Short Communication

High-cell-density cultivation of *Nitrosomonas europaea* in a membrane bioreactor
for performing protein purification and characterization studies

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Advancements in DNA sequencing techniques in the past decade have dramatically accelerated the accumulation of genomic information on prokaryotes. At present, genome sequences of 50 and 11 bacterial and archaeal phyla, respectively, are available in public databases (Land et al., 2015). Genome annotation and sequence similarity searches have highlighted large number of prokaryotic genes encoding functionally uncharacterized proteins and have indicated that approximately 50% proteins encoded by prokaryotic genomes have not been functionally characterized (Blattner et al., 1997; Galperin and Koonin, 2010). Characterization of these uncharacterized proteins is a huge challenge for understanding prokaryotic biology and physiology. For this purpose, purification of specific proteins has been conventionally carried out, and the biological functions of uncharacterized proteins has been investigated (Dietl et al., 2015; Hiratsuka et al., 2008). Although well-established techniques are available for protein purification, characterization of uncharacterized proteins requires large volumes of cultures, especially for microorganisms with a low growth yield. For instance, purification and characterization of proteins from *Nitrosomonas europaea*, an autotrophic ammonia-oxidizing bacterium, requires culture volumes of 100–1,000 L (Lawton et al., 2013; Yamanaka and Shinra, 1974). Cultivation of microorganisms at such a large scale is laborious and often unaffordable because of technical limitations (e.g., disinfection). Cultivation of microorganisms at a high cell density allows the scaling down of culture volumes required for performing such experiments. Some studies have cultivated bacterial cells at a high cell density by using a chemostat reactor equipped with a membrane (Meulepas et al., 2009; Oshiki et al., 2013; Tappe et al., 1996). In a reactor, fresh medium was continuously supplied with full retention of the cells by membrane filtration, resulting in an increase of cell density in the culture. In the case of *N. europaea*, the cells were cultivated in a membrane bioreactor (MBR) at 10⁹ cells/mL, which was 1 to 2 orders of magnitude higher than that observed in batch culture (Tappe et al., 1996). However, characterization of proteins by using a high-cell-density culture has not been performed to date. Therefore, it is unclear whether protein purification and characterization by using cells cultivated in a high-cell-density culture are affordable. The present study aimed to: (1) cultivate *N. europaea* at a high cell density by using an MBR; (2) characterize the whole protein expression in this high-cell-density culture; and (3) determine the affordability of protein purification and characterization by using cells cultivated in the high-cell-density culture.

* N. europaea* cells (NBRC 14298) were cultivated at 30°C in a 1-L MBR equipped with a hollow fiber membrane unit (300 polyethylene tubes; pore size 0.1 μm; tube diameter 1 mm; length 70 mm; Fig. 1) and having a continuous supply of inorganic culture medium (per liter: 3.3 g [NH₄]₂SO₄, 2.94 g KH₂PO₄, 0.18 g MgSO₄·7H₂O, 0.027 g CaCl₂·2H₂O, 0.467 g NaH₂PO₄, 0.4 g Na₂CO₃, 1.5 mg FeSO₄, and 0.133 mg CuSO₄·5H₂O [pH 8]). The liquid...
High-cell-density cultivation method

The volume of the culture was adjusted to 600 ml by using a liquid level sensor. The culture was continuously mixed and purged with air filtered using a 0.2-μm membrane filter. The inorganic medium was continuously supplied after filtration with another hollow fiber membrane unit. After 1,029 h of cultivation, a portion of the culture was removed daily by using a peristaltic pump (designated as P) at a dilution rate of 0.00014 h⁻¹.

The growth of *N. europaea* cells in the MBR was almost arrested when OD₆₀₀ of the culture reached 0.7. Moreover, the growth was not completely recovered after removing the cell culture, as described in previous studies (Chapman et al., 2006; Tappe et al., 1996). In the high-cell-density culture, the concentration of NH₃ was high (>39 mM), whereas that of NO₂⁻ was lower than its inhibitory concentration (Zart and Bock, 1998). Factor(s) deciding the upper limit of cell density in the MBR culture need to be examined in another study.
propanesulfonate, 2% [w/v] Triton-X, 7 M urea, 2 M thiourea, and 60 mM dithiothreitol) and by incubating them on ice for 1 h. Supernatant obtained after centrifugation at 25,000 × g and 4°C for 1 h was used as the total protein fraction. Proteins in the total protein fraction were separated on an 8% polyacrylamide gel containing SDS at 100 V for 90 min, and the gel was stained with CBB Protein Safe Stain solution (Takara Bio, Shiga, Japan). SDS-PAGE patterns of the total protein fraction obtained from the high-cell-density culture were not similar to those of the total protein fraction obtained from the batch culture (Fig. 3). For example, proteins with a high molecular weight (>245 kDa) and proteins not denatured by SDS were abundant in the fraction obtained from the high-cell-density culture, suggesting that operational conditions in the MBR were optimal for cultivating bacterial cells expressing large concentrations of specific proteins.

The high-cell-density culture was used for performing protein purification and characterization to demonstrate its utility for investigating the functions of specific proteins in cells cultivated using this culture. In the present study, we partially purified *N. europaea* hydroxylamine oxidoreductase (HAO), a soluble octahem cytochrome c protein, by using a method described previously (Shimamura et al., 2008). *N. europaea* oxidizes ammonia to hydroxylamine and hydroxylamine to nitrite by using ammonia monooxygenase and HAO, respectively (Arp et al., 2007). After 1,556 h of cultivation, 535 ml of *N. europaea* cell suspension (370 mg-wet) was removed from the MBR and was used for protein purification. The cell suspension was centrifuged at 33,540 × g and 4°C for 10 min and was washed twice with 20 mM PO4 buffer (pH 7). The cell pellet obtained was suspended in 4 ml PO4 buffer containing 2% (w/v) N-octyl β-D-glucopyranoside and was incubated at 4°C for 5 h with gentle rotation. Next, the cell suspension was ultracentrifuged at 143,000 × g and 4°C for 60 min, and the supernatant obtained was used as a crude cell extract. Proteins in the crude extract were charged to Q Sepharose XL media (GE Healthcare, Little Chalfont, UK) equilibrated with 20 mM PO4 buffer (pH 7). The protein fraction containing HAO was eluted by increasing the NaCl concentration to 0.3 M, which was ultrafiltered (molecular weight cutoff, 30 kDa; Merck), and was equilibrated using PO4 buffer (pH 7) containing 0.15 M NaCl. Next, the protein fraction was resolved by performing gel filtration chromatography with Superdex 200 media (GE Healthcare) equilibrated with 20 mM phosphate buffer (pH 7) containing 0.15 M NaCl. A symmetrical peak containing HAO was collected, and concentrated by ultrafiltration to be 0.8 mg-BSA ml⁻¹ (Table S1). The purity of *N. europaea* HAO was examined by performing native PAGE. Two μg of the purified protein fraction was separated on a 10% polyacrylamide gel and was detected by staining the gel with the CBB Protein Safe Stain solution, as mentioned previously. PAGE of the protein fraction produced a single protein band of approximately 200 kDa (Fig. 4), which corresponded to the molecular weight of *N. europaea* HAO (Hooper et al., 1978). Dominance of *N. europaea* HAO in the purified protein fraction was examined by performing spectrometry at 350–650 nm by using a UV-VIS spectrometer UV-2700 (Shimadzu). The purified protein reduced with sodium dithionite produced Soret, β-band, and α-band peaks at 418, 524, and 552 nm, respectively, and an additional peak at 468 nm (Fig. S2). These peaks were previously reported for *N. europaea* HAO by Hooper et al. (1978), indicating the abundance of *N. europaea* HAO in the purified protein fraction. NH2OH reduction activity of the purified protein was examined by performing in-gel activity staining. For this, the native polyacrylamide gel after electrophoresis was anaerobically incubated with a staining solution containing 50 mM Tris-HCl (pH 8), 3 mM sodium...
dithionite, 0.6 mM methyl viologen (MV), and 5 mM NH$_3$OH at 20°C. The incubation was performed in an anaerobic glove box containing <1 ppm oxygen. After 30 min of incubation, the gel was removed from the staining solution and the development of colorless bands on the gel, due to MV oxidation and NH$_3$OH reduction, was examined. NH$_3$OH reduction activity was observed in the region of the polyacrylamide gel containing the protein band (Fig. 4), indicating that functional HAO can be purified from cells cultivated in a high-cell-density culture.

The present study showed that an MBR can be used for cultivating bacterial cells at a high density. Protein concentration in *N. europaea* cells cultivated in the MBR was 31-times higher than that in the batch culture. Although the previous protein purification from *Nitrosomonas europaea* required >100 L of batch culture, high-cell-density cultivation can be performed to significantly scale down the culture volume. Bacterial cells cultivated using an MBR can be used for protein purification, as indicated by the purification of functional HAO from *N. europaea* cultivated in the high-cell-density culture. Thus, high-cell-density cultivation is a powerful tool, while the operation condition of MBR needs to be fine-tuned to express specific proteins in bacterial cells.

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Supplementary Materials

Supplementary figures and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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