0.009	0.102±0.01
t1.7	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*



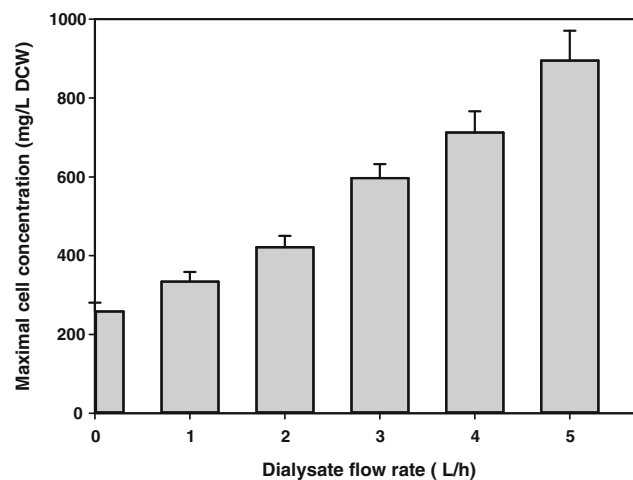
### 388 Dialysis fermentations of *N. europaea*

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  $2.8 \pm 0.6$  mmol/g DCW/h at the most  
 412 rapid phase of growth (between 60 and 80 h of the fer-  
 413 mentation).

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 ( $\sim 44$  mmol/L; see above) could well be high  
 419 enough to limit further growth if more ammonium would be  
 420 supplied to the system.

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 concentra-  
 tions remained below 5 mmol/L (in both chambers) through-  
 out this set of fermentations (data not shown). The exponen-  
 tial phase of growth was proportionally prolonged with increas-  
 ing 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 sig-  
 nificantly ( $p < 0.1\%$ ) increase with the maximal cell concen-  
 tration (Table 1). On the contrary, the maximal cell concentra-  
 tion 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 in-  
 crease 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*

438 *N. europaea* (Tappe et al. 1996; Chapman et al. 2006). This  
439 substantial increase may either be due to the removal of nitrite  
440 from the biomass and/or to the continuous supply of (fresh)  
441 ammonium for energy, growth, and anabolism. Further exper-  
442 iments with altered ammonium input would be needed to dis-  
443 tinguish the two potential effects.

444 Due to its geometry and construction, the type of dialysis  
445 membrane bioreactor used in this study is available at labora-  
446 tory scale only. However, technical-scale dialysis applications  
447 employing external membrane modules with minimal resi-  
448 dence time and the provision of oxygen are readily commer-  
449 cially available (Fuchs et al. 2002). Using *E. coli*, these fer-  
450 mentation systems proved to be as efficient as their lab-scale  
451 counterparts in obtaining high cell concentrations. It remains  
452 to be seen if such scaled-up dialysis bioreactor could also be  
453 employed for the production of viable *N. europaea* cultures  
454 with high biomass yield.

## 455 Conclusions

456 In this work, we have demonstrated a methodological im-  
457 provement of the cultivation of the slow-growing nitrifying  
458 bacterium *N. europaea* that resulted in significantly increased  
459 final cell concentrations. In their natural environment, soil-  
460 borne nitrifying bacteria have to cope with low nutrient con-  
461 centrations. By contrast, industrial wastewaters typically con-  
462 tain high amounts of ammonium, and its conversion to nitrite  
463 may limit the growth of the very microorganisms performing  
464 it. Our work showed that *N. europaea* can nevertheless be  
465 grown to relatively high cell concentrations (up to 900 mg/L  
466 DCW) in the presence of ammonium at concentrations com-  
467 parable of that in industrial wastewater by employing contin-  
468 uous fermentation technology coupled with medium dialysis.

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## AUTHOR QUERIES

### **AUTHOR PLEASE ANSWER ALL QUERIES.**

- Q1. Please check if the affiliations are presented correctly.
- Q2. Stein & Arp (1998) was not cited anywhere in the text. Please provide a citation. Alternatively, delete the item from the list.

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