



A simple culture technique of *Rhodobacter azotoformans* EBN-7 for public use: application to NH_4^+ -N removal in shrimp aquaculture water

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Abstract

Photosynthetic bacteria (PSB) attract considerable interest as useful microorganisms; nevertheless, a generalized culture technique has not been previously reported owing to difficulty in their cultivation. Therefore, a simple culture technique suitable for public use was investigated. Among the PSB tested, the strain *Rhodobacter azotoformans* EBN-7 was the most suitable for scale-up production because it showed the highest specific growth rate (0.20 h^{-1}) on basal medium. In scale-up cultivation (500 L), *R. azotoformans* EBN-7 showed 4.50×10^{10} colony-forming units mL^{-1} (number of viable cells), dry cell weight of 26.8 g/L, and a specific growth rate of 0.15 h^{-1} . Cultivation using this final culture broth (as seed culture) in a 15 L simple reactor was successful, with maintenance of cell activity evident. For use as seed culture, the maximum allowable preservation period of *R. azotoformans* EBN-7 at 4°C was 3 months. When *R. azotoformans* EBN-7 cultivated in a simple technique was applied to shrimp aquaculture water, NH_4^+ -N was reduced from 0.61 mg/L to 0.24 mg/L (by 60.7%) in 4 days in comparison with the control. Thus, this simple culture technique using *R. azotoformans* EBN-7 has the potential for a good removal efficiency of NH_4^+ -N, making seed culture easier and suitable for public use.

Keywords: *Rhodobacter azotoformans* EBN-7, Seed culture, Simple culture technique, Scale-up production, Shrimp aquaculture water

Introduction

Recently, environmental issues closely associated with human health have become matters of primary concern, as the quality of life becomes higher along with the enhancement of the living standard. Across industries, eco-friendly management, especially

for waste and water quality, and establishment of sustainability policies have gained importance. Microorganisms have been receiving increasing attention owing to their potential in replacing agricultural chemicals such as chemical fertilizers and pesticides (Dwivedi et al., 2021; Sharma et al., 2021). The culture of beneficial microorganisms is being researched in more than 130 labora-

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tories across Korea, and the cultured microorganisms are coming into wide use (Cho et al., 2019). Photosynthetic bacteria (PSB), lactic acid bacteria, actinomyces, and filamentous fungi are some examples of such microorganisms (Higa, 1996). PSB are diversely utilized: to increase plant growth by soil amendment (Lee & Song, 2010; Sunayana et al., 2005); to prevent animal diseases by improving barn environment (Han et al., 2008); to purify water; to remove odors (Do et al., 2003; He et al., 2010; Lu et al., 2019b); to produce single-cell proteins (Kornochalart et al., 2014; Saejung & Thammaratana, 2016); and to produce polysaccharides, carotenoids, chlorophylls, coenzyme Q₁₀, and 5-aminolevulinic acid (Meng et al., 2017; Wang et al., 2017; Zhou et al., 2015b). Moreover, vitamin B12 produced by PSB is used for the treatment of anemia and neuritis and can also be used as an animal growth-stimulating feed source (Sasaki et al., 2005). Moreover, the technology of clean hydrogen production by PSB is noteworthy because hydrogen produced from organic waste is considered a promising substitute energy source (Cai et al., 2019; Moreira Silva et al., 2016). As a high-value material, microorganisms are also used in foods, medicines, and cosmetics (Kars & Ceylan, 2013; Lu et al., 2019a; Sasaki et al., 2005). Particularly, the *Rhodobacter* species are used to treat organic compounds at high concentrations in wastewater because it prefers organic compounds to inorganic compounds as hydrogen donors for photosynthesis (Cheong et al., 1997; Lee, 1971). This bacterial genus is also used as a component in food and fertilizers.

PSB have been extensively used in environmental bioremediation and bioenergy production owing to their versatile metabolic abilities. They also play a role in biofertilization, biostimulation, and biocontrol mechanisms to promote plant growth (Lee et al., 2021). However, culturing PSB is challenging and expensive because of difficulties in culturing them without contamination, which necessitates quality control (Cho et al., 2006). This leads to problems in scaling up for mass production, which makes it complicated to make PSB available for diverse applications. Therefore, although PSB have diverse applications in many fields, they have not come into widespread use. To

date, *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* are the species currently registered as a subsidiary feed in Korean law of animal feed standards (The Korea Law Information Center, 2016). To ensure distribution of good-quality PSB for public use, more research on cultural characteristics and scale-up cultivation of PSB is indispensable, and a simple culture technique that can be used by non-experts needs to be developed.

Therefore, in the present study, a simple culture technique for a photosynthetic bacterium was developed, and its suitability for environmental fields was demonstrated not only to simplify the cultivation of useful PSB but also to disseminate this technique to the public. Using this technique, owing to the maintenance of high cell activity, is expected to be cost effective over the long term in comparison with purchasing microbial products available in the market on several occasions. In this study, the cultivation of the standard species *R. capsulatus* was first investigated on biomass analysis, and then compared with three other species of useful PSB to select a species suitable for scale-up cultivation. Subsequently, the selected species was cultivated in a 500 L reactor to identify the optimum culture conditions for scale-up. Then, these cultivated PSB were used as seed culture for cultivation in a 15 L simple reactor to develop an easier culture technique suitable for public use. Moreover, the cultivated PSB were preserved at 4 °C, and then the preserved PSB were used as seed culture to perform cultivation in a 250 L simple reactor to determine the optimum preservation period that allows cell reactivation. Finally, the selected PSB species cultivated by a simple culture technique was tested its potential in the removal of NH₄⁺-N in shrimp aquaculture water.

Materials and Methods

Photosynthetic bacterium and culture

The species of PSB used in this study was *R. capsulatus* BR, which was obtained from Ecobiznet Company (Chuncheon, Korea). *R. capsulatus* BR was cultivated on various culture media (Table 1). To eliminate the possibility of contamination,

Table 1. Composition of various media for the cultivation of *Rhodobacter capsulatus* BR

Medium	Composition (per L)
Basal	2.7 g DL-malic acid, 0.8 g ammonium phosphate, 1 g tryptone, 3.76 g monosodium glutamate, 2 g yeast extract, 0.5 g K ₂ HPO ₄ , 0.5 g KH ₂ PO ₄ , and 2.1 mL trace elements (per L, 3 g FeSO ₄ ·7H ₂ O; 0.01 g H ₃ BO ₃ ; 0.01 g Na ₂ MoO ₄ ·2H ₂ O; 0.02 g MnSO ₄ ·H ₂ O; 0.01 g CuSO ₄ ·5H ₂ O; 0.01 g ZnSO ₄ ; and 0.5 g ethylenediamine tetraacetic acid); pH = 6.8
MYC	1 g DL-malic acid; 3 g yeast extract; and 2 g casamino acid; pH = 6.8
van Niel's	10 g yeast extract, 1 g K ₂ HPO ₄ , and 0.5 g MgSO ₄ ; pH = 6.8

red colonies of *R. capsulatus* BR formed on a solid MYC culture medium containing 1.5% agar was transferred with a loop to a 1 L flask (with a working volume of 600 mL) using the MYC culture medium; the flask was incubated for 5 d at 30 °C and 14.86×g under a light intensity of 1,000 Lux (Saejung & Thammaratana, 2016). The condition of cells was periodically observed under a microscope, and the optical density (OD) and cell viability were also measured to assess cell growth during the cultivation period.

Examination of photosynthetic bacterial species for scale-up cultivation

Scale-up cultivation of PSB is requisite to ensure their effective use in diverse applications. Accordingly, the suitability of *R. capsulatus* for the scale-up cultivation was explored, and its cultural characteristics were compared with those of other generally utilized PSB species in environmental fields (Ansari et al., 2021; He et al., 2010)—*Rhodobacter azotoformans* EBN-7, *R. sphaeroides* BL6, and *R. sphaeroides* GB1—which were obtained from the Ecobiznet Company.

Red colonies of each PSB species formed on a solid MYC culture medium was transferred with a loop to a 1 L flask (with a working volume of 600 mL) using the MYC culture medium, and then the flask was incubated for 5 d at 30 °C and 14.86×g under a light intensity of 1,000 Lux. To increase cell activity, each bacterial species was preliminarily incubated in the MYC culture medium three times for adaptation. The carefully adapted seed culture was used for the scale-up cultivation, and the selection of species was based on the specific growth rate, number of viable cells, and cell purity.

Pilot-scale cultivation

After a PSB species and its optimum culture medium were selected, pilot-scale cultivation was performed in a 500 L bioreactor (KoBio Tech, Chuncheon, Korea). The seed culture was prepared in a 1 L flask incubated at 30 °C and 14.86×g under a light intensity of 1,000 Lux. During cultivation, the cell activity was periodically observed under a microscope, and the color tone of cell broth was compared with the red–green–blue (RGB) color chart to check the possibility of contamination. When the seed culture reached a stage at which high cell activity and high cell purity were maintained, a 500 L bioreactor (with a working volume of 250 L) was inoculated with 3% (*v/v*) inoculum and incubated at 30 °C for 5 d under a light intensity of 1,000 Lux. During the cultivation, aeration and agitation rates were ad-

justed on the basis of cell growth and consumption of dissolved oxygen (DO). Air was initially supplied at 55 L/min, and this flow rate was gradually increased to 70, 85, and 100 L/min at 24, 48, and 72 h, respectively. Simultaneously, agitation speeds were gradually increased to 2.19, 3.06, and 3.55×g at 24, 48, and 72 h, respectively.

Simple technique for seed cultivation

With the aim of developing a simple technique for cultivating the seed culture of PSB, cultivation was performed using a simple reactor to explore whether this technique is applicable to seed culturing. The simple reactor was a 15 L, open-type, rectangular glass tank (with a working volume of 10 L) with a continuous air supply at 0.05 vvm with a submersible pump (10 W; UP100, Hyup-Shin Water Pump, Seoul, Korea). The culture medium used in this study was the MYC culture medium (initial pH 7; Ecobiznet Company). The 500 L bioreactor was inoculated with 1% (*v/v*) PSB inoculum and incubated at 30 °C and 0.11×g for 3 d under a light intensity of 1,000 Lux. The final culture broth was tested for its suitability as seed culture.

The possibility of preserving PSB for a long period at 4 °C is important for its practical use. To assess the optimal preservation period, the PSB cultivated in a 500 L bioreactor was preserved at 4 °C for various periods (1, 2, 3, and 4 months, respectively) and then cultivated in a simple pilot-scale reactor (250 L; Ecobiznet Company) to investigate the optimal preservation period to maintain cell activity at 4 °C. The 250 L reactor was made of type 304 stainless steel, and the installed apparatus included those for aeration, agitation, and temperature control with cooling and sterilization. With the touch of a button, sterilization process (with increase in temperature up to 100 °C) started for 2 h after the reactor was filled with the MYC culture medium. Then, the reactor temperature was reduced to 30 °C, followed by inoculation with 1% (*v/v*) seed culture preserved at 4 °C for different periods. This cultivation was automatically continued for 3 d at 30 °C and 0.11×g with an aeration rate of 0.05 vvm under a light intensity of 1,000 Lux according to the reactor system program initially inputted. In this manner, the effect of preservation at 4 °C on cell activity was investigated to determine the optimal preservation period that allows for cell reactivation.

Application of simple culture technique

PSB cultivated using a simple technique was applied to environmental fields to explore its suitability as seed culture. The culti-

vated PSB species was *R. azotiformans* EBN-7, and it was used in a shrimp aquaculture farm (Ganghwa-gun, Incheon, Korea). Before breeding shrimps, *R. azotiformans* EBN-7 was added to the aquaculture farm at 100 mL/ton (with viable cells of $1.75 \pm 0.23 \times 10^9$ CFU/mL) every week to improve water quality, and the concentration of $\text{NH}_4^+\text{-N}$ was measured as an index using the indophenol method (The Korea Law Information Center, 2022).

Analyses

The change in OD of cells during the cultivation period was examined by measuring the absorbance. The OD was determined at 660 nm using a UV-VIS spectrophotometer (Hach, Berlin, Germany) against distilled water as a control. Cell viability were periodically assessed by counting red colonies formed on the solid MYC culture medium, represented as CFU per mL. The pH of the culture broth was also measured. All measurements were performed in triplicate.

Results and Discussion

Growth profile of *Rhodobacter capsulatus* BR

The species *R. capsulatus* is one of the most used PSB with wide applications. Therefore, the growth of *R. capsulatus* BR was first studied on various culture media (Fig. 1). The lag phase was approximately 20 h in all cultivation experiments on various culture media, which was followed by exponential growth, and the maximum growth was observed when a stationary phase was reached (Fig. 1A). The best growth was achieved when *R. capsulatus* BR was cultivated on the basal medium. When cultivated on the basal medium, the number of viable cells of *R. capsulatus* BR maximally reached 4.75×10^9 after 89 h, which was relatively higher than that previously reported— 3×10^8 under dark-aerobic conditions or 3×10^9 under photo-anaerobic conditions (Costa et al., 2017). Although pH change was not significant during the lag phase, pH increased as cells grew exponentially (Fig. 1B), indicating that the change in pH was closely related to cell growth. The trend of change in OD was somewhat different from that in cell number (Fig. 1C). The OD started to increase after 24 h incubation, and thereafter it increased steadily even after the stationary phase of cell growth. Photosynthetic pigments are known secondary metabolites that are not essential for survival but are useful to increase bacterial viability under environmental stress (Orlandi et al., 2021). In the present study, there was a time gap between the maximum

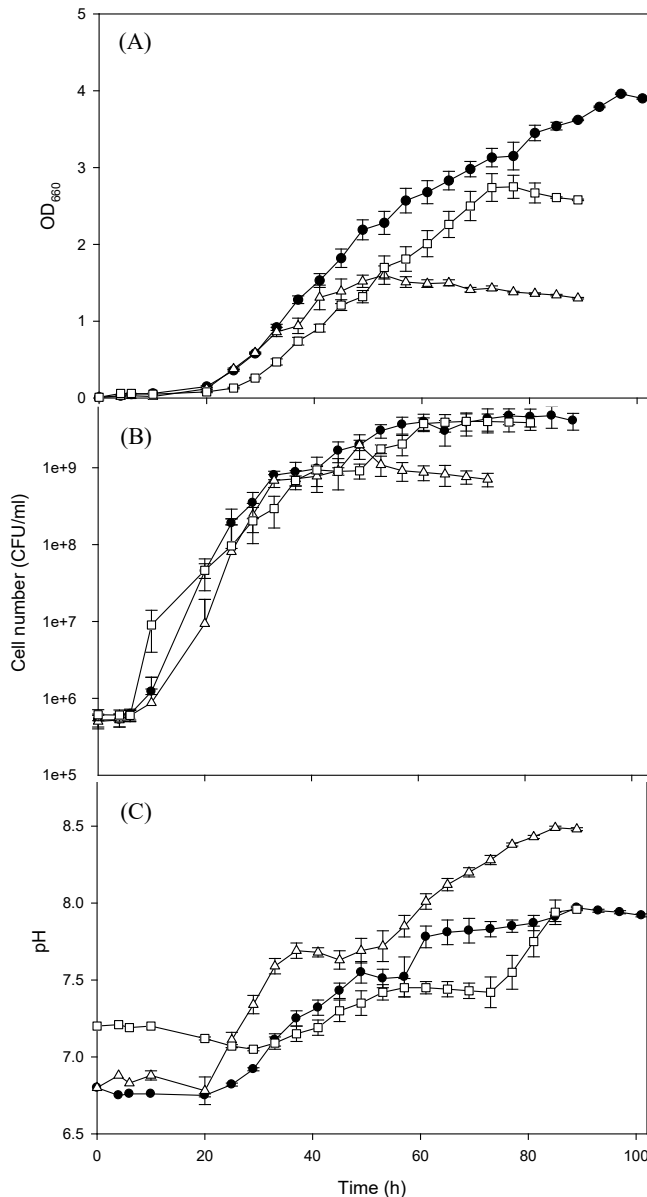


Fig. 1. Growth profiles. OD₆₆₀ (A), number of viable cells (B) and pH (C) of *Rhodobacter capsulatus* BR cultured on various media (basal, closed circles; MYC, open triangles; and van Niel’s, open rectangles). OD, optical density.

cell number and the maximum OD, which signifies a time gap between cell growth and the production of the red pigment by *R. capsulatus* BR. To identify the optimal culture medium for *R. capsulatus* BR, the maximum OD at 660 nm was determined. The maximum OD values were 3.96, 2.75, and 1.60 for the basal medium, van Niel’s medium and MYC medium, respectively. The growth profile of *R. capsulatus* BR on the basal medium

indicates that the maximum specific growth rate was 0.12 h^{-1} . On the basis of the above parameters, growth characteristic of *R. capsulatus* BR were distinctly identified.

Color tone of *Rhodobacter capsulatus* BR

The color tones of *R. capsulatus* BR cultivated on various culture media were explored (Fig. 2). When *R. capsulatus* BR was cultivated on both the basal medium and the MYC medium, distinct changes in the color tone were revealed after approximately 20 h. According to the RGB color chart, the color tone changed from gold (#FFD700) to light coral (#F08080). This result was consistent with previous findings on PSB cultivation in which the color of the culture broth changed within 24 h (Meng et al., 2017). The change in the color tone to red is caused by the production of carotenoids or bacteriochlorophyll *a* and *b* that absorb the

light beyond 800 nm wavelength (Kim et al., 2005; Madukasi et al., 2011; Maoka, 2020). In contrast, the color tone did not perceptibly change when *R. capsulatus* BR was cultivated on van Niel's medium. This retardation in the change in color tone was presumed to result from low growth on this medium, as seen in Fig. 1. The highest red tone (red; #ff0000) was observed in the MYC culture broth at 24 h (Fig. 2B). This difference in red tone may be the effect of casamino acid included only in the MYC medium, indicating that a special nitrogen source is probably critical to exhibit the highest red tone. Because casamino acid is reported to contain a stimulating factor for the growth of PSB (Kumagai et al., 1968), the active growth of *R. capsulatus* BR by the addition of casamino acid may result in a redder tone. All culture media turned red (red; #ff0000) after 29 h cultivation. Subsequently, however, the color tone of *R. capsulatus* BR was different in different media: crimson (#DC143C) in the MYC medium and dark red (#8B0000) in both basal and van Niel's media. Although the cultivation of *R. capsulatus* BR in the MYC medium revealed the highest red tone, the red color tone turned pale at the end of cell growth period owing to low cell growth (as observed in Fig. 1C).

Morphology of *Rhodobacter capsulatus* BR

The morphology of *R. capsulatus* BR cultivated on various culture media for 101 h was explored by microscopy. The cells grown on the basal medium were relatively long (3–4 μm) and 1 μm wide, whereas those grown on the MYC medium were longer (4–5 μm) and less wide (0.5 μm). Cells grown on van Niel's medium were shorter (1–2 μm) and oval (1 μm width). Thus, the morphology of *R. capsulatus* BR varied with the composition of the culture medium. The strain *R. capsulatus* has been reported to exhibit pleiomorphism dependent on environmental conditions. For example, a globular cell shape (below pH 7) changes to a rod shape above pH 7, and the cells lengthen as pH increases (van Niel, 1944). In this study, the strain *R. capsulatus* exhibited morphological changes on different carbon sources. During the cultivation period, cells often show aggregation and settle down, with a change in red tone, when they are contaminated or not properly cultured. Therefore, periodic observations of cell morphology are indispensable because contamination is the primary concern in the scale-up cultivation of PSB. The cultural characteristics (growth profile, color tone, and morphological observations) of *R. capsulatus* BR suggested that the basal medium was more suitable than MYC or van Niel's medium for *R. capsulatus* BR culture.

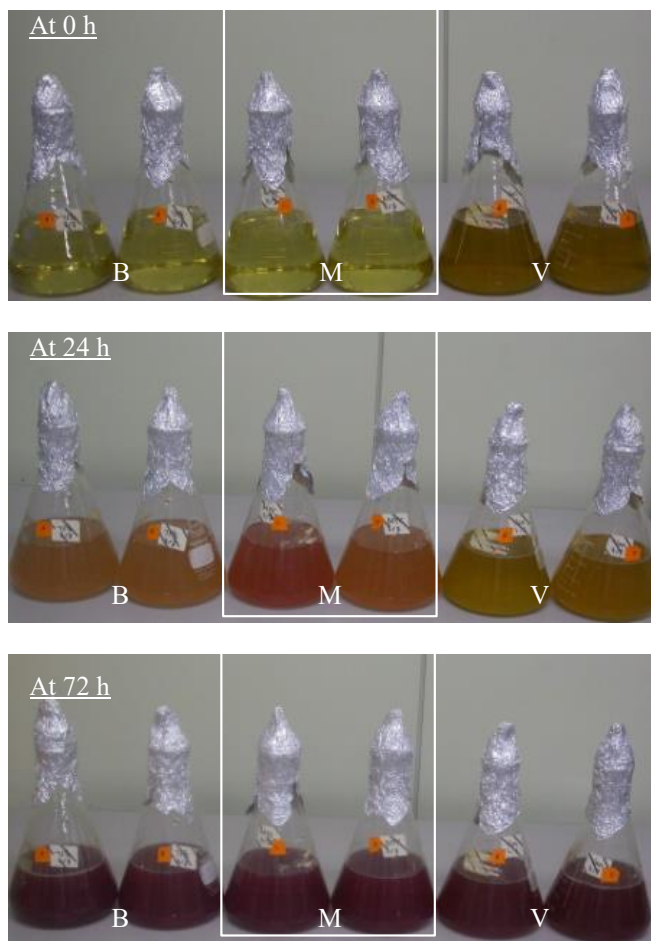


Fig. 2. Change in the color tone of *Rhodobacter capsulatus* BR cultivated on various culture media (B, basal medium; M, MYC medium; and V, van Niel's medium) during cultivation.

Growth characteristics of test photosynthetic bacteria

After the basal medium was determined to be an appropriate medium, four test PSB species were cultivated in a 1 L flask using the basal medium to explore their growth characteristics (Fig. 3). The four PSB species showed logarithmic growth after a lag phase of approximately 15 h. Compared with the previous cultivation experiment, the duration of the lag phase was reduced owing to preliminary adaptation in the same culture medium. The measured maximum OD₆₆₀ values were as follows: 6.67 for *R. azotoformans* EBN-7 after 109 h; 3.58 for *R. capsulatus* BR after 154 h; 5.15 for *R. sphaeroides* BL6 after 141 h; and 6.30 for *R. sphaeroides* GB1 after 154 h (Fig. 3A). On the basis of the OD₆₆₀ values, the specific growth rate of *R. azotoformans* EBN-7 was calculated to be 0.20 h⁻¹, which was superior to that of *R. capsulatus* BR (0.13 h⁻¹), *R. sphaeroides* BL6 (0.17 h⁻¹) or *R. sphaeroides* GB1 (0.18 h⁻¹). These specific growth rates are fairly good in comparison with previously reported values: the specific growth rate of *R. sphaeroides* was 0.08 h⁻¹ when cultivated in a medium containing succinate as a C source and (NH₄)₂SO₄ as an N source (Jeon & Kim, 2010), and that of *R. sphaeroides* was 0.12 h⁻¹ when cultivated in the modified Sistrom's minimal medium containing trace elements and vitamins (Orsi et al., 2019). Thus, the basal medium provided test PSB with sufficient nutrition, and *R. azotoformans* EBN-7 was selected to be used for scale-up cultivation.

In the cultivation of all four PSB species, pH tended to increase gradually with cell growth (Fig. 3B). However, the tendency of increase in pH diminished after 80 h, except in *R. azotoformans* EBN-7. The time point at which the increase in pH diminished was almost consistent with that at which the increase in OD₆₆₀ was reduced, particularly in the cultivation of *R. capsulatus* BR. After 154 h, the maximum pH values of 8.53, 8.09, 8.02 and 7.85 were recorded for *R. azotoformans* EBN-7, *R. sphaeroides* BL6, *R. capsulatus* BR, and *R. sphaeroides* GB1, respectively. The highest numbers of viable cells of four PSB species were 8.75 × 10⁹ after 80 h for *R. azotoformans* EBN-7, 6.25 × 10⁹ after 130 h for *R. capsulatus* BR, 1.10 × 10¹⁰ after 92 h for *R. sphaeroides* BL6, and 8.75 × 10⁹ after 130 h for *R. sphaeroides* GB1, respectively (Fig. 3C). The cultivation time to reach the highest number of viable cells was lower than that required to reach the maximum OD₆₆₀ owing to the difference in cell materials used to measure these parameters. In all species, the highest number of viable cells reached over 1 × 10⁹ between 32 h and 40 h. In a parallel experiment, the maximum dry-cell weight of *R. azotoformans* EBN-7 was observed to be 3.56 g/L after 109

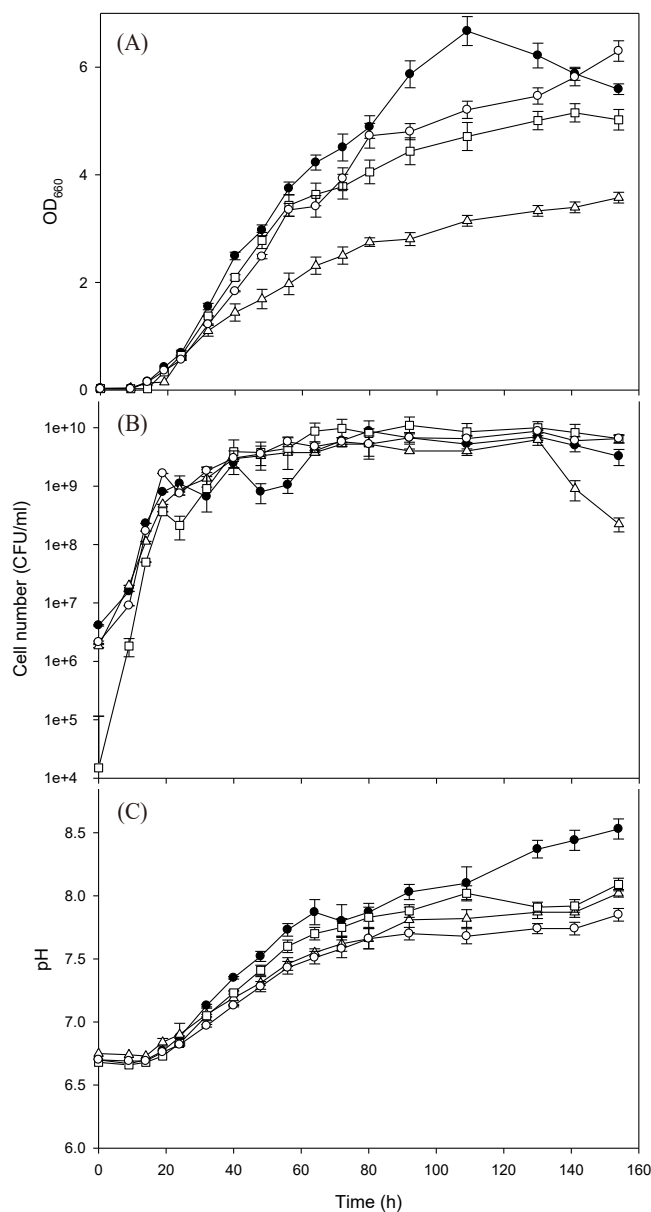


Fig. 3. Changes in OD₆₆₀ (A), number of viable cells (B) and pH (C) of four test photosynthetic bacteria species (*Rhodobacter azotoformans* EBN-7, closed circles; *Rhodobacter capsulatus* BR, open triangles; *Rhodobacter sphaeroides* BL6, open rectangles; and *R. sphaeroides* GB1, open circles) cultivated on basal medium. OD, optical density.

h, whereas those of *R. sphaeroides* BL6, *R. capsulatus* BR, and *R. sphaeroides* GB1 were 3.49, 2.47, and 4.88 g/L, respectively, after 154 h. This tendency of maximum dry-cell weight was similar to that of maximum OD₆₆₀ value for each PSB species. Therefore, there was a time lag between the point at which dry-cell

weight or OD₆₆₀ reached the maximum value and the point at which the highest number of viable cells was observed.

Color tone of test photosynthetic bacteria

The four PSB species started to reveal a red tone after 19 h of cultivation, reached its maximum (Indian red; #CD5C5C) between 32 and 40 h, and thereafter the change in the red tone was not significant. The cultivation time to reach the highest red tone was similar to that required to reach the maximum number of viable cells (over 1×10^9 CFU/mL). After 154 h of cultivation, *R. capsulatus* BR culture broth was tinged with dark red (dark red; #8B0000), whereas *R. sphaeroides* GB1 culture broth was tinged with light red or orange red (crimson; #DC143C). The species *R. azotoformans* EBN-7 and *R. sphaeroides* BL6 lay almost halfway between the two red tones.

Microscopic observation of test photosynthetic bacteria

The morphological characteristics of the four PSB species cultivated on the basal medium were observed under a microscope at 154 h of cultivation (Fig. 4). The species *R. azotoformans* EBN-7 and *R. sphaeroides* BL6 were short rods (1 μ m wide and 1–2 μ m long), whereas the other two species were relatively longer rods (1 μ m wide and 3–4 μ m long for *R. capsulatus* BR; and 1 μ m wide and 1.5–2 μ m long for *R. sphaeroides* GB1, respectively). The motility of the longest rod, *R. capsulatus* BR, was inferior to that of the other three species. In addition, *R. capsulatus* BR formed 1–2 chains. On the basis of these findings, the species *R. azotoformans* EBN-7 was found to be the most appropriate for scale-up cultivation, with the highest specific growth rate (0.20 h^{-1}). Therefore, *R. azotoformans* EBN-7 was used for scale-up cultivation in the subsequent experiments.

Scale-up cultivation of *Rhodobacter azotoformans* EBN-7

Following the selection of an ideal PSB species and an optimal

culture medium, scale-up cultivation was performed in a 500 L reactor for 96 h (Fig. 5). At approximately 15 h after inoculation, *R. azotoformans* EBN-7 cells grew exponentially. This growth tendency was consistent with that observed in laboratory-scale cultivation. The pH reached 8.14 after 16 h and thereafter was intentionally maintained at 7. This pH was decided on the basis of the pH (8.1) at the maximum OD₆₆₀ in laboratory-scale cultivation to avoid the retardation in cellular growth when the optimum pH was not maintained. The DO level decreased rapidly from the beginning of cultivation, and increased to 70% saturation immediately after simultaneously increasing the air flow rate and agitation speed at 24 h. However, DO level again declined rapidly owing to active cell growth and did not recover after 48 h, indicating that cells consumed more oxygen than that supplied. Further increase in aeration rate and agitation speed had an adverse effect on biomass concentration owing to cell aggregation. At the end of cultivation at 96 h, the number of viable cells reached 4.50×10^{10} (Fig. 5). Therefore, the scale-up cultivation resulted in a slightly higher number of viable cells with a slightly lower specific growth rate (0.15 h^{-1}) than those obtained during cultivation in a 1 L flask. This slightly reduced value in the specific growth rate during scale-up experiments has also been reported in previous studies. The specific growth rate was decreased from 0.21 h^{-1} to 0.18 h^{-1} when *R. capsulatus* cultivated in a 1 L flask was transferred to a 500 L bioreactor (Cho et al., 2006). In addition, the specific growth rate of *Rhodospseudomonas palustris* decreased from 0.12 h^{-1} (in 1 L culture) to 0.068 h^{-1} (in 5 L culture) in batch-type bioreactors (Kim & Lee, 2000). This denotes the difficulty in scale-up cultivation.

Effect of preservation at 4°C on cell activity

Prior to application to environmental fields, mass-cultivated PSB must be preserved. Moreover, it is cost effective if they can be preserved simply by storing at 4°C without reduction in cell

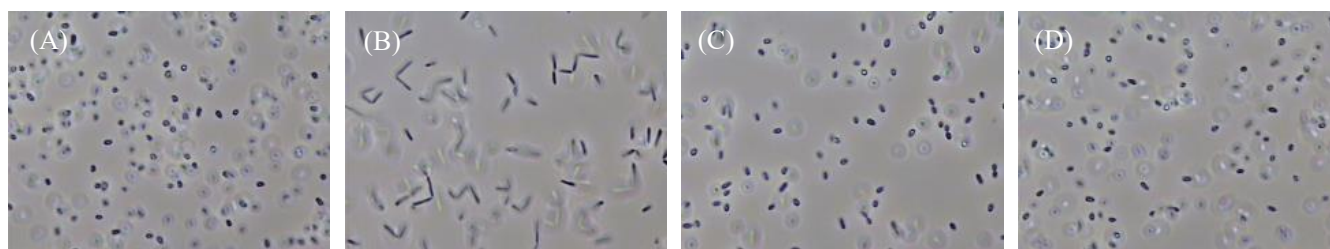


Fig. 4. Microscopic photographs of four live photosynthetic bacterial species cultivated on basal medium at 154 h (1,000 \times). *Rhodobacter azotoformans* EBN-7 (A), *Rhodobacter capsulatus* BR (B), *Rhodobacter sphaeroides* BL6 (C), and *R. sphaeroides* GB1 (D).

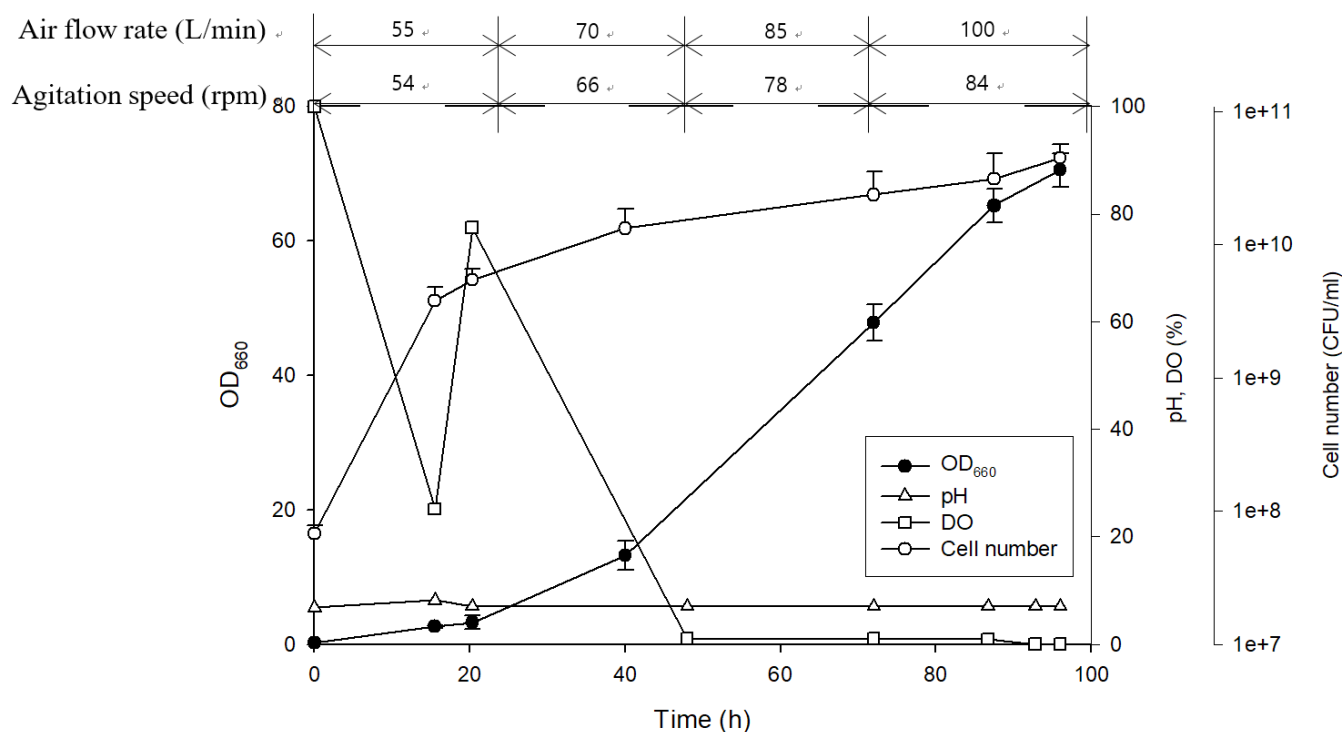


Fig. 5. Changes in OD at 660 nm (OD₆₆₀) and number of viable cells with pH and DO during the cultivation of *Rhodospirillum rubrum* EBN-7 in a 500 L reactor. OD, optical density; DO, dissolved oxygen.

activity. Therefore, *R. azotoformans* EBN-7 preserved at 4°C for different periods was used as seed culture for cultivation in a 250 L bioreactor to assess the extent of cell reactivation (Fig. 6). All cultivation systems using seed cultures preserved for different periods exhibited OD₆₆₀ in the range of 1.02–1.56 and the number of viable cells in the range of 6.25 × 10⁸–1.75 × 10⁹. When used as seed culture, the cell reactivation ability of *R. azotoformans* EBN-7 decreased with increasing storage period, resulting in a decrease in both levels of cell growth and the number of viable cells in comparison with control PSB (not preserved). This phenomenon was severer when cells preserved for 4 months were seeded. The effect of preservation period on cultivation was also observed on color tone. The final culture broths exhibited a dark red (#8B0000) color after cells preserved for 1 to 3 months were seeded. However, the final culture broth exhibited the an Indian red (#CD5C5C) color after cells preserved for 4 months were seeded. Moreover, the cultivation system using seed culture preserved for 4 months exhibited two-phase separation (transparent in the upper phase and thick in the lower phase) and developed an odor. This likely occurred because of the prolonged maintenance under dark and anaerobic conditions. In conclusion, for use as seed culture, the

maximum allowable period of preservation at 4°C for *R. azotoformans* EBN-7 was 3 months.

Application of a simple culture technique

R. azotoformans EBN-7 was cultivated in a simple 15 L reactor to develop a simple culture technique for seed culture. *R. azotoformans* EBN-7 cultivated in a 500 L reactor was used as seed culture. As the seeded *R. azotoformans* EBN-7 grew, the color turned red (crimson; #DC143C) after 3 d (Fig. 7). This indicates that *R. azotoformans* EBN-7 easily proliferated in the reactor, which was confirmed by microscopic observation. After *R. azotoformans* EBN-7 was cultivated by the simple technique, it was applied to a shrimp aquaculture farm for the removal of NH₄⁺-N from the water to test its potential. In the control group, the initial concentration of NH₄⁺-N in the shrimp aquaculture water was 0.25 mg/L; the concentration of NH₄⁺-N increased to 0.27 mg/L after 6 d and to 0.61 mg/L after 10 d (Fig. 8). In the treatment group, the cultivated *R. azotoformans* EBN-7 was applied at day 6 (0.27 mg/L of NH₄⁺-N), and the NH₄⁺-N concentration was reduced to 0.24 mg/L at day 10. Therefore, the treatment of shrimp aquaculture water with the cultivated

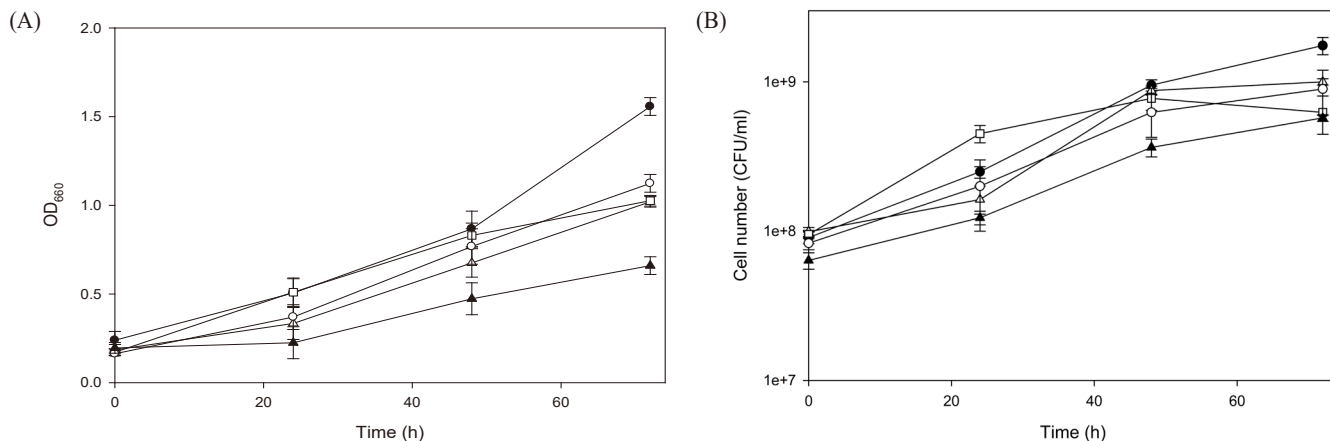


Fig. 6. Changes in OD at 660 nm (OD₆₆₀) (A) and number of viable cells (B) during cultivation using the seed culture variously preserved for 0 month (closed circles), 1 month (open triangles), 2 months (open rectangulars), 3 months (open circles) and 4 months (closed triangles), respectively. OD, optical density.

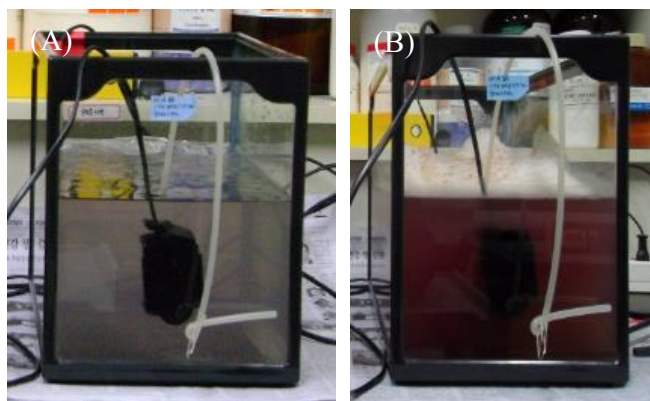


Fig. 7. Change in color tone in a simple reactor during the cultivation of *Rhodobacter azotoformans* EBN-7: at day 0 (A) and at day 3 (B).

R. azotoformans EBN-7 resulted in a 60.7% reduction in 4 d in comparison with the control. In aquaculture water treatment, ammonia-oxidizing archaea and anaerobic ammonia-oxidizing bacteria play a key role in the removal of nitrogen substances (Lu et al., 2021). Among those microorganisms, PSB were reported to reduce ammonia accumulation in an aquaculture pond. The NH₄⁺-N content in the treatment group decreased by 29.81 % in comparison with control when *R. palustris* was applied to grass carp aquaculture water (Zhang et al., 2014). The potential ability of PSB in NH₄⁺-N removal were also reported in other applications. Idi et al. (2015) demonstrated the ability of *R. sphaeroides* ADZ101 in the treatment of a synthetic wastewater containing

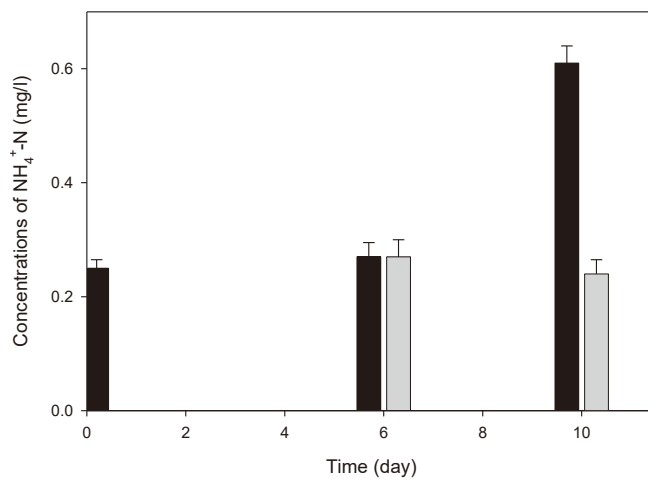


Fig. 8. Application of *Rhodobacter azotoformans* EBN-7 (grey bars) cultivated using a simple technique to an aquaculture farm against control (black bars). The photosynthetic bacteria were added to the treatment system at day 6.

NaNO₃ as the sole nitrogen source. During the 7 d cultivation, the strain *R. sphaeroides* ADZ101 maximally removed 62% of NH₄⁺-N from an initial concentration of 52 mg/L under anoxic growth conditions. In addition, Zhou et al. (2015a) reported that 83.2% NH₄⁺-N was removed after 4 d when an ammonia-tolerant photosynthetic bacterium (ISASWR2014) was applied to chicken manure wastewater, which indicates a higher NH₄⁺-N removal efficiency than that in our study. This difference may be caused by the difference in PSB species used

for treatment. Moreover, the seed culture of PSB species used in this study was cultivated using a simple technique, and thus its quality may be somewhat lower than the PSB cultivated in a bioreactor under optimal conditions. Nevertheless, the cultivation of *R. azotoformans* EBN-7 using a simple culture technique exhibited a relatively good removal efficiency of $\text{NH}_4^+\text{-N}$. This enables seed culture prepared by a simple culture technique to be easily used for public.

Conclusion

PSB are diversely utilized owing to their versatile metabolic abilities; nevertheless, their cultivation is difficult. Therefore, a generalized culture technique has not been previously reported. Considering the importance of cultivation, a simple culture technique suitable for public use was investigated. The strain *Rhodobacter azotoformans* EBN-7 was found to be the most appropriate for scale-up cultivation, with the highest specific growth rate (0.20 h^{-1}) on basal medium, and it showed 4.50×10^{10} colony-forming units mL^{-1} (number of viable cells), dry cell weight of 26.8 g/L , and a specific growth rate of 0.15 h^{-1} in scale-up cultivation (500 L). As seed culture, this final culture broth was successfully cultivated in a 15 L simple reactor, with maintenance of cell activity evident. The maximum allowable preservation period of *R. azotoformans* EBN-7 at 4°C was 3 months for use as seed culture. In this study, cultivation of *R. azotoformans* EBN-7 in a simple reactor and preservation of *R. azotoformans* EBN-7 at 4°C for < 3 months exhibited its potential in the removal of $\text{NH}_4^+\text{-N}$ by 60.7% (from 0.61 to 0.24 mg/L) in shrimp aquaculture water, allowing its use as seed culture without reduction in cell growth potential. Therefore, this simple technique showing a good removal efficiency of $\text{NH}_4^+\text{-N}$ would considerably contribute to the dissemination of useful PSB for varied applications.

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate

This article does not require IRB/IACUC approval because there are no human and animal participants.

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