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SHORT COMMUNICATION

Nitrifying Soil Bacterium *Nitrosomonas europaea*: Operational Improvement of Standard Culture Medium

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Abstract

Nitrosomonas europaea is the most extensively studied ammonia-oxidizing bacterium (AOB), being a ubiquitous player in the conversion of ammonia in the soil environment. The precipitation of constituents of the standard culture medium, American Type Culture Collection (ATCC) #2265, further hinders both the study of *N. europaea* vital role in the nitrogen cycle and the development of biotechnological applications, as the presence of inorganic debris severely compromises scale-up procedures and downstream processing. The standard formulation was analyzed; the precipitate was identified as being struvite. Struvite is spontaneously formed when magnesium, phosphate, and ammonium are available in a high pH solution. This combination is common and transversal to a series of AOB media formulations. Therefore, a non-precipitating medium was developed. The modified medium does not require filter sterilization, which reduces 99% of its production cost. The kinetic performance of *N. europaea* was evaluated in the standard and modified medium. A precise and direct quantification of the bacterium cell growth profile was achieved using fluorescent in situ hybridization (FISH) and flow cytometry, which was correlated with other metabolic parameters. The performance of *N. europaea* was not impaired in the modified medium.

Keywords *Nitrosomonas europaea* · Struvite · Soil-nitrifying bacteria · Inorganic precipitation

1 Introduction

Nitrogen is the most abundant element in the Earth atmosphere, being also one of the most important backbone components of some essential biomolecules, such as nucleotides and proteins (Camargo and Alonso 2006). Moreover, it is an indispensable nutrient for the plants and the soil microorganisms. Nitrification is an essential process in the nitrogen cycle of all soils, and it is responsible for the biological conversion of ammonia (NH_3) to nitrate (NO_3^-). One of the major bottlenecks in the nitrification process is the first oxidation step of the most reduced form of nitrogen: NH_3 to nitrite (NO_2^-) (Amoo and Babalola 2017; Alfaro et al. 2018; Stein 2019), which is performed by the ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Aoyagi et al. 2018). *Nitrosomonas europaea* is the most extensively studied AOB (Stein 2019). Nevertheless, the knowledge gathered on this key bacterium is still insufficient, and its essential action may be further optimized. One of the main problems to be considered in the study of *N. europaea* is the choice of an optimum culture medium. In this regard, optimization of culture medium has been studied early by several authors (Hommes et al. 2003; Papp et al. 2016). However, pure *N. europaea* culture studies are hindered due to the spontaneous inorganic precipitation that occurs in its standard culture medium, American Type Culture Collection (ATCC) #2265 broth. The presence of precipitates in the culture also compromises the research and development of innovative biotechnologies that require *N. europaea* biomass free of debris. The design of a culture medium with negligible or absent precipitation of its constituents is an essential solution for accurate studies and feasible scale-up processes. A non-precipitating medium allows maximum bioavailability of all the elements comprised in the medium recipe and their accurate

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quantification. In this study, the formulation of the standard *N. europaea* culture medium, ATCC #2265, was extensively examined. The spontaneous precipitation process was identified, and an easy solution was proposed in a new formulation, herein named as modified medium. *N. europaea* growth kinetics in the standard and in the modified medium was thoroughly analyzed. To accurately determine the cell concentration, fluorescent in situ hybridization (FISH) and flow cytometry were carried out. This work describes, for the first time, the identification of the inorganic precipitate found in the standard culture medium, as well as the exhibited strategy to prevent it, by adjusting its formulation.

2 Materials and Methods

2.1 Microorganism and Culture Conditions

The bacterial strain *N. europaea* NCIMB 11850 was used. All cultures were performed in dark to avoid bacterial growth photoinhibition (Pan et al. 2018). All flasks cultures were inoculated with 10% of their volume. In all experiments, a culture medium evaporation rate was on the average estimated at approximately 20 μL day^{-1} (data not shown) and taken in account to avoid metabolite overestimation. Three independent assays were performed, each possessing three replicates.

The used media were the recommended standard medium ATCC #2265 culture broth and a modified medium based on the ATCC #2265. The standard ATCC #2265 broth was prepared using three different aqueous solutions independently sterilized by filtration using a 0.2 μm cellulose acetate filter: solution 1—25 mmol L^{-1} ammonium sulfate, 3 mmol L^{-1} monopotassium phosphate, 1.5 mmol L^{-1} magnesium sulfate heptahydrate, 2.4 mmol L^{-1}, calcium chloride, 1 μmol L^{-1} iron (II) sulfate (dissolved in 8.4 μmol L^{-1} ethylenediaminetetraacetic acid, pH 7.0), and 8.4 μmol L^{-1} copper (II) sulfate; solution 2—40 mmol L^{-1} monopotassium phosphate and 3.9 mmol L^{-1} monosodium phosphate, with the pH adjusted to 8.0 with sodium hydroxide 10 mol L^{-1}; and solution 3—3.8 mmol L^{-1} sodium carbonate. In the end, the three solutions were combined and once again aseptically sterilized by filtration through 0.2 μm cellulose acetate filter. The modified medium uses the following constituents dissolved in deionized water in four separate solutions: solution A—25 mmol L^{-1} ammonium sulfate, 43 mmol L^{-1} monopotassium phosphate, and 3.9 mmol L^{-1} monosodium phosphate, with the pH adjusted dropwise to 8.0 with sodium hydroxide 10 mol L^{-1}; solution B—2.4 mmol L^{-1} calcium chloride; solution C—3.8 mmol L^{-1} sodium carbonate; and solution D—1 μmol L^{-1} iron (II) sulfate (dissolved in 8.4 μmol L^{-1} ethylenediaminetetraacetic acid, pH 7.0) and 8.4 μmol L^{-1} copper (II) sulfate. All solutions were autoclaved at 121 °C for 20 min and aseptically mixed once chilled. The culture conditions were 120 rpm shaking at 28 °C. A pre-inoculum was prepared 10 to 15 days before each culture.

2.2 Fluorescence In Situ Hybridization Probe Specifications and Cytometry Analysis

N. europaea cell concentration was determined by FISH followed by quantification by flow cytometry. *N. europaea* cells were hybridized with Nsm156 (S-Nsm156Sa-19) which is specific for the members of the *Nitrosomonas* genus (Mobarry et al. 1996). The Nsm156 sequence is 5′-TATT AGCACATCTTTCGAT-3′ labeled at its 5′ extremity with an amino linker to 6-carboxyfluorescein (6-FAM) fluorophore. Following the FISH hybridization protocol, the samples were analyzed in a EC800 Flow Cytometry Analyzer (Sony Biotechnology Inc.) equipped with an argon-blue laser (488 nm). The green fluorescence signal was detected by a FL1 photomultiplier tube with a band-pass filter (BP 525/50 nm). A flow rate of 10 μL min^{-1} with a sample volume of 100 μL and a maximum of 15,000 counts per sample were established as setting parameters for acquisition.

2.3 Microscope Imaging and Scanning Electron Microscopy

Microscopic observation of the *N. europaea* cultures allowed the visualization of the precipitates present in the standard culture medium, and the epifluorescence microscopy was used to confirm FISH hybridization. Culture samples were observed with an epifluorescence microscope (BX51, Olympus) coupled with a camera (DP71, Olympus), using contrast phase and epifluorescence (FITC filter – EX 470–490 nm/EM 516 nm). The culture medium precipitates obtained through evaporation at room temperature (RT = 25 °C) were observed using a stereoscopic microscope (8Z-8T, Olympus) coupled with a video camera (CCD AVC-D5CE, Sony).

2.4 X-Ray Diffraction Analysis

X-ray diffraction (XRD) analysis was used to identify the precipitate present in the standard ATCC #2265 broth. Standard ATTC #2265 and modified culture media were evaporated at RT, and their salts were analyzed through XRD. The X-ray diffraction patterns were registered using a Philips Geiger Counter diffractometer (PW1710, Philips). The X-ray tube conditions were Cu 40 kV, 30 mA. The starting angle and ending angle were 2.01° and 64.97°, respectively. The step width was 0.02°, the time per step was 1.25 s, the wavelength was 1.541838 Å, and the receiving slit had 0.2 mm.

2.5 Kinetic Analysis

The *N. europaea* kinetics in the two different media was followed using several parameters, namely ammonia nitrogen (N–NH_3) concentration, ammonium nitrogen (N–NH_4^+) concentration, nitrite nitrogen concentration (N–NO_2^-), pH, and cell quantification. The N–NH_4^+ was evaluated using the Nessler reagent (Gao et al. 2018). N–NO_2^- was quantified by LCK 342 kits (Hach). Flow cytometry analysis was performed on *N. europaea* bacterium hybridized with FISH probe. Each sample, once collected in aseptic conditions, was immediately centrifuged at 16160 *g* for 20 min at RT; the supernatant was discarded, and the cells were fixed with glutaraldehyde 2.5% (*v*/*v*) and stored in the dark at 4 °C until further use. When the FISH protocol was performed, the cells were centrifuged at 16000 *g* for 20 min (RT); the supernatant was discarded, and the cells were resuspended in a solution composed of 4 mol L^{-1} sodium chloride, 20 mmol L^{-1} of Tris-hydrochloric acid (pH 8.0), and 0.2 nmol L^{-1} of Nsm156 FISH probe in sterile Milli-Q water. The hybridization occurred for 2 h at 46 °C. Then, the cells were centrifuged for 20 min at 16160 *g* (RT); the supernatant was discarded, and the cells were resuspended in the washing solution, which comprised 0.55 mol L^{-1} of sodium chloride and 17.5 mmol L^{-1} of Tris-hydrochloric acid in sterile Milli-Q water. FISH hybridization and all subsequent steps were performed in the dark or under dim light conditions.

The N–NH_3 concentration was estimated through Eq. 1 using the mean value of N–NH_4^+ concentration and the mean value of pH for each sampling point (Oliveira et al. 2015).

$$[\mathrm{N{-}NH_3}] = \frac{\left[\mathrm{N{-}NH_4^+}\right] \times 10^{\mathrm{pH}}}{\exp\left(\frac{6344}{T}\right) + 10^{\mathrm{pH}}} \quad (1)$$

In this equation, [N–NH_3] represents the N–NH_3 concentration (mg L^{-1}), [N–NH_4^+] represents N–NH_4^+ (mg L^{-1}), and *T* the temperature in kelvin at a given time. Zero-order reaction (Eq. 2) was used to determine the rate of decrease of N–NH_3 and N–NH_4^+ concentration (Austin et al. 2019). In addition, Eq. 2 was used to determine the N–NO_2^- generation.

$$r = k[A]^0 \quad (2)$$

Where *r* corresponds to the rate, *k* corresponds to the zero-order constant, and $[A]^0$ to the initial concentration of the sum of NH_3 and NH_4^+, or the concentration of N–NO_2^-.

The analysis was performed using GraphPad Prism 5 (Graphpad Software Inc.) and Microsoft Office Excel software (Microsoft).

3 Results

Spontaneous inorganic precipitation occurs in the recommended standard ATCC medium for *N. europaea* culture, even prior the inoculation. Figure 1a displays the observation of an orthorhombic crystal typically found in the standard ATCC #2265 culture medium. Stereoscopic microscope image of the orthorhombic crystals obtained after evaporation of the standard ATCC #2265 broth is showed in Fig. 1b. Regarding these orthorhombic structures and analyzing the culture medium formulation, magnesium ammonium phosphate hexahydrate ($MgNH_4PO_4{\cdot}6H_2O$), generally known as struvite, was found to be a good candidate for the possible precipitate. The identification of the orthorhombic crystals was possible through XRD. Figure 2 displays XRD spectra obtained from the salts that resulted from the evaporation of the standard ATTC medium (Fig. 2a), modified medium (Fig. 2b), and the superimposed profile of struvite. The struvite XRD profile has a high homology with the standard ATCC medium XRD profile, whereas the modified medium XRD spectrum has nearly no superimposition, especially between 14° and 26°. Moreover, the orthorhombic structures are absent in the modified medium. Therefore, evidences found in the XRD spectra, namely in the homology between standard ATCC precipitates and the struvite, lead us to conclude that the orthorhombic crystals found in the ATCC medium are in fact struvite. Consequently, as expected, the removal of magnesium from the formulation prevented the struvite formation.

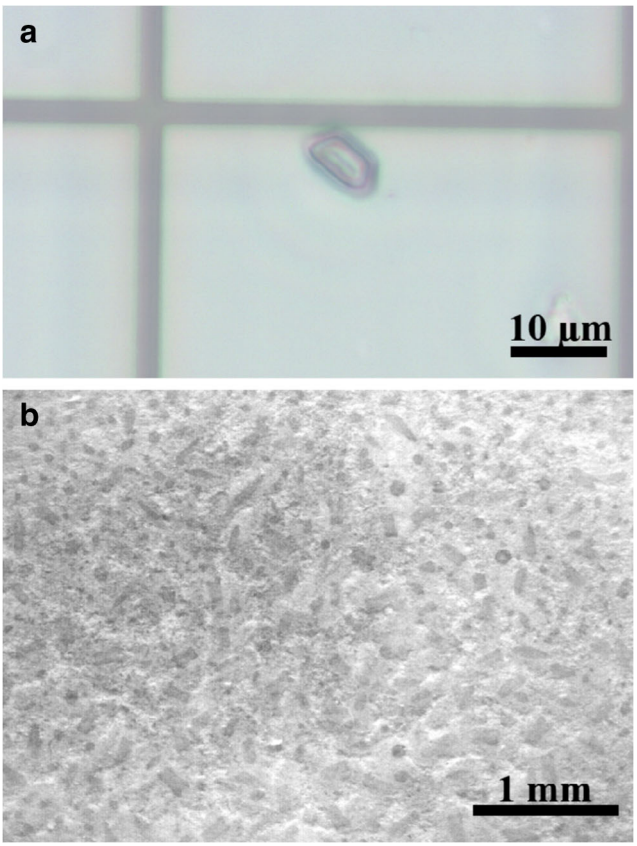

Fig. 1 **a** An orthorhombic precipitate typically found in the standard ATCC #2265 broth. **b** Orthorhombic crystals obtained after evaporation of the standard ATCC #2265 broth

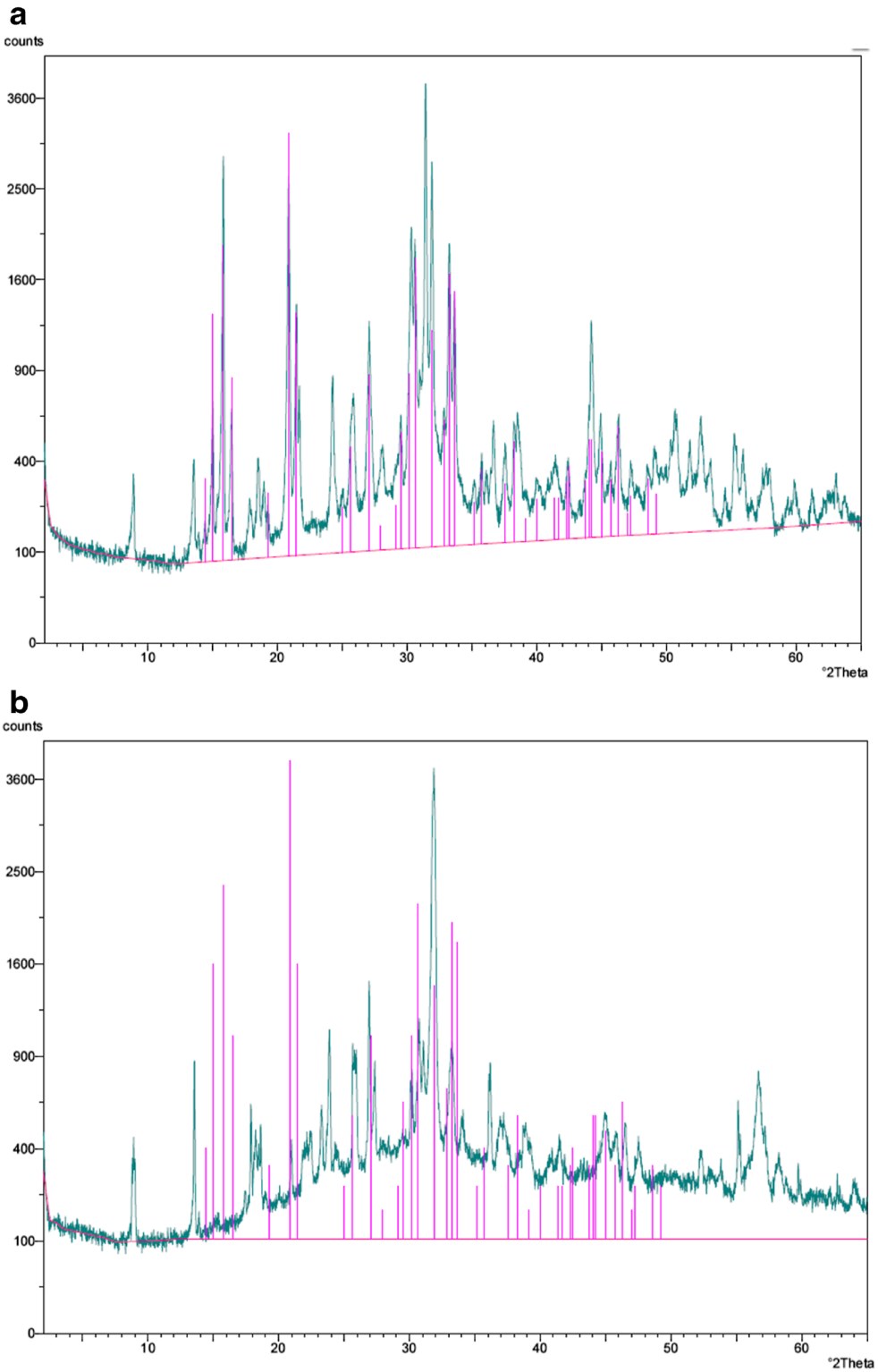

Fig. 2 XRD profiles of the salts obtained from the evaporation at room temperature of ATCC #2265 broth (**a**) and modified medium (**b**). The vertical pink lines correspond to the struvite diffraction pattern (powder diffraction file (PDF) No. 15-0762)

The modified medium also displayed an important operational improvement. Instead of filter sterilization as recommended by the ATCC, the modified culture medium components were autoclaved. Due to the inherent limitations on the precise quantification of *N. europaea* cells, the bacterium cells were hybridized with a fluorescent probe and individually counted using flow cytometry (Coskuner et al. 2005; Manser et al. 2005). The applied FISH protocol was effective in staining the bacterium as shown in Fig. 3. The *N. europaea* performance in each medium is displayed in Fig. 4. *N. europaea* growth was monitored through cell concentration (Fig. 4a). It is clearly observable that *N. europaea* cell concentration

profile is similar throughout assay in both culture media. The exponential phase endured approximately 144 h in both media. The estimated specific growth rate was 0.040 h^{-1} and 0.044 h^{-1} in the standard ATCC and modified medium, respectively. At the stationary phase, the maximum cell concentration was also similar, approximately 1.7×10^5 and 1.8×10^5 cell μL^{-1} in the standard ATCC and modified medium, respectively. Figure 4b displays the N–NO_2^- production kinetics, which is equivalent in both media. The generation of N–NO_2^- was exponential for approximately 192 h. The maximum N–NO_2^- mean concentration in the standard ATCC #2265 broth was 260 mg L^{-1} and 256 mg L^{-1} in the modified medium. In Fig. 4c, which represents the sum of mean N–NH_4^+ and mean N–NH_3 profile, it is noticeable that the concentration of N–NH_3 was also similar in both media, even though struvite precipitation occurred in standard ATCC #2265 broth. Cell growth plateau was roughly reached at 144 h. However, both N–NO_2^- generation and N–NH_3 consumption reached their plateau approximately at 192 h. NH_3 oxidation follows a zero-order reaction; k was determined for the sum of N–NH_3 and N–NH_4^+, as well as for N–NO_2^- (Austin et al. 2019). Calculated k was highly similar. For the standard ATCC medium, the k for N–NH_3 and N–NH_4^+ was 2.02 ± 0.08 mg L^{-1} h^{-1}, and the k for N–NO_2^- was 2.05 ± 0.18 mg L h^{-1}. The estimated k for modified medium was 2.07 ± 0.10 mg L^{-1} h^{-1} and 1.98 ± 0.09 mg L^{-1} h^{-1}. Overall, all constant rates were roughly 2 mg L^{-1} h^{-1} in both media. The consistency of the estimated k is in accordance with the stoichiometry of NH_3 oxidation performed by AOB (Eq. 3) (Austin et al. 2019). The pH profile is represented in Fig. 4d. *N. europaea* displayed a similar profile in both culture medium formulations, exhibiting during the exponential growth a pH decrease rate of approximately 0.012 h^{-1}.

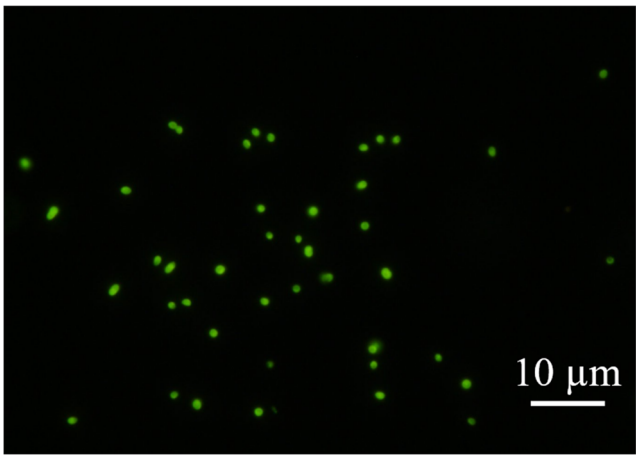

Fig. 3 Epifluorescence microscopy image of *N. europaea* cultured in modified medium after hybridization with Nsm156 labeled with 6-FAM fluorophore

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (3)$$

4 Discussion

Several publications referred the use of ammonium (as energy source), phosphate (simultaneously as buffer solution and phosphate source), and magnesium (as divalent cation) as components of *Nitrosomonas* spp. culture media with an alkaline pH level of approximately 8.0 (Montrás et al., 2008; Papp et al. 2016). These comprise the required conditions for the struvite formation, which follows the equation described in Eq. 4) (Barbosa et al. 2016).

$$HPO_4^{2-} + Mg^{2+} + NH_4^+ + 6H_2O \rightarrow MgNH_4PO_4 + 6H_2O + H^+ \quad (4)$$

Nevertheless, the inevitable spontaneous struvite precipitation phenomena, which is further stimulated by agitation, are not referenced (Barbosa et al. 2016). Struvite precipitates, once present in the culture medium formulation, will inevitably impair all downstream processing. The molar ratio for struvite formation is 1 mol Mg^{2+}: 1 mol NH_4^+: 1 mol PO_4^{3-}. It means that the standard ATCC #2265 broth may have up to 1.5 mmol L^{-1} of biologically unavailable N–NH_3 (Barbosa et al. 2016). Moreover, as displayed in Eq. 2, struvite crystallization induces a pH drop due to proton formation, which affects the concentration of available N–NH_3 (Barbosa et al. 2016). Therefore, due to the protons released into the medium when NH_3 is oxidized (Eq. 3) in addition to the protons released during struvite crystallization, the pH profile was unexpectedly slightly lower in the standard ATTC culture medium.

The typical shape of struvite crystals is orthorhombic, such as the precipitates found in the ATCC #2265 broth (Ariyanto et al. 2014). Therefore, to thoroughly analyze the implications of struvite formation, a modified medium was used, which was identical to the standard ATTC medium, with a single modification in its formulation—it did not contain magnesium sulfate. The presence of magnesium is described as vital for bacteria growth (Kitjaruwankul et al. 2016), working as a cofactor alongside adenosine triphosphate (ATP) and responsible for most of its biological interactions due its uniquely large hydrated radius (Kitjaruwankul et al. 2016). Magnesium is also referred as being imperative for lipopolysaccharide (LPS) stabilization; however, this role may be performed by the calcium cation present in both formulations (Kim et al. 2016). Being a chemolithoautotrophic, *N. europaea* displays

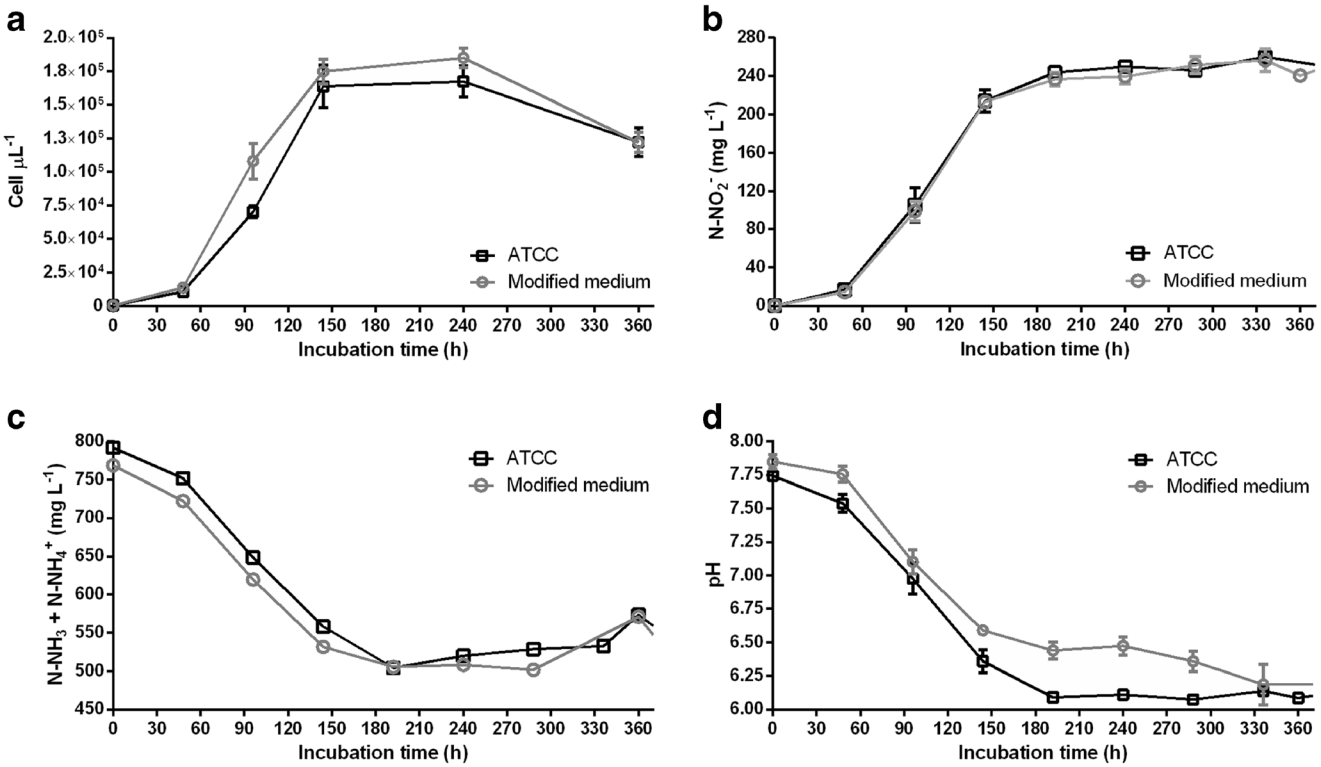

Fig. 4 *N. europaea* kinetic profiles in ATCC #2265 broth and modified medium. **a** Cell concentration, **b** Nitrite nitrogen concentration. **c** Sum of the mean ammonia nitrogen and mean ammonium nitrogen. **d** pH variation

a highly complex and versatile biosynthetic machinery (Drozd 2018). Therefore, despite the indications that pointed towards *N. europaea* growth arrest once magnesium was withdrawn from the culture medium formulation, the growth and kinetic analysis performed prove otherwise. The strategy used to accurately estimate the cell concentration was the flow cytometry since *N. europaea* cell growth is difficult to quantify through classic microbial methodologies, due to its long generation times (Mobarry et al. 1996). Several methods were used to monitor the growth of *N. europaea*; however, all shown inherent disadvantages. The quantification of *N. europaea* colony-forming units (CFU) presents as main hurdles the small size of the colonies, which take more than 3 weeks to become identifiable under stereoscopic microscope or a microscope, thus making this procedure highly prone to error or contamination. Direct correlation of $N–NO_2^-$ concentration and cell concentration has been widely used as feasible method to overcome this limitation; nonetheless, it is an indirect measurement (Cruvellier et al. 2016). As displayed in Fig. 4, *N. europaea* at 144 h of incubation apparently arrested cell division dedicating its energy, resulting from NH_3 oxidation, solely to cell maintenance in a stationary phase. Therefore, this correlation is only valid in the exponential phase. The use of dry weight to estimate cell concentration requires high culture volumes due to the low biomass concentration usually achieved, especially in the early incubation periods. Moreover, if inorganic precipitates are present in the culture medium, the dry weight will be overestimated, which compromises the accuracy of the method. Inorganic precipitation also affects downstream correlations between dry weight and optical density (Papp et al. 2016).

For both tested media, all kinetic profiles were very similar. In standard ATCC culture, albeit its lower pH values and the presence of struvite crystals, none of the analyzed parameters were impaired throughout the incubation, in comparison to the values obtained in the modified medium. The achieved specific growth rates are similar and near the estimated maximum specific rate for *N. europaea*, stated as approximately 0.06 h^{-1} (Ramírez et al., 2009). Finally, the obtained modified medium was stable, with no inorganic precipitation. The absence of filter sterilization of the culture medium and of magnesium sulfate represented saving cost approximately 99% in its production. Such a considerable saving is due to the price of the 0.2 µm sterile filters that should be used to produce the standard ATCC #2265 broth. This strategy should be tested for other bacteria that require culture medium formulations possessing the combination of magnesium, ammonium, phosphate, and a high pH value, namely *Nitrosomonas eutropha*, *Nitrosomonas communis*, and *Nitrosomonas nitrosa* that are cultured in the recommended medium of the Leibniz Institute of German Collection of Microorganisms and Cell Cultures DSMZ 1583. In addition are *Nitrosococcus oceani* that is cultured in the recommended ATCC medium #928 and *Nitrosospira multiformis* cultured in the ATCC medium #929.

5 Conclusion

The difficult estimation of *N. europaea* cell concentration may be accurately determined by using FISH allied to flow cytometer analysis. Several AOB culture media possess an identical combination magnesium, ammonium, phosphorous, and a high pH described in their formulation as the standard ATCC #2265 broth, which inevitably will result in spontaneous precipitation of struvite. The tested modified medium formulation for the culture of *N. europaea* allows further studies, scale-up methodologies, and downstream procedures, without the presence of precipitates, and does not affect the bacterium behavior. In addition, the alternative methodology to produce modified medium resulted in a considerable cost reduction (approximately 99%).

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