Thesis for the Degree of Doctor of Philosophy

# Isolation, cultivation and cell-viability maintenance of useful bacteria suitable for treatment of aquaculture wastewater

by

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School of Marine Fisheries Life Science

(Major in Biotechnology)

The Graduate School

Pukyong National University

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# (양식장 폐수처리에 적합한 유용 미생물의 분리, 배양 및 세포 활성유지)

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by

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A dissertation

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# 양식장 폐수처리에 적합한 유용 미생물의 분리, 배양 및 세포 활성유지

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## 요약

수산물은 건강에 이로운 단백질원으로서 많은 사람이 선호하는 식품으로 그 수요가 매년 증가하였다. 이에 따라 어획량이 이 수요를 감당하기 어려워짐으로써, 양식을 통한 수산물 생산이 크게 증가하고 있으며, 양식 산업은 미래의 식품을 책임질 4차 산업으로의 중요성이 주목받고 있다. 그러나 양식 산업의 발전은 수산식품의 소비를 증가시키게 되었고, 이에 따른 폐기물 및 폐수 발생량도 증가하게 되어, 환경보존 및 양식 수산물 생산 증대를 위한 효율적 폐기물/폐수처리의 필요성이 생겼다.

효율적이며 친환경적인 양식장 폐기물/폐수처리를 위해, 미생물을 이용한 폐수처리 기술들이 많이 개발되고 있으며, 갯지렁이 내장 속에 서식하는 미생물들과 광합성 세균들은 해수 양식장에서 배출되는 폐수를 처리하는데 있어 아주 유용한 미생물로 알려져 있다. 따라서 본 연구에서는 양식장 폐수처리에 적합한 유용 미생물들을 분리하고, 대량배양 및 단순배양 기술로 이 미생물들의 폐수 처리 용이성을 높이고, 또한 세포 활성유지 기술을 이용하여 실제 새우양식장 폐수처리에 투입하였을 때의 처리 효율을 높이고자, 유용 미생물을 이용한 양식장 폐수처리의 실용화 가능성을 연구하였다.

Chapter 1에서는, 새우양식장 바닥에 서식하면서 퇴적물을 정화하는 능력을 가진 갯지렁이(*Marphysa sanguinea*) 내장으로부터 유용 미생물들을 분리하여, 그 미생물들의 폐수처리 능력을 알아보았다. 갯지렁이 내장으로부터 얻은 샘플을 6

가지 다른 영양배지에서 배양하여, colony 모양 및 현미경 관찰을 통하여 다른 특성을 가진 균주를 총 87개 분리하였고, 이 균주 중 성장이 우수하고, 단백질 및 지질 분해능이 있는 균주를 탐색한 다음, 상호간 길항작용이 없는 균주를 최종적으로 8개 얻었다. 여러 가지 성분을 포함하는 양식장 폐수 특성에 맞추기 위하여, 최종적으로 얻은 8개 균주를 다양한 구성을 가진 12개의 다른 미생물 조합으로 설계한 다음 유사 양식장 폐수에 적용했더니, 단백질 분해능이 우수하며 생균수($4.2 \times 10^6$ CFU/mL)가 높은 미생물 조합은 *Bacillus paramycoides*, *B. pumilus*, *Stenotrophomonas sp.*, *B. paranthracis* 2종 및 *Micrococcus luteus*를 함유하는 6개 균주의 조합이었다. 이 복합미생물군을 이용한 새우양식 폐수 처리 결과, 처리 12시간 만에 COD와 TN이 각각 51.0%, 44.6% 제거되었고, C/N비는 10으로 거의 일정하게 유지되어, 이 복합미생물군이 안정적으로 폐수를 처리할 수 있음을 보여주었다. 따라서 갯지렁이 내장에서 분리한 유용 미생물들은 새우양식장 폐수처리에 적합함을 확인할 수 있었다.

Chapter 2에서는, 수질정화 능력이 우수하며, 농축산분야에서 생물학적 복원에 뛰어난 역할을 하는 유용 미생물로서 보고된 광합성세균은 고부가가치의 미생물이지만 순수배양의 어려움으로 단순하게 배양하기가 까다로워 이 미생물의 활용도를 떨어뜨리고 있다. 따라서 이에 관한 배양기술의 개발 및 개발 기술의 보급 필요성이 크므로, 이 연구에서는 현장 투입에 보다 용이하게 사용할 수 있는 배양 방법 및 용이하게 배양된 광합성세균의 실제 새우양식장 폐수처리에 있어서의 처리효율에 대해 알아보았다. 사용한 광합성세균 중, scale-up 생산에 가장 적합한 균주는 *Rhodobacter azotoformans* EBN-7이었으며, Basic medium에서 가장 높은 비증식속도(0.20/h)를 나타내었다. *R. azotoformans* EBN-7 균주는 500 L 대규모 배양에서, $4.50 \times 10^{10}$ CFU/mL, 건조 세포 중량 26.8 g/L 및 비증식속도(0.15/h)를 나타내었다. 여기서 얻은 최종 배양액을 종균으로 사용하여 15 L 단순 배양기에서 배양한 결과, 배양 3일 만에 광합성세균이 우점하는 것을 현미경 관찰을 통해 확인하였다. 현장 폐수처리에 투입할 활성 있는 종균 생산을 위한 *R. azotoformans* EBN-7의 최대 허용 보존기간은 4 ℃에서 3개월로 조사되었다. 250 L 간이 배양기에서 배양한 *R. azotoformans* EBN-7을 새우양식장 폐수에 적

용했을 때, $NH_4^+$-N은 대조군 대비 60.7%로 크게 감소하였다. 따라서 새우양식장 폐수처리에 있어, 단순 배양기술에 의해 배양된 *R. azotoformans* EBN-7 균주가 효과적으로 사용될 수 있음을 알 수 있었다.

Chapter 3에서는, 유용 미생물의 폐수처리를 위한 현장 투입 시, 세포의 활성을 길게 유지할 수 있는 방법들을 알아보았다. 이 연구에 사용한 유용 미생물은 광합성세균이었고, 미생물 고정화 및 동결건조 기술을 이용하여 광합성세균의 세포활성이 길게 유지할 수 있도록 하였고, 또한 현장 적용을 통해 폐수처리 효율을 조사하였다. 광합성세균 고정화에 사용되는 고정용액들 중, 0.1 M $CaCl_2$는 환경오염이 없고, 기질 확산성, 세포 충전에 유리한 크기 (4 mm) 및 비용 효율성 측면에서 가장 효과적인 2가 금속 이온 용액으로, 이 용액을 사용하여 제조된 비드는 비교적 높은 생균수($1.91 \times 10^9$ CFU/mL)를 갖는다. 생성된 광합성세균 비드의 보관방법으로는 4 ℃, 3.5% NaCl 용액에서 가장 높은 세포 생존율을 나타내었다. 동결건조 방법에서는 탈지유(9%)와 포도당(2%)을 동결보호 첨가제로 첨가했을 때, 가장 높은 세포 생존력이 확인되었다. 최적 조건으로 제조된 양식장 저질개선용 광합성세균 비-드와 양식수 처리용 동결건조 분말을 18주간의 새우 사육 양식장에 적용했을 때, 대조군에 비해 $NH_4^+$는 55% 제거되었고, $NO_3^-$ 및 $NO_2^-$는 10 % 제거되었다. 따라서 최적 조건의 세포 고정화 및 동결건조 방법은 광합성세균의 활성을 더 길게 유지시킬 수 있었으며, 그 결과 효과적인 *in situ* 새우양식장 물 처리가 가능함을 보였다.

이상의 Chapters 1-3의 연구를 통해, 갯지렁이 내장에 서식하는 미생물들의 적절한 복합미생물조합이 유용하게 해수 양식장 폐수처리에 사용될 수 있음을 확인하였고, 배양에 어려움이 있는 광합성 세균의 간단 배양기술을 통한 종균 생산 방법은, 양식장 폐수처리 현장 투입 시, 광합성 세균의 유용성을 더 용이하게 할 수 있었으며, 미생물 고정화 및 동결건조 조건의 최적화는 광합성 세균의 현장 투입 시, 보다 안정적으로 길게 세포활성을 유지할 수 있어, in situ 양식장 물 처리에 아주 유용함을 확인할 수 있었다. 따라서 본 연구에서 얻은 결과들로부터, 지속가능한 수산양식에 있어서의 유용 미생물과 관련 기술들의 개발 및 실용화의 중요성을 확인하였다.

# CHAPTER I

# Identification of bacteria isolated from rockworm viscera and application of isolated bacteria to shrimp-aquaculture wastewater treatment

# Abstract

Useful microorganisms were isolated from the viscera of the rockworm(*Marphysa sanguinea*), which inhabits the bottom of the shrimp aquaculture and has the ability to purify sediment, and investigated the wastewater treatment ability of the microorganisms. Samples obtained from the viscera of rockworms were cultured in 6 different nutrient media, and a total of 87 isolates with different characteristics were isolated through colony shape and microscopic observation. Among these isolates, isolates with excellent growth and protein and lipid degradation. After searching, 8 isolates without mutual antagonism were finally obtained.

In order to match the characteristics of farm wastewater containing various components, the finally obtained 8 isolates were designed as 12 different microbial combinations with various compositions and then applied to similar aquaculture wastewater. As a result, the microbial combination with excellent protein degrading ability and high viable cell number($4.2 \times 10^6$ CFU/mL) was a combination of 6 isolates including *Bacillus paramycoides, B. pumilus, Stenotrophomonas sp.,* 2 *B. paranthracis, and Micrococcus luteus.* As a result of wastewater treatment of shrimp farming using this complex microorganism, COD and TN were removed by 51.0% and 44.6%, respectively, after 12 hours of treatment, and the C/N ratio was maintained almost constant at 10, so that this complex microorganism group was stably, it has been shown that wastewater can be treated.

# 1. Introduction

Aquaculture is a potential solution for providing poor and food-insecure populations around the world with relatively cheap proteins (Kobayashi et al., 2015). This important role of aquaculture made it become the fastest growing sector in the past few decades (Ngo et al., 2016). As the consumption of fish recognized as healthy foods steadily increases, farmed fish production for human consumption had surpassed wild capture fish at last (World Bank, 2014). Although aquaculture brings benefits to humans, its flourish causes some problems. This is because a large amount of waste and wastewater is generated from the aquaculture industry, negatively exerting environmental impacts on aquatic ecosystems. These impacts include the pollution of nearby waters, alteration in habitat, transmission of diseases and parasites to wild populations, accidental release of exotic species, etc. (Diana, 2012). Aquaculture wastewater generally possesses different composition as well as concentration, compared with that of municipal or industrial wastewater. Marine aquaculture wastewater contains a large amount of nitrogen derived from fish excreta and feed residue (Schwartz and Boyd, 1994). In shrimp aquaculture, 5345 - 7157 $m^3$ of wastewater can be typically discharged per a ton of shrimp production (Anh et al., 2010). Therefore, developed countries have intensified strict regulations for effluent discharges from aquaculture, but most developing countries have not been ready for this policy yet (Ngo et al., 2016). In view of environmental and social aspects, an

efficient treatment of the effluent discharges is essential to develop sustainable aquaculture.

Polychaetes are characterized to be involved in purifying deposits through their feeding behavior, and thereby mitigate the environmental impact from pollutions. This role of polychaetes was reported in the bioremediation of deposited waste at the bottom of fish ponds (Kinoshita et al., 2008). Therefore, polychaetes are ecologically important taxa particularly in marine benthic communities (Giangrande et al., 2005). Their degradation ability for organic pollutants is derived from gut microbes including *Capitella teleta* (Hochstein et al., 2019) and *Osedax* (Rouse et al., 2004). A polychaete species, *Nereis*, was reported to efficiently utilize unconsumed feed and fecal materials collected from a marine recirculating system (Bischoff et al., 2009). In addition, *Bacillus* sp. strain EBW4 isolated from a polychaete, *Perinereis aibuhitensis*, exhibited the potential ability to degrade organic hydrocarbons (Shin et al., 2013). Moreover, polychaetes are often used for detecting marine pollution as indicators, and show excellent potential not only in accumulation of bioavailable heavy metals from benthic habitats (Rainbow, 1995), but also in tolerance for high levels of organic compounds in soft bottoms (Tomassetti and Porrello, 2005).

As polychaete species, rockworms are filter feeders showing potential ability to degrade environmental pollutants. It was reported that extracellular enzymes produced by bacteria present in the excrement of a rockworm, *Marphysa sanguinea* exhibited some

potential degradation of polycyclic aromatic hydrocarbons (Onozato et al., 2012). Besides, rockworms are used as live bait for fishing, and they are an important nutrient source for stimulating gonad maturation and spawn in both fish and crustaceans for hatching. However, the total supply of rockworms does not meet the demand, since the natural production of rockworms is somewhat limited not only due to their poor tolerance at low temperatures, but also due to excessive hunting (Predevelli, 1994). Therefore, rockworms attract much attention recently for the purpose of aquaculture production as well as the development of related rearing technology. Rockworms exhibit that their food intake is inactive in a larval stage, whereas their food intake is active in a juvenile stage. According to the study of earthworm (Aira et al., 2016), bacterial community in the earthworm gut changes significantly when earthworms are fed on diverse substrates. Therefore, a gradual change in the rockworm gut flora is presumed at the juvenile stage. This gut flora has also been known to play an important role in the immune system, influencing the rockworm life cycle (McFall-Ngai et al., 2013). Therefore, rockworms are considered to be beneficial resources for water purification as well as nutritiously valuable resources.

Considering the potential ability of rockworms in the degradation of environmental pollutants, microorganisms inhabited in the rockworm viscera are good candidates for wastewater treatment. Therefore, symbiotic viscera microorganisms are considered to be usefully applied to purify shrimp aquaculture effluent (Robinson et al., 2015). A total of

43 phylums, 105 classes, 263 orders, 606 families, and 1265 genera were identified in the shrimp farm biofloc. Bacteroidetes was the most dominant with 26.5%, followed by Alphaproteobacteria, Chloroflexi and Actinobacteria(National Fisheries Research & Development Institute, 2014). However, there has been little study on the role of microorganisms inhabited in the rockworm viscera. In this study, therefore, native bacteria were isolated from rockworm (*M. sanguinea*) viscera to utilize their potential ability of water purification. Among the isolates, ones showing potential ability were first sorted and identified. After then, the identified isolates were applied to the shrimp aquaculture wastewater to evaluate their potential ability.

# 2. Materials and Methods

## 2.1. Isolation of microorganisms and culture conditions

Rockworms (*Marphysa sanguinea*) in different growth stages were collected from the Fisheries Sciences Technology Center (Pukyong National University, Goseung, Korea). The collected rockworms were washed with PBS buffer solution (1X, pH 7.4, Thermofisher Scientific, USA) and 70% (v/v) ethanol in order. After skins of the washed rockworms were cut lengthwise, internal parts were dissolved in the PBS buffer solution to achieve microorganisms present in viscera. The prepared sample was centrifuged at 15,520 $x\,g$ for 10 min. To detect microorganisms even being in little existence, the supernatant was poured onto various solid culture media: nutrient broth agar (Neogen, USA); skim milk agar (Bioshop, Canada); laminarin agar (TCI, Japan); alginate agar (Sigma-Aldrich, USA); spirit blue agar (Sigma-Aldrich, USA); and phenol red tween agar (Sigma-Aldrich, USA) media. The nutrient broth agar medium was consisted of 8 g/L nutrient broth and 15 g/L agar (Junsei, Japan). The skim milk agar medium was consisted of 10 g/L skim milk and 15 g/L agar. The laminarin agar medium was contained 1 g/L laminarin, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NaCl, 0.1 g/L $CaCl_2$, 2 g/L $(NH_4)_2SO_4$, 0.5 g/L $KH_2PO_4$, 0.5 mL/L mineral solution, 0.5 mL/L vitamin solution, and 15 g/L agar (pH 6.8). The alginate agar medium was consisted of 0.5 g/L peptone (Bioshop, Canada), 0.1 g/L sodium alginate, and 15 g/L agar (pH 6.8). The spirit

blue agar medium was consisted of 32.15 g/L SBA and 30 mL/L tributyrin (TCI). The phenol red tween agar medium was contained 8 g/L nutrient broth, 10 mL/L tween, 0.018 g/L phenol red and 15 g/L agar (pH 6.8). All agar plates were incubated at 25 ℃ for 72 h. Colonies formed on each agar medium were sorted out by both morphological difference under microscopic observation and colonial shape. The sorted colonies were finally streaked onto fresh nutrient broth agar media, and then each isolated colony was used for identification.

## 2.2. Characterization and identification of isolates

To characterize each sorted colony, Gram staining, stabbing into nutrient broth agar medium and microscopic observation were performed. In addition, degradation ability of each colony on polymers was conducted using carboxymethyl cellulose agar (Sigma-Aldrich) skim milk agar and spirit blue agar media. The carboxymethyl cellulose agar medium was consisted of 2 g/L peptone, 5 g/L yeast extract, 1 g/L CMC, and 15 g/L agar (pH 6.8). Ten microliters of each isolate grown in a late-log growth phase were dropped into 8 mm paper discs (Advantec, Japan), and then incubated at 25 ℃ for 24 h. After incubation, the sizes of clear zones formed on carboxymethyl cellulose agar and skim milk agar media were measured for cellulose and protein degradation ability, respectively, and formation of blue colonies on spirit blue agar medium was observed for lipid degradation

ability. All the isolates were incubated at 25 ℃ for 24 h after cross-streaked on the nutrient broth agar medium. To check the occurrence of antagonism among the isolates, possibility of any hindrance in cell growth was observed after the incubation. In this process strains that inhibit other strains were excluded.

To identify the isolates, 16S rRNA sequence analysis was conducted at Cosmogenetech (Seoul, Korea). The used primers were universal oligonucleotides corresponding to the 27F (5′ AGA GTT TGA TCC TGG CTC AG 3′), 1492R (5′ GGT TAC CTT GTT ACG ACT T 3′). The sequences of isolates were matched with the 16S-based ID in EzBioCloud (https://www.ezbiocloud.net/), and the identified 16S rRNA sequence of each isolate was used to constitute a phylogenetic tree using MEGA version 7. The sequences were first aligned with Clustal W using BioEdit program (Version 7.2.0), and then a phylogenetic tree was constituted by neighbor-joining tree analysis with 1,500 bootstrap replicates.

## 2.3. Treatment of shrimp aquaculture wastewater

To examine the potential degradation ability of microorganisms present in rockworm viscera, selected isolates were applied to treat shrimp aquaculture wastewater. Shrimp aquaculture wastewater was simulated using formulated diet for Whiteleg shrimp (*Litopenaeus vannamei*). The Whiteleg shrimp diet was grinded and sieved through 38 μm mesh. The Whiteleg shrimp diet prepared in this way consisted

of: 38% crude protein; 5% crude lipid; 2.7% phosphorus; 1.2% calcium; 4.0% crude fiber; and 17% crude ash. The shrimp aquaculture wastewater was prepared by dissolving 1 g Whiteleg shrimp diet in 500 mL distilled water. Afterwards, the shrimp aquaculture wastewater was autoclaved at 121 ℃ for 15 min. Prior to the treatment of shrimp aquaculture wastewater, isolates were preliminarily adapted in the shrimp aquaculture wastewater at 25 ℃ for 24 h. The adapted isolates (10 %, v/v) were seeded into 1 L flask containing 500 mL autoclaved shrimp aquaculture wastewater, and the flask was incubated at 25 ℃ and 130 rpm for 48 h. Samples were periodically taken and reaction parameters were determined.

## 2.4. Analytical methods

During the treatment of shrimp aquaculture wastewater, reaction parameters (pH), optical density (OD), viable cell number, chemical oxygen demand (COD) and total nitrogen (TN) were determined to examine the treatment ability of isolates. The pH and OD of each sample collected from the flask were measured by pH meter (Mettler Toledo, Switzerland) and spectrophotometer (Hanson Technology Co., Korea) at 600 nm, respectively. The collected sample was also appropriately diluted with sterile distilled water, spread on the nutrient broth agar medium, and incubated at 25 ℃ for 48 h to determine viable cell numbers. The viable cells formed on the nutrient broth agar medium were counted and represented as colony forming unit (CFU)

per ml. The concentrations of $COD_{Cr}$ and TN in samples were determined using a HS 2000 Water-quality Analyzer (Humas Co., Ltd, Korea). All the analyses were conducted in triplicate.

# 3. Results and Discussion

## 3.1. Isolation of potential microorganisms

The screening of potential microorganisms from rockworm viscera was successfully done. Colonies differently formed in their shapes on six agar media were first sorted out, and their morphological difference was carefully observed under microscope. The colonies displaying unique characteristics were serially picked from the six agar media (nutrient broth agar (NB); skim milk agar (SM); laminarin agar (LA); alginate agar (AL); spirit blue agar (SB); and phenol red tween agar (PR) media), and each isolate was named after an originally isolated spot of such agar medium in order. The finally isolated colonies were 87. Considering aquaculture environment, these microbial species was further screened based on their ability of polymer degradation at 25 ℃ (Wyban et al., 1995). Only 25 colonies were eligible for these criteria: sufficient growth ($OD_{600}$ > 0.8) at 25 ℃, potential degradation ability (> 0.5 cm clear zone) toward protein or degradation ability of lipid. Afterwards, antagonism test was performed between the 25 isolates, and 12 colonies were excluded due to their inhibition to growth of other microbial strains. Among the remaining 13 isolates (shown in Table 1), strains of LA9, SB13, SM16, SM19, and PR21 were finally excluded due to their inability of lipid degradation.

**Table 1.** Characteristics of 13 isolates and their polymer-degrading ability

| Isolates | Gram staining | Cell morphology | Motility | Cell dimensions (L x W, in μm)[a] | Degradation ability | |
|---|---|---|---|---|---|---|
| | | | | | Protein | Lipid |
| AL1 | + | rod | + | 5.41 × 1.30 | + | + |
| AL4 | + | rod | + | 3.06 × 0.61 | + | + |
| AL5 | − | rod | + | 3.56 × 0.52 | + | + |
| AL6 | + | rod | + | 5.42 × 1.37 | + | + |
| LA9 | + | rod | − | 3.56 × 0.54 | + | − |
| LA10 | + | rod, 4−5 chains | + | 4.03 × 1.19 | + | + |
| SB13 | − | rod, 1−2 chains | + | 2.93 × 0.50 | + | − |
| NB14 | − | rod, 1−2 chains | + | 2.90 × 0.42 | + | + |
| PR15 | − | tetrad-forming cocci | + | 1.02 × 1.03 | + | + |
| SM16 | − | rod, 1−2 chains | + | 3.57 × 0.48 | + | − |
| SM19 | − | rod | + | 3.02 × 0.36 | + | − |
| PR21 | + | cocci | + | 0.75 × 0.75 | + | − |
| AL22 | − | rod | + | 2.21 × 0.54 | + | + |

[a] L and W mean the length and width of cell, respectively.

To select appropriate mixed microorganisms suitable for the treatment of shrimp aquaculture wastewater containing complex components, 12 different combinations of isolates were examined based on degradability of polymers (protein and lipid) that are generally present in shrimp aquaculture wastewater (Iber and Kasan, 2021). This experiment was done because the mixed culture is more advantageous than single culture in the degradation of complex components present in nature (Jankowska et al., 2015). In particular, mixed cultures of microorganisms could have better utilization of the substance in nature, since a broad range of enzymes are synthesized by the mixed cultures and such enzymes are able to degrade various polymers. In this respect, combined isolates cultivated in 1 g/L shrimp aquaculture wastewater at 25 ℃ for 48 h were tested for their polymer-degrading ability. All test groups exhibited degradation abilities on both protein and lipid (Table 2). Particularly, Groups 2 and 8 exhibited a higher protein degradation activity. In addition, the viable cell number of Group 2 was better than that of Group 8. This indicates that proliferation of mixed culture in Group 2 was the most active due to its high degradation of polymers present in shrimp aquaculture wastewater. Meanwhile, the mixed culture in Group 5 exhibited the highest number of viable cells, but was excluded in this study due to its low ability of protein degradation. Therefore, Group 2 composed of six isolates was selected to apply to shrimp aquaculture wastewater treatment.

**Table 2.** Polymer-degrading ability and viable cell number in each test group after 24 h cultivation at 25 ℃

| Test group | Composition of combined isolates | Degradation ability on | | Viable cells (CFU/mL) |
|---|---|---|---|---|
| | | Protein (cm)[a] | Lipid | |
| 1 | AL1, AL4, AL5, AL6, LA10 | 1.6 ± 0.3 | +[b] | 5.3 ± 0.02 × $10^6$ |
| 2 | AL1, AL4, AL5, AL6, LA10, PR15 | 2.0 ± 0.2 | + | 4.2 ± 0.03 × $10^6$ |
| 3 | AL1, AL4, AL5, AL6, LA10, NB14 | 1.8 ± 0.1 | + | 3.3 ± 0.02 × $10^6$ |
| 4 | AL1, AL4, AL5, AL6, LA10, AL22 | 1.7 ± 0.3 | + | 2.1 ± 0.04 × $10^6$ |
| 5 | AL1, AL4, AL5, AL6, LA10, NB14, AL22, PR15 | 1.4 ± 0.4 | + | 1.7 ± 0.09 × $10^7$ |
| 6 | AL4, AL5, AL6, LA10 | 1.8 ± 0.2 | + | 3.2 ± 0.03 × $10^6$ |
| 7 | AL4, AL5, AL6, LA10, PR15 | 1.7 ± 0.2 | + | 2.2 ± 0.10 × $10^6$ |
| 8 | AL4, AL5, AL6, LA10, NB14 | 2.1 ± 0.3 | + | 1.0 ± 0.02 × $10^4$ |
| 9 | AL4, AL5, AL6, LA10, AL22 | 1.1 ± 0.3 | + | 1.7 ± 0.06 × $10^6$ |
| 10 | AL4, AL5, AL6, LA10, NB14, PR15, | 1.7 ± 0.1 | + | 4.2 ± 0.03 × $10^5$ |
| 11 | AL4, AL5, AL6, LA10, PR15, AL22 | 1.6 ± 0.3 | + | 2.0 ± 0.08 × $10^6$ |
| 12 | AL4, AL5, AL6, LA10, NB14, AL22 | 1.8 ± 0.2 | + | 2.3 ± 0.01 × $10^5$ |

[a] Mean diameter of each clear zone.

[b] '+' means degradation ability of lipid.

## 3.2. Characterization and identification of isolates

Since the Group 2 exhibited the best polymer-degrading activity, the characteristics of each isolate included in the Group 2 was examined to distinguish it when applied. The isolates AL1, AL4, AL6 and LA10 were Gram positive and rod bacteria, whereas the isolate AL5 was Gram negative and rod bacterium. In particular, the isolate LA10 was a chain-forming bacterium. Unlike these bacteria, the isolate PR15 was observed to uniquely form tetrad cocci. All isolates were motile and different in cell dimensions. Colors of colonies formed on the nutrient broth agar medium were ivory (AL1, AL6 and LA10), pale yellow (AL4), and yellow (AL5 and PR15).

The 16S rRNA sequence analysis was conducted for the six selected isolates consisting of Group 2. Homology searches showed that the isolates AL1, AL4, AL5, AL6, LA10 and PR15 were the most closely matched to *Bacillus paramycoides* (100%), *Bacillus pumilus* (99.7%), *Stenotrophomonas rhizophila* (100%), *Bacillus paranthracis* (99.9%), *Bacillus paranthracis* (99.9%) and *Micrococcus luteus* (99.5%), respectively (Table 3). Based on partial 16S rRNA sequences of each isolate, a neighbor-joining phylogenetic tree was drawn with other phylogenetically related strains (Fig. 1). *Bacillus* species are known to produce diverse extracellular enzymes, and thus, microorganisms showing potential proteolytic activity in industrial fields belong to the genera *Bacillus* (Pant et al., 2015). *B. paramycoides* was reported to have some potential ability to treat hospital wastewater (Rashid et al.,

2020), to degrade organophosphate compounds (Ren et al., 2020) and to remove toxic selenite from contaminated sites (Borah et al., 2021). *B. pumilus* was reported to produce potential xylanase (Kapoor et al., 2008), to efficiently degrade toxic aromatic compounds (Surendra et al., 2017) and to improve the health and growth rate of striped catfish when it was used with *B. amyloliquefaciens* as a dietary additive (Thy et al., 2017). *B. paranthracis* was also reported to degrade not only a pharmaceutical pollutant, diclofenac (Chopra and Kumar, 2020), but also polycyclic aromatic hydrocarbons) (Wang et al., 2020). Moreover, *Stenotrophomonas* sp. was also reported to produce various extracellular enzymes, such as proteases, lipases, nucleases, chitinases and elastases (Ryan et al., 2009). *M. luteus* was reported to have potential ability of degradation for cyclic organic compounds (pyridine (Sims et al., 1986) and nitrobenzene (Zheng et al., 2009)) and hydrocarbons (Tuleva et al., 2009; Shin and Kajiuchi, 2002), enabling it to be used in bioremediation (Kanjilal et al., 2015). Therefore, the above previously reported results imply that the six selected isolates in this study were eligible for the treatment of shrimp aquaculture wastewater.

**Table 3.** Result of identification for the six selected isolates

| Isolate | Length (bp) | GenBank Accession No. | Identification | Similarity (%) |
|---|---|---|---|---|
| AL1 | 1440 | MAOI01000012 | *Bacillus paramycoides* | 100 |
| AL4 | 1472 | ABRX01000007 | *Bacillus pumilus* | 99.7 |
| AL5 | 1469 | CP007597 | *Stenotrophomonas rhizophila* | 100 |
| AL6 | 1438 | MACE01000012 | *Bacillus paranthracis* | 99.9 |
| LA10 | 1439 | MACE01000012 | *Bacillus paranthracis* | 99.9 |
| PR15 | 1231 | CP001628 | *Micrococcus luteus* | 99.5 |


**Fig. 1.** A phylogenetic tree of 6 isolates, based on neighbor-joining tree analysis. 0.05 denotes the genetic distance. (A) isolate

AL1, (B) isolate AL4, (C) isolate AL5, (D) isolate AL6, (E) isolate LA10, and (F) isolate PR15.

## 3.3. Application of six isolates to shrimp aquaculture wastewater

The six selected isolates were applied to 1 g/L shrimp aquaculture wastewater to investigate their potential degradation ability on substances mainly from uneaten feed and feces. During the treatment of shrimp aquaculture wastewater, pH started to increase from 6.21 to 7.45 for first 12 h, and afterwards it was maintained (Fig. 2A). At the same time, cell density ($OD_{600}$) rapidly increased from 1.37 to 2.34 for first 12 h, and the rapid increase was also shown in number of viable cell from $5.90 \times 10^4$ to $2.06 \times 10^5$ CFU/mL). Moreover, this tendency was exactly reflected in the reductions of $COD_{Cr}$ and TN as result of biodegradation by the isolates (Fig. 2B). This indicates that biodegradation occurred actively for first 12 h with cell growth. During the first 12 h, the removal percentages of $COD_{Cr}$ and TN were 51.0% and 44.6%, respectively. The ratio of $COD_{Cr}$ to TN was maintained at 10.0, except for treatment time at 8 h. This was because the reduction rate of TN by the mixed culture surpassed that of $COD_{Cr}$ between 3 h and 8 h. According to the number of viable cells in the above result (Table 2), the 6 isolates in group 2 are shown to be in a log state where they can grow more. Therefore, when comparing the removal efficiencies of COD and TN for 36 h, the mixed isolates of this study showed excellent SAW treatment efficiency. For example, Luo et al. (2012) reported that shrimp wastewater treatment using three phase by variety of organisms included bacteria, and $COD_{Cr}$ and TN removal

rate for 96 h only by bacteria shown maximum 39.5 ± 0.93% and 38.6 ± 1.12%, respectively (Luo et al., 2012).

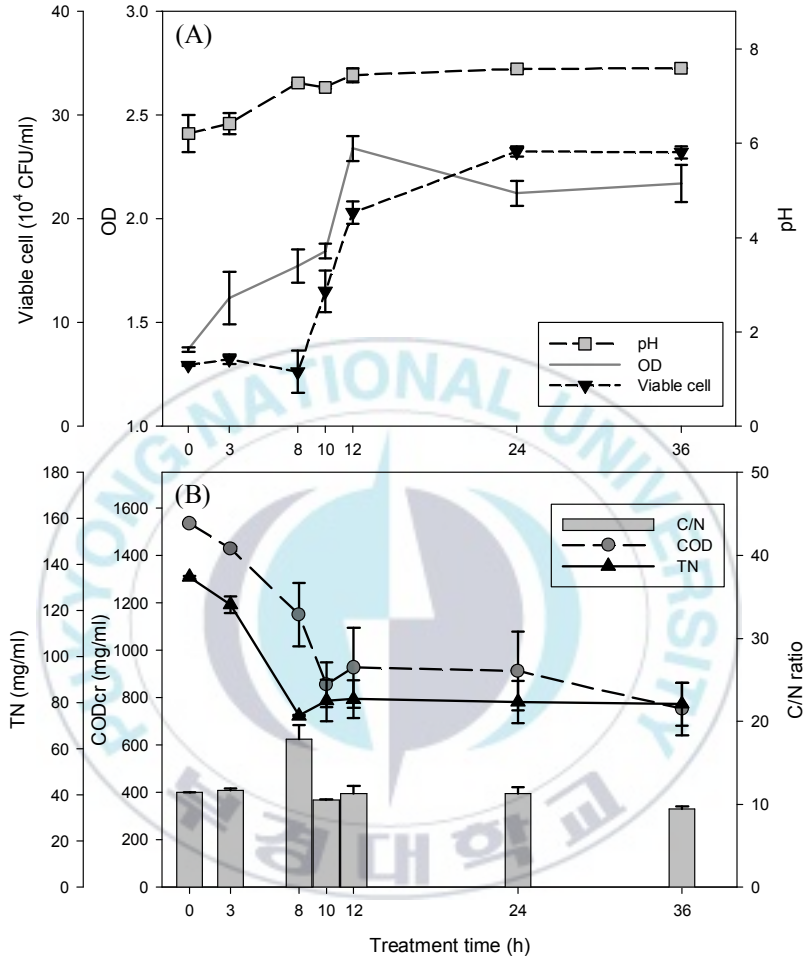


**Fig. 2.** Changes in reaction parameters during the treatment of 1 g/L SAW by the six selected isolates at 25 ℃. (A) Profiles of pH, optical cell density and viable cell numbers and (B) Concentrations of $COD_{Cr}$ and TN, and C/N ratio.

In this study, change of C/N ratio during the biodegradation was in a range of 9.4 – 11.4. Efficiency of biological wastewater treatment is affected by C/N ratio and others, such as pH, concentrations of fat and lipid, etc. (Sunny and Mathai, 2015). Therefore, C/N ratio is an important parameter affecting the biodegradation process in wastewater treatment (Gao et al., 2010). For carbon utilization, microorganisms have some difficulty to synthesize their necessary enzymes under too little N, whereas the microbial growth is inhibited under too much N, particularly in the form of ammonia (Fontenot et al., 2007). In general, the C/N ratios of various wastewaters have been known to be 20:1 to 30:1 (Fontenot et al., 2007). However, efficient treatment of wastewater has been known to take place at the C/N ratio of 10:1 (Fontenot et al., 2007). Considering the above results, shrimp aquaculture wastewater was efficiently biodegraded by the six isolates in this study, since C/N ratio was maintained at around 10:1 without significant change.

Since wastewater of residual feed from shrimp farms contains high nitrogenous concentrations (Roy et al., 2010; Iber and Kasan, 2021), C/N ratio is indispensably optimized for efficient treatment. Removal of nitrates on various C/N ratios (5:1, 10:1, 20:1 and 30:1) was studied for shrimp aquacultural wastewater, and the most effective nitrate removal was achieved at a C/N ratio of 10:1 (Roy et al., 2010). A C:N ratio of 10:1 was reported to produce best results in terms of maximum nitrogen and carbon removal from shrimp aquaculture wastewater performed in a sequencing batch reactor (Fonenot et al.,

2007). A COD:TKN ratio of 10:1 was also reported to be optimum for high efficiency of nitrogen removal in activated sludge process under very low operating oxygen concentration, with oxidation-reduction potential for aeration control (Chevakidagarn et al., 2012). On the other hand, C/N ratio of 15:1 was reported to display the highest removal of ammonia (98.7%) in fish farm wastewater (Bakar et al., 2015). All these results indicate that optimizing C/N ratio is an important factor for efficient removal of nitrogen-containing residues (Nguyen et al., 2018; Roy et al., 2010; Bakar et al., 2015). As a result, C/N ratio was remained at 10:1 for 36 h in this study, and it is appropriate for efficient nitrate removal.

In this study, shrimp aquaculture wastewater was efficiently biodegraded (removal efficiencies of 51.0% $COD_{Cr}$ and 44.6% TN) by the mixed culture of six isolates at a C/N ratio of 10:1 for 12 h. Afterwards, further degradation by the mixed culture was not distinctly displayed. Efficiency of biological wastewater treatment has known to be dependent upon several essential factors, such as continuous aeration, constant waste removal and additional carbon source for growth of treatment bacteria (Iber and Kasan, 2021). The SAW treatment in this study was performed in 1 L flask containing 500 mL SAW, indicating treatment under limited oxygen. Although the six isolates maximally reached $2.06 \times 10^5$ CFU/mL in the SAW treatment (shown in Fig. 2), they could grow up to $4.2 \times 10^6$ CFU/mL for 24 h (shown in Table 2) under more favorable conditions. Therefore, more considerable reduction of residual COD and TN would

be taken place with continuous aeration (Avnimelech, 2009; Iber and Kasan, 2021). This inference can be adequately supported from the previous report. Boopathy et al. (2007) performed biological treatment of shrimp farm wastewater under anoxic and aerobic modes, and they obtained greater reduction of COD during the aerobic mode than in the anoxic mode (Boopathy et al., 2007).

# 4. Conclusion

Useful microorganisms were isolated from the viscera of the rockworm(*Marphysa sanguinea*) and their wastewater treatment ability was investigated. Samples obtained from the intestines of rockworm were cultured in 6 different nutrient media, and a total of 87 isolates with different characteristics were isolated through colony shape and microscopic observation. Of these isolates, 8 isolates with excellent growth, protein and lipid degrading ability, and no antagonism were finally obtained. As a result of designing these 8 isolates into 12 different combinations of microorganisms with various compositions and applying them to similar farm wastewater, the microbial combination with excellent protein degrading ability and high viable cell number($4.2 \times 10^6$ CFU/mL) was a combination of 6 isolates including *Bacillus paramycoides, B. pumilus, Stenotrophomonas sp.,* 2 *B. paranthracis, and Micrococcus luteus.* As a result of wastewater treatment of shrimp farming using this complex microorganism, COD and TN were removed by 51.0% and 44.6%, respectively, after 12 hours of treatment, and the C/N ratio was maintained almost constant at 10, so that this complex microorganism group was stably, it has been shown that wastewater can be treated. Therefore, it was confirmed that useful microorganisms isolated from rockworm intestines are suitable for wastewater treatment of shrimp aquaculture.

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# CHAPTER II

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

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# Abstract

Photosynthetic bacteria 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 photosynthetic bacteria 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) on basal medium. In scale-up cultivation (500 L), *R. azotoformans* EBN-7 showed $4.50 \times 10^{10}$ colony-forming units per mL (number of viable cells), dry cell weight of 26.8 g/L, and a specific growth rate of 0.15/h. 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 ℃ 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.

# 1. 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 laboratories across Korea, and the cultured microorganisms are coming into wide use (Cho et al., 2018). 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 and 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., 2019); to produce single-cell proteins (Kornochalert et al., 2014; Saejung and Thammaratana, 2016); and to produce polysaccharides, carotenoids, chlorophylls, coenzyme $Q_{10}$, and 5-aminolevulinic acid (Meng et al., 2017; Wang et al., 2017; Zhou et al., 2015). 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 (Caia et al., 2019; Moreira Silva et al., 2016). As a high-value material, microorganisms are also used in foods, medicines, and cosmetics (Kars and Ceylan, 2013; Lu et al., 2019; 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, 2022). 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 ℃, 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 in the removal of $NH_4^+$-N in shrimp aquaculture water.

# 2. Materials and Methods

## 2.1. Photosynthetic bacterium and culture

The species of PSB used in this study was *R. capsulatus* BR, which was obtained from Ecobiznet Company (Chuncheon, Gangwon-do, Korea). *R. capsulatus* BR was cultivated on various culture media (Table 1). To eliminate the possibility of contamination, 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 180 rpm under a light intensity of 1000 Lux (Saejung and 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.

**Table 1.** Composition of various media for the cultivation of *R. 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 $K_2HPO_4$, 0.5 g $KH_2PO_4$, and 2.1 mL trace elements (per L, 3 g $FeSO_4 \cdot 7H_2O$; 0.01 g $H_3BO_3$; 0.01 g $Na_2MoO_4 \cdot 2H_2O$; 0.02 g $MnSO_4 \cdot H_2O$; 0.01 g $CuSO_4 \cdot 5H_2O$; 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 $K_2HPO_4$, and 0.5 g $MgSO_4$; pH = 6.8 |

## 2.2. 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) *R. 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 ℃ and 180 rpm under a light intensity of 1000 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.

## 2.3. 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 Co., Chuncheon, Gangwon-do, Korea). The seed culture was

prepared in a 1 L flask incubated at 30 ℃ and 180 rpm under a light intensity of 1000 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 ℃ for 5 d under a light intensity of 1000 Lux. During the cultivation, aeration and agitation rates were adjusted 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 66, 78, and 84 rpm at 24, 48, and 72 h, respectively.

## 2.4. 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 Co., 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 ℃ and 15 rpm for 3 d under a light intensity of 1000 Lux. The final culture broth was tested for its suitability as seed culture.

The possibility of preserving PSB for a long period at 4 ℃ is important for its practical use. To assess the optimal preservation period, the PSB cultivated in a 500 L bioreactor was preserved at 4 ℃ 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 ℃. 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 ℃) started for 2 h after the reactor was filled with the MYC culture medium. Then, the reactor temperature was reduced to 30 ℃, followed by inoculation with 1% (v/v) seed culture preserved at 4 ℃ for different periods. This cultivation was automatically continued for 3 d at 30 ℃ and 15 rpm with an aeration rate of 0.05 vvm under a light intensity of 1000 Lux according to the reactor system program initially inputted. In this manner, the effect of preservation at 4 ℃ on cell activity was investigated to determine the optimal preservation period that allows for cell reactivation.

## 2.5. Application of simple culture technique

PSB cultivated using a simple technique was applied to environmental fields to explore its suitability as seed culture. The cultivated PSB species was *R. azotoformans* EBN-7, and it was used in a shrimp aquaculture farm (Ganghwa-gun, Incheon, Korea). Before breeding shrimps, *R. azotoformans* EBN-7 was added to the aquaculture farm at 100 mL/ton (with viable cells of 1.75 ± 0.23 × $10^9$ CFU/mL) every week to improve water quality, and the concentration of $NH_4^+$-N was measured as an index using the indophenol method (The Korea Law Information Center, 2022).

## 2.6. 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 Co., Ltd, 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 colony-forming units (CFU) per mL. The pH of the culture broth was also measured. All measurements were performed in triplicate.

# 3. Results and Discussion

## 3.1. 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 $4.75 \times 10^9$ after 89 h, which was relatively higher than that previously reported $3 \times 10^8$ under dark-aerobic conditions or $3 \times 10^9$ 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 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. On the basis of the above parameters, growth characteristic of *R. capsulatus* BR were distinctly identified.

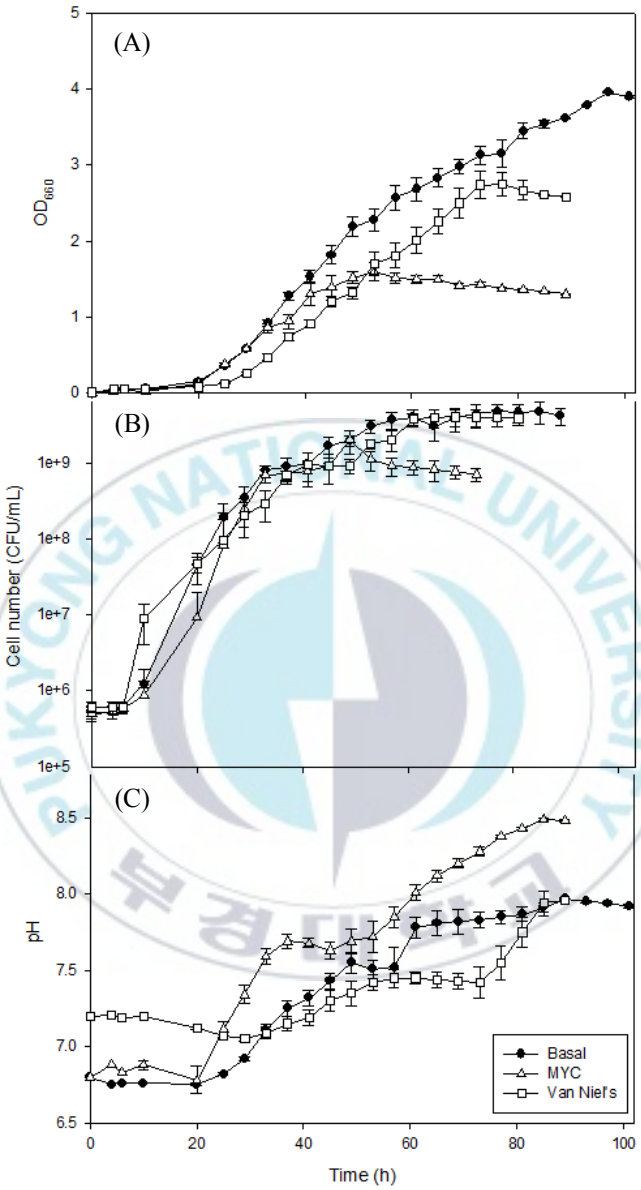


**Fig. 1.** $OD_{660}$ (A), number of viable cells (B) and pH (C) of *Rhodobacter capsulatus* BR cultured on various media.

## 3.2. 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; Takashi, 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 (Tadashi 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).


**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.

## 3.3. 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.

## 3.4. 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 $OD_{660}$ 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 $OD_{660}$ values, the specific growth rate of *R. azotoformans* EBN-7 was calculated to be 0.20/h, which was superior to that of *R. capsulatus* BR (0.13/h), *R. sphaeroides* BL6 (0.17/h) or *R. sphaeroides* GB1 (0.18/h). These specific growth rates are fairly good in comparison with previously reported values: the specific growth rate of *R. sphaeroides* was 0.08/h when cultivated in a medium containing succinate as a C source and $(NH_4)_2SO_4$ as an N source (Jeon and Kim, 2010), and that of *R. sphaeroides* was 0.12/h 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.

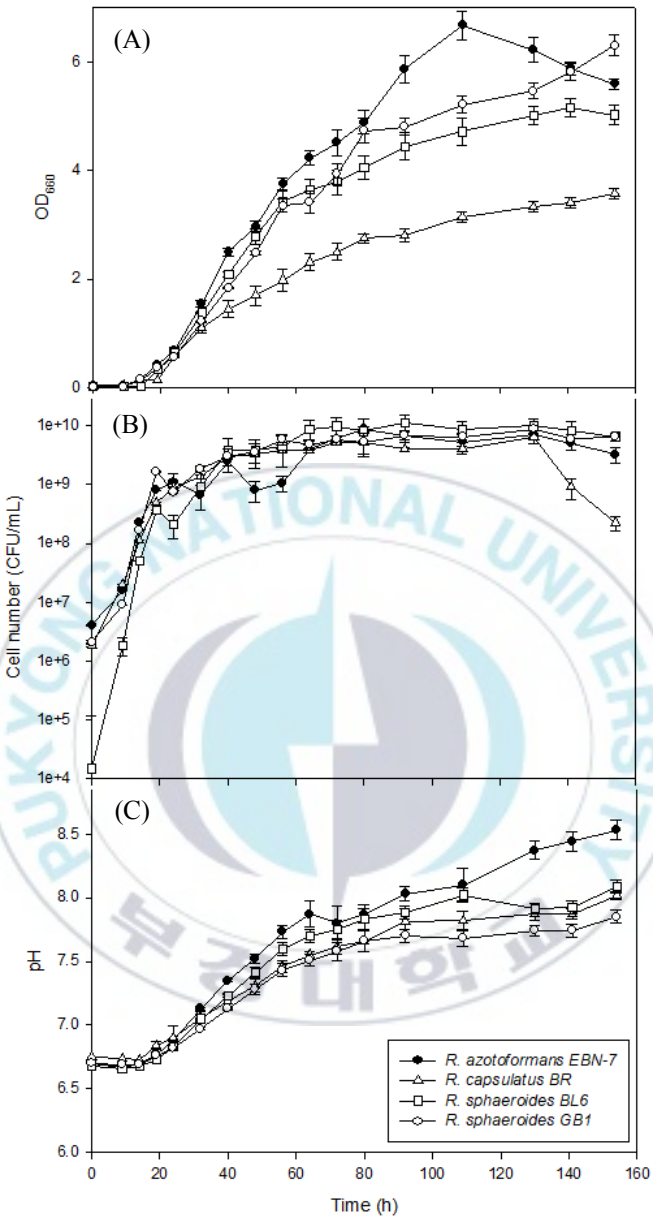


**Fig. 3.** Changes in $OD_{660}$ (A), number of viable cells (B) and pH (C) of four test PSB species cultivated on basal medium.

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 $OD_{660}$ 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 $8.75 \times 10^9$ after 80 h for *R. azotoformans* EBN-7, $6.25 \times 10^9$ after 130 h for *R. capsulatus* BR, $1.10 \times 10^{10}$ after 92 h for *R. sphaeroides* BL6, and $8.75 \times 10^9$ 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 $OD_{660}$ owing to the difference in cell materials used to measure these parameters. In all species, the highest number of viable cells reached over $1 \times 10^9$ 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 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 $OD_{660}$ value for each PSB species. Therefore, there was a time lag between the point at which dry-cell weight or $OD_{660}$ reached the maximum value and the point at which the highest number of viable cells was

observed.

### 3.5. 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 $1 \times 10^9$ 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.

### 3.6. 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 μm wide and 1−2 μm long), whereas the other two species were relatively longer rods (1 μm wide and 3−4 μm long for *R. capsulatus* BR; and 1 μm wide and 1.5−2 μm 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). Therefore, *R. azotoformans* EBN-7 was used for scale-up cultivation in the subsequent experiments.

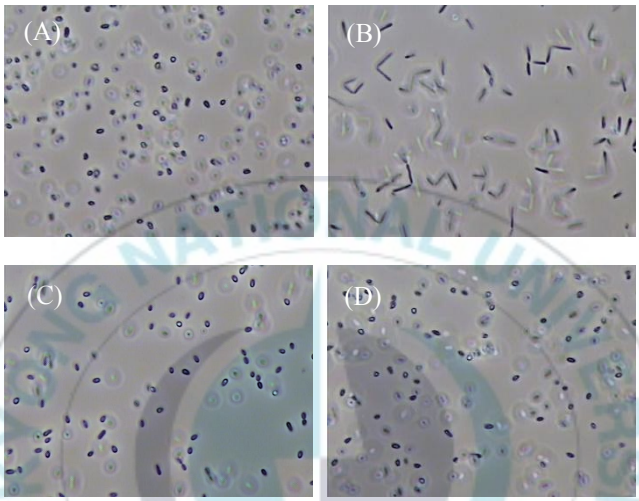


**Fig. 4.** Microscopic photographs of four photosynthetic bacterial species cultivated on basal medium at 154 h (1,000×). (A) *Rhodobacter azotoformans* EBN-7, (B) *R. capsulatus* BR, (C) *R. sphaeroides* BL6, and (D) *R. sphaeroides* GB1.

## 3.7. 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 $OD_{660}$ 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 $4.50 \times 10^{10}$ (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) 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 to 0.18/h 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 *Rhodopseudomonas palustris* decreased from 0.12/h (in 1 L culture) to 0.068/h (in 5 L culture) in batch-type bioreactors (Kim and Lee, 2000). This denotes the difficulty in scale-up cultivation.

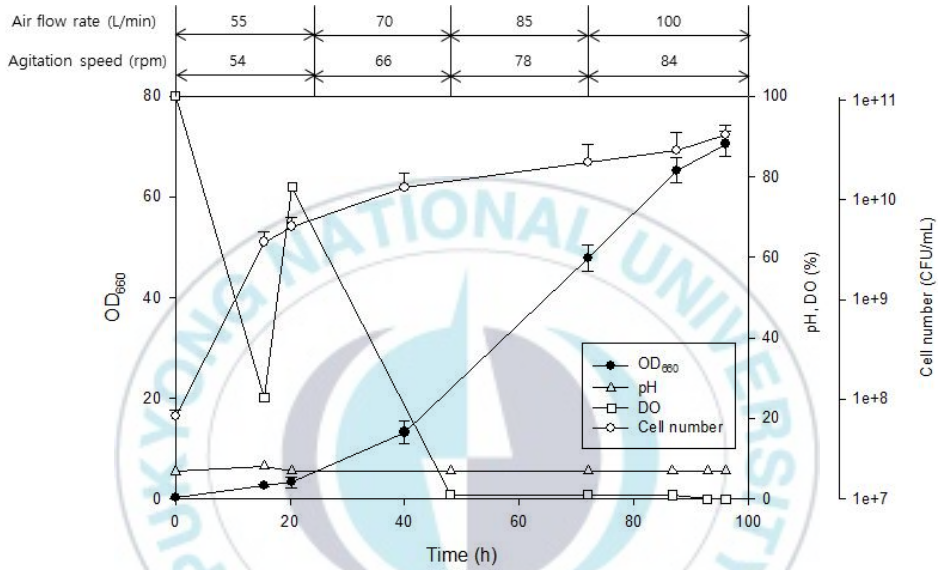


**Fig. 5.** Changes in optical density at 660 nm ($OD_{660}$) and number of viable cells with pH and dissolved oxygen (DO) during the cultivation of *R. azotoformans* EBN-7 in a 500 L reactor.

## 3.8. Effect of preservation at 4 ℃ 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 ℃ without reduction in cell activity. Therefore, *R. azotoformans* EBN-7 preserved at 4 ℃ 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 $OD_{660}$ in the range of 1.02−1.56 and the number of viable cells in the range of $6.25 \times 10^8$−$1.75 \times 10^9$. 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 ℃ for *R. azotoformans* EBN-7 was 3 months.

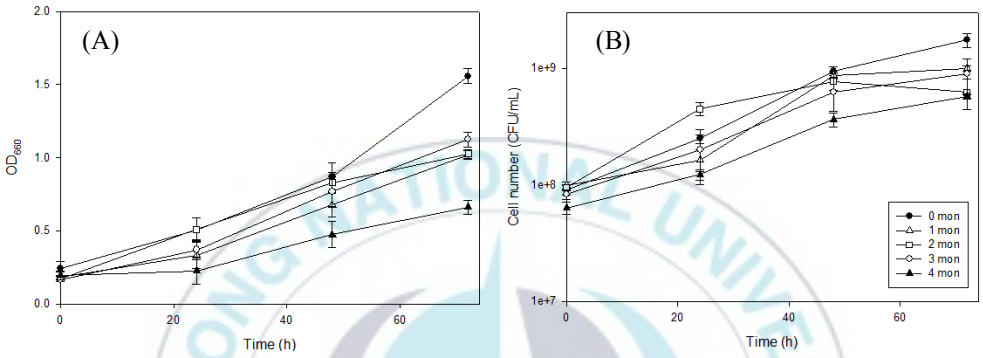


**Fig. 6.** Changes in optical density at 660 nm ($OD_{660}$) (A) and number of viable cells (B) during cultivation using the seed culture variously preserved for 0 month, 1 month, 2 months, 3 months and 4 months, respectively.

## 3.9. 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 $NH_4^+$-N from the water to test its potential. In the control group, the initial concentration of $NH_4^+$-N in the shrimp aquaculture water was 0.25 mg/L; the concentration of $NH_4^+$-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 $NH_4^+$-N), and the $NH_4^+$-N concentration was reduced to 0.24 mg/L at day 10. Therefore, the treatment of shrimp aquaculture water with the cultivated *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, photosynthetic bacteria were reported to reduce ammonia accumulation in an aquaculture pond. The $NH_4^+$-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 photosynthetic bacteria in $NH_4^+$-N removal were also reported in other applications. Idiet et al. (2015) demonstrated the ability of *R. sphaeroides* ADZ101 in the treatment of a synthetic wastewater containing $NaNO_3$ as the sole nitrogen source. During the 7 d cultivation, the strain *R. sphaeroides* ADZ101 maximally removed 62% of $NH_4^+$-N from an initial concentration of 52 mg/L under anoxic growth conditions. In addition, Zhou et al. (2015) reported that 83.2% $NH_4^+$-N was removed after 4 d when an ammonia-tolerant photosynthetic bacterium (ISASWR2014) was applied to chicken manure wastewater, which indicates a higher $NH_4^+$-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 $NH_4^+$-N. This enables seed culture prepared by a simple culture technique to be easily used for public.

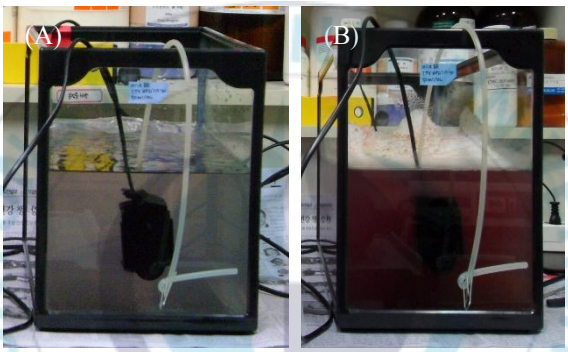


**Fig. 7.** Change in color tone in a simple reactor during the cultivation of *Rhodobacter azotoformans* EBN-7: at day 0 (A) and at day 3 (B).

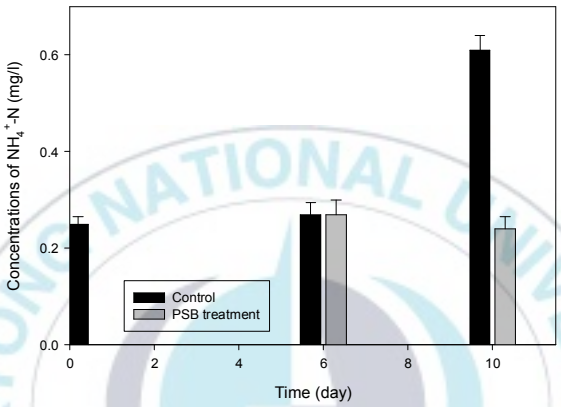


**Fig. 8.** Application of *Rhodobacter azotoformans* EBN-7 cultivated using a simple technique to an aquaculture farm against control. The photosynthetic bacteria were added to the treatment system at day 6.

# 4. Conclusion

Photosynthetic bacteria 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) on basal medium, and it showed $4.50 \times 10^{10}$ colony-forming units per mL (number of viable cells), dry cell weight of 26.8 g/L, and a specific growth rate of 0.15/h 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 ℃ 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 ℃ for <3 months exhibited its potential in the removal of $NH_4^+$-N by 60.7% (from 0.61 mg/L 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 $NH_4^+$-N would considerably contribute to the dissemination of useful PSB for varied applications.

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# CHAPTER III

# Improvement in microbial stability of photosynthetic bacteria via optimised cell immobilisation and lyophilisation: Application to the treatment of shrimp aquaculture wastewater

The full content of this chapter has been published in Journal of Marine Bioscience and Biotechnology 2022;14(2):112-123.

# Abstract

Photosynthetic bacteria (PSB) play an important role in water purification, and their application is beneficial for sustainable aquaculture. However, it is difficult to maintain the microbial stability of PSB from sub-culturing to preservation. Since enhancement in microbial stability of PSB is critical, optimised conditions for cell immobilisation and lyophilisation were investigated. In PSB immobilisation, 0.1 M $CaCl_2$ was the most effective divalent metal ion solution in terms of cost-effectiveness, resulting in beads with 4 mm diameter and high loading ($1.91 \times 10^9$ CFU/mL) of viable cells. Maintenance of cell viability, external appearance, and colour of PSB beads were best in 3.5% NaCl during storage. In lyophilisation, addition of skim milk (9%) and dextrose (2%) as cryoprotective additives retained the highest cell viability. When optimally manufactured beads and lyophilised powder of PSB was applied to shrimp aquaculture water, $NH_4^+$, $NO_3^-$ and $NO_2^-$ were more effectively removed, by 55%, 100% and 100%, respectively, over an 18-week shrimp breeding period, relative to controls. Thus, microbial stability of PSB via optimised cell immobilisation and lyophilisation was successfully enhanced, enabling wide application.

# 1. Introduction

Biological treatment of wastewater is an eco-friendly method for degrading pollutants dissolved in diverse types of effluents(Hussain et al., 2021). Biological wastewater treatment uses microorganisms, such as bacteria, fungi, algae and protozoa, and pollutants as nutrients are degraded and converted to $CO_2$, $CH_4$, and other compounds, fulfilling the requirement for appropriate disposal. Biological treatment is widely applied to treat municipal sewage and industrial wastewater containing organics, since it is effective and more economical than many mechanical or chemical processes. To achieve optimal removal of organic substances from wastewater, it is treated aerobically or anaerobically, and sometimes it goes through a combination of aerobic and anaerobic processes(Narayanan and Narayan, 2019). Biological treatment processes are also divided into two cell growth phases: suspended growth and attached growth. Selection of the type of biological treatment depends on various factors, such as compliance with regulations for environmental discharge quality.

Increasing the cell density of useful microorganisms in treatment tanks can reduce treatment time in biological treatments. Microbial immobilisation can be efficient in this regard, since it can enhance the performance and economics of various fermentation processes (Zhu, 2007). Microbial immobilisation can generate not only a higher cell density, but also a reduction in adverse interactions between microorganisms (Lu et al., 2020). Other advantages include

enhancement of microbial stability and reduction in reactor volume, compared with suspended culture systems (Elkahlout et al., 2016). Moreover, easy separation of the biomass from the liquid, facile product recovery, and specific metabolic enhancements can be achieved using cell immobilisation (Zhu, 2007). To immobilise microorganisms, various types of supports have been employed, such as natural polymer gels including agar, carrageenan, alginate and chitosan, and synthetic polymers including polyacrylamide, polyurethane and polyvinyl (Elkahlout et al., 2016; Lopez et al., 1997; Katzbauer et al., 1995; Tian et al., 2009). Alginate, a major carbohydrate comprising the cell wall of brown seaweeds, includes two anionic monomers: β-D-mannuronic acid and α-L-guluronic acid residues. Since this property of alginate is appropriate for cell immobilisation, cells entrapped in alginate beads are widely used in water purification processes due to their elastically porous characteristics (Rocher et al., 2008).

Photosynthetic bacteria (PSB) are widely distributed in terrestrial and aquatic environments. They can act as primary producers and they perform diverse roles in nature. PSB are applied in bioremediation, since they can utilise various kinds of pollutants, such as pesticides, heavy metals, dyes, crude oil and odours, with minimum requirement for nutrients, providing a cheap, alternative approach to conventional treatment methods (Idi et al., 2015). Their actions can clean contaminated environments and simultaneously generate valuable products.

PSB are commonly used for water purification since they perform an important function. However, microbial stability of PSB decreases during sub-culturing and preservation, limiting their functionality and wide application in various types of wastewater treatment. To compensate for a lack of treatment efficiency, PSB are immobilised within suitable supports, improving their treatment efficiency (Kim et al., 2005; Xu et al., 2012; Wang and Liao, 2005; Jeong et al., 2009). Prior to practical use, the general preservation method for immobilised PSB is to submerge beads in water, seal tightly in a storage container, and maintain at 4°C. However, this method does not retain the viability of cells in beads, and the commercial value of beads therefore decreases as the circulation is extended during marketing and transportation. This problem limits the dissemination of useful PSB to the public, hence a method to maintain the microbial stability of PSB is urgently needed.

In addition to preservation of immobilised cells, shelf life of PSB can be extended by lyophilisation with cryoprotective additives. This method has been known to be successful with no further loss in cell viability during 2 - 3 years of storage at 9 ℃, especially for oxygenic species of *Rhodospirillaceae* sp. grown under heterotrophic conditions, but it is not suitable for anoxygenic *Rhodospirillaceae* sp. that are not able to grow aerobically in darkness (Malik, 1988). Therefore, this method can be suitably applied to heterotrophically-growing purple non-sulphur bacteria to maintain not only their cell viability, but also their water purification ability.

From sub-culturing to preservation, it is difficult to maintain the microbial stability of PSB as useful microorganisms for water purification, preventing the wide dissemination and deployment of PSB. Therefore, the present study was performed to explore a suitable method to maintain the microbial stability of PSB over a long time. For this purpose, an appropriate divalent solution for solidifying PSB beads was first investigated, then an appropriate preservative solution for PSB beads was sought to preserve beads for a long time without considerable loss of cell viability. Appropriate cryoprotective additives for lyophilisation of PSB were also explored. Finally, optimally manufactured beads and lyophilised powder of PSB were applied to shrimp aquaculture water to demonstrate their water purification ability in practical use.

# 2. Materials and Methods

## 2.1. Photosynthetic bacteria and culture media

PSB used in this study were *Rhodobacter capsulatus* for cell immobilisation and *Rhodobacter sphaeroides* for powdering by lyophilisation; they were obtained from Ecobiznet Company (Chuncheon, Gangwon-do, Korea). *R. capsulatus* and *R. sphaeroides* were selected due to potential for water purification (Jeong et al., 2009) and high maintenance of cell viability (Liu et al., 2015), respectively, and these characteristics were expected to help remove pollutants generated mainly from uneaten feed and faeces in aquaculture. Each PSB was cultivated on basal agar medium containing the following (per L): agar, 15 g; DL-malic acid, 2.7 g; ammonium phosphate, 0.8 g; monosodium glutamate, 3.76 g; tryptone, 1 g; yeast extract, 2 g; $K_2HPO_4$, 0.5 g; $KH_2PO_4$, 0.5 g; trace elements, 2.1 mL (pH 6.8). Trace elements included (per L): 3 g $FeSO_4 \cdot 7H_2O$; 0.01 g $H_3BO_3$; 0.01 g $Na_2MoO_4 \cdot 2H_2O$; 0.02 g $MnSO_4 \cdot H_2O$; 0.01 g $CuSO_4 \cdot 5H_2O$; 0.01 g $ZnSO_4$; 0.5 g ethylenediamine tetraacetic acid. Red colonies that grew on solid medium after 3 days were used as a seed inoculum for flask culture. Red colonies were transferred with a loop to 1 L flasks (working volume 600 mL) containing sterile basal medium, and flasks were incubated at 30 ℃ with shaking at 180 rpm under 1000 lux light for 3 days. Colonies formed on solid medium were transferred to fresh solid medium every 2 weeks to keep cells

active, and stored at 4 ℃ until used.

## 2.2. Cell immobilisation

To develop a stable format for photosynthetic cells (*R. capsulatus*) with maintenance of cell viability during storage, PSB were immobilised in Na-alginate. Cells used in the immobilisation were grown until the late-exponential phase in a 1 L flask. Cultivated cells were collected, centrifuged at 10,000 × g for 10 min, and suspended in distilled water (DW). Cell immobilisation was then conducted according to the method performed by Chen et al. (1998). The suspended solution containing 200 mg/mL PSB was mixed with 1.5 % Na-alginate, and the mixed solution was transferred to a 1 L syringe-type bead maker (Ecobiznet company, Chuncheon, Gangwon-do, Korea) set at a height of 20 cm. A small amount of the mixed solution was dropped vertically through the needle plump (equipped with a 1 mm nozzle) into a beaker containing 0.1 M $CaCl_2$ solution; globular beads (~4 mm) formed in the beaker were tempered for 10 min and washed with sterile DW. These Na-alginate beads were tinged with red, and stored at 4 ℃ until use.

To compare the properties of beads solidified in a typical $CaCl_2$ solution, empty beads (not loaded with PSB cells) were also solidified by dropping 1.5% Na-alginate solution into several other divalent cation solutions ($MgCl_2$, $SrCl_2 \cdot 6H_2O$, $SnCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$ and $NiCl_2 \cdot 6H_2O$). Furthermore, beads

containing a red pigment (Watercolor Paints, DONG-A, Korea) instead of cells were dried at room temperature for 24 h, and dried beads were immersed in DW for 3 h to examine elasticity (shrinking and swelling) of the solidified beads. The degree of restoration of beads to the original state was evaluated by measuring bead size using Digital Vernier Callipers (Mitutoyo Korea, Gyeonggi-do, Korea).

## 2.3. Preservation of immobilised PSB

The resulting Na-alginate beads were sieved to remove water, and 3.25 kg of beads were placed into a 7 L airtight plastic container for preservation. To explore an appropriate preservative strategy for beads, various preservative solutions were prepared and compared with unsterile DW as a control. After 3.25 kg of beads were placed in a plastic container (7 L), 2 L of the prepared solutions were poured in at a volume ratio of 7:3 (beads to solution) until soaking the beads fully. The prepared preservative solutions were divided into five groups: an unsterile DW solution containing 3.5% NaCl (Experimental group 1); an unsterile DW solution containing 1% PEG#200, 1.02% sucrose and 0.03% NaCl (Experimental group 2); a sterile DW solution (Experimental group 3); a sterile DW solution containing 3.5% NaCl (Experimental group 4); and a sterile DW solution containing 1% PEG#200, 1.02% sucrose and 0.03% NaCl (Experimental group 5). Unsterile DW served as a negative control group. Alginate beads immersed in the various preservative solutions were stored at 30 ℃

under darkness to investigate changes in appearance or leakage of beads. Samples of stored beads were taken on day 0, 3, 7 and 13, and their external appearance, pH, and viable cell number at an appropriate dilution were determined after vortexing.

## 2.4. Lyophilisation of PSB

To extend the storage period of PSB with maintenance of cell viability, 3 L of culture broth cultivated for 3 days was lyophilised using a freeze-dryer (PVTF20R, Ilshinbiobase, Gyeonggi-do, Korea) for 48 h to generate a powder. Prior to lyophilisation, various cryoprotective agents were added to the final culture broth as follows: 270 g of skim milk (9%, w/v) with 60 g of dextrose (2%, w/v; Experimental group 1); 150 g of skim milk (5%, w/v) with 60 g of dextrose (2%, w/v; Experimental group 2); 150 g of skim milk (5%, w/v) with 60 g of dextrose (2%, w/v) and 15 g of erythritol (0.5%, w/v; Experimental group 3); 150 g of skim milk (5%, w/v) with 90 g of MSG (3%, w/v), 30 g of dextrose (1%, w/v) and 15 g of erythritol (0.5%, w/v; Experimental group 4). The final culture broth without any cryoprotective additive served as a control. The resultant powder after lyophilisation was stored at 4 ℃, and the powder weight and number of cells present in the powder were measured to calculate the survivability of PSB using the following equation:

Survivability (%) =

$$\frac{\text{Cell numbers (CFU/g) x powder weight (g)}}{\text{Total volume of initial culture broth with preservative (L) x Cell numbers (CFU/mL) x } 10^3 \text{ (mL/L)}} \times 100 \quad (1)$$

## 2.5. Application of PSB in the field

To explore the microbial stability of the preserved PSB, both immobilised and lyophilised PSB were applied to fields, and field tests were performed at a shrimp aquaculture farm (Ganghwa-gun, Incheon, Korea). Shrimp breeding was performed for 18 weeks in a 33,057.9 $m^2$ farm after providing 500,000 shrimp seeds (at a rate of 15 seeds/$m^3$). For shrimp seed breeding, Shrimpower shrimp feed (Ecobiznet Company) was fed 1−2 times per day in the initial growth stage, 5−7 times per day after the middle growth stage, and the total amount of feed was 14,000 kg.

To control water quality, preserved PSB were applied. For 18 weeks, 500 g of lyophilised powder-type PSB was added every 2 weeks. Also, the amount of PSB beads used in the field study was gradually increased, since PSB beads were used to treat uneaten feed remaining at the bottom of the shrimp farm. Therefore, 20 L of PSB beads was added every 2 weeks during week 6 to week 10, 30 L of PSB beads was added every 2 weeks during week 10 to week 14, and 2.5 L of PSB beads was added every week during week 14 to week 18 to control sediment quality. To examine the effect of PSB, changes

in water quality were observed during shrimp seed breeding.

## 2.6. Analysis

To determine the viable cell number included in alginate beads, 10 beads were sampled and transferred to 5 mL of 0.1 M sodium citrate solution, followed by vortexing for 20 min to disintegrate beads. To determine the viable cell number present in powdered PSB, 1 g of powder was dissolved in 10 ml DW and analysed. Samples for the determination of viable cells were streaked on an agar plate containing (per L) agar (15 g), DL-malic acid (1 g), yeast extract (3 g) and casamino acid (2 g, pH 6.8) at appropriate dilutions. After a 1 day incubation, the number of colonies formed on the agar plate was counted, and the number of viable cells was calculated based on the dilution ratio. Finally, the number of viable cells was expressed as colony forming units (CFU) per mL of sample.

# 3. Results and Discussion

## 3.1. Cell immobilisation

Beads of *R. capsulatus* immobilised in 1.5% Na-alginate solution were globular in shape, ~4 mm in diameter, and tinged with red (Fig. 1). The viable cell number entrapped in beads was $1.91 \times 10^9$ CFU/mL on average. In the experiment comparing the properties of beads, the solidified beads displayed somewhat different properties when Na-alginate beads were dropped into various divalent cation solutions (Fig. 2). In particular, bead formation did not occur in 0.1 M $MgCl_2$ solution because the Na-alginate solution became soft after dropping. Except for $MgCl_2$ solution, other divalent cation solutions assisted the formation of globular beads. Although bead size was not significantly different, beads solidified in 0.1 M $CoCl_2$ were the largest and softest.

In the experiment testing bead elasticity (shrinking and swelling), beads (loaded with red pigment) shrunk to different sizes when dried (Fig. 3A). Beads also exhibited differences in the degree of restoration in DW (Fig. 3B). In particular, beads solidified in 0.1 M $SnCl_2 \cdot 2H_2O$ were broken to pieces during drying or restoration. The best restoration ability was observed for beads solidified in 0.1 M $CoCl_2$ solution; the original beads were 4 mm in diameter, and they were significantly shrunken after 24 h of drying, and restored to 6 mm in diameter with a globular shape after 3 h of immersion in DW. This result may reflect the soft quality of the original beads, consistent

with the results of analysis of their properties shown in Fig. 2.

This property of beads formed in 0.1 M $CoCl_2$ solution was re-examined with loading of PSB inside beads for practical use, and the same phenomenon was observed (Fig. 3C). To utilise the superior elasticity of these beads for practical use, they were repeatedly shrunk and swollen 10 times. The weight of beads was gradually reduced, indicating some leakage of PSB. However, leakage of PSB was not significant after three cycles. Therefore, beads solidified in 0.1 M $CoCl_2$ solution were suitable for practical application based on their capacity for repeated use (Fig. 4). Although bead formation in 0.1 M $CoCl_2$ solution was found to be suitable to maintain the stability of PSB activity over a long time, cobalt (Co) can remain *in situ* when beads are repeatedly used in aquatic and soil environments. Bioremediation strategies for remediation of Co toxicity attracts much attention, since Co accumulation in agricultural fields and water bodies has significant ramifications for crop plants (Mahey et al., 2020). Considering the toxic effects of Co accumulation in nature, use of $CoCl_2$ solution for bead formation should be avoided. On the other hand, $CaCl_2$ is advantageous because the unit cost of beads production was cheaper than those of beads solidified using other divalent cation solutions when European-Pharmacopoeia-grade divalent cation solutions were used to solidify beads.

Moreover, the size of beads can affect diffusivity of substrates as well as cell loading. Speirs et al. (1995) reported that calcium alginate beads entrapping *Gluconobacter suboxydans* were shrunk rapidly in air

and fully restored in water, and oxygen diffusivity into beads was dependent upon bead size. Lee et al. (2020) investigated the optimum size of alginate beads in photo-bioreactor experiments for nutrient (total nitrogen and total phosphorus) removal in wastewater. Among beads of different sizes (2.0, 3.5 and 5 mm), the highest nutrient removal was obtained with 3.5 mm beads in which microalgae (*Chlorella vulgaris* and *Chlamydomonas reinhardtii*) were immobilised. Considering cost-effectiveness, safety without environmental pollution, and bead size for substrate diffusivity and cell loading, the most appropriate solution for the solidification of *R. capsulatus* beads was 0.1 M $CaCl_2$.

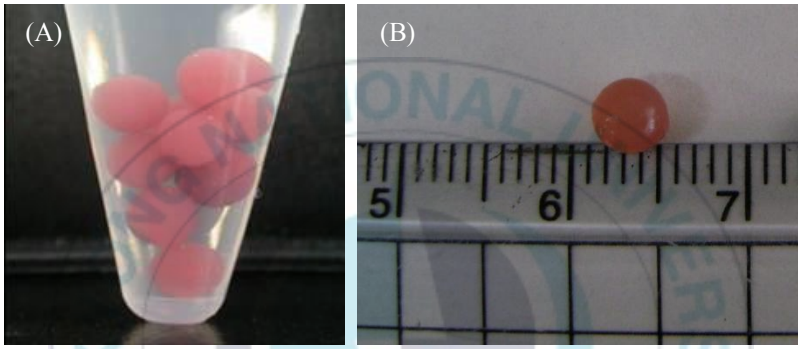


**Fig. 1.** Na-alginate beads of *Rhodobacter capsulatus* (A) Beads stored in DW, (B) Bead size in millimetres.

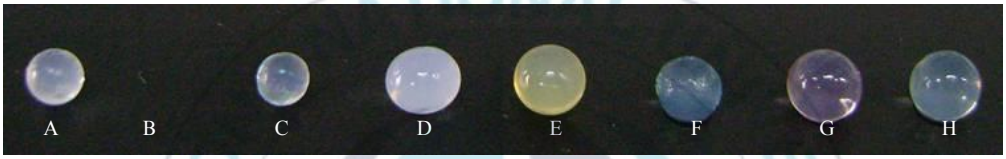


**Fig. 2.** Beads solidified in 0.1 M divalent cation solutions (A) $CaCl_2$, (B) $MgCl_2$, (C) $SrCl_2 \cdot 6H_2O$, (D) $SnCl_2 \cdot 2H_2O$, (E) $FeCl_3 \cdot 6H_2O$, (F) $CuCl_2 \cdot 2H_2O$, (G) $CoCl_2 \cdot 6H_2O$, (H) $NiCl_2 \cdot 6H_2O$.

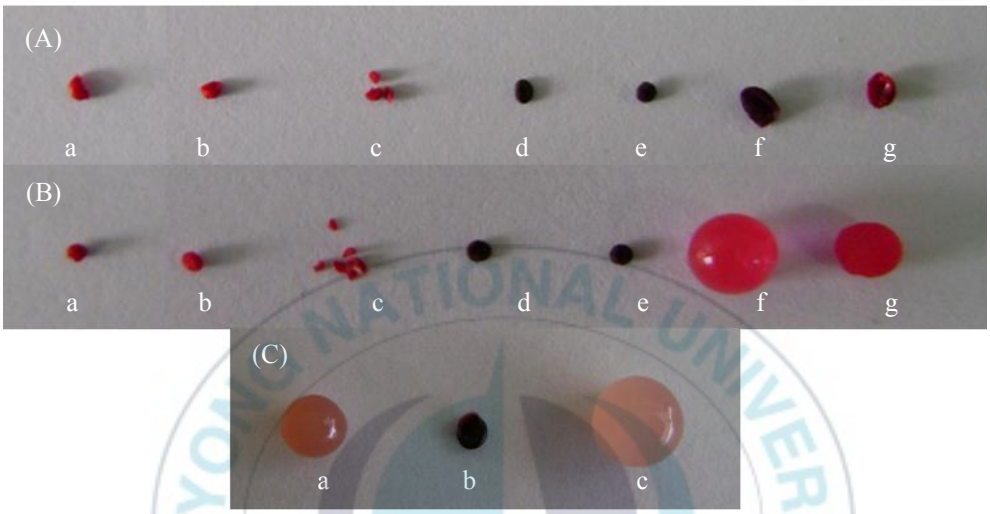


**Fig. 3.** Results of elasticity tests for beads solidified in 0.1 M divalent cation solutions

(A) Beads dried for 24 h: (a) $CaCl_2$, (b) $SrCl_2 \cdot 6H_2O$, (c) $SnCl_2 \cdot 2H_2O$, (d) $FeCl_3 \cdot 6H_2O$, (e) $CuCl_2 \cdot 2H_2O$, (f) $CoCl_2 \cdot 6H_2O$ and (g) $NiCl_2 \cdot 6H_2O$,

(B) Beads restored in DW after drying,

(C) Beads formed in 0.1 M $CoCl_2$ with loading of PSB: (a) original; (b) completely dried; (c) completely restored.

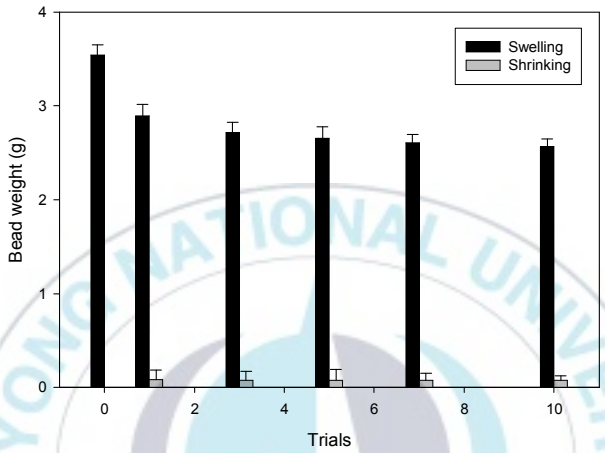


**Fig. 4.** Results of elasticity testing of beads formed in 0.1 M $CoCl_2$ with loading of a red pigment after 10 cycles of shrinking and swelling.

## 3.2. Storage of PSB in beads

The number of viable cells present in beads stored in various preservative solutions was observed for 14 days. To select an appropriate preservative solution in a shorter time, beads were stored at 30°C in the dark to accelerate putrefaction. The viable cell number in each experimental group was similarly changed from $10^9$ to $10^5-10^7$ CFU/mL after 14 days of storage, alongside controls, whereas leaked viable cells were increased from $10^4$ to $10^6-10^7$ CFU/mL (Fig. 5). There was no significant difference (in the viable cell number in beads and leaked viable cells) between the control group ($2.83 \times 10^6$ and $2.83 \times 10^6$ CFU/mL) and Experimental group 3 ($2.13 \times 10^5$ and $7.25 \times 10^6$ CFU/mL), Experimental group 1 ($6.00 \times 10^7$ and $1.83 \times 10^7$ CFU/mL) and Experimental group 4 ($5.50 \times 10^5$ and $1.08 \times 10^7$ CFU/mL), as well as Experimental group 2 ($8.63 \times 10^5$ and $1.28 \times 10^7$ CFU/mL) and Experimental group 5 ($5.38 \times 10^5$ and $8.75 \times 10^6$ CFU/mL). These results indicate that use of sterile DW for bead preservation did not significantly affect the viability of cells in beads. Viable cells were more protected when protective solution (3.5% NaCl or a mixed solution of 1% PEG#200, 1.02% sucrose and 0.03% NaCl) were added, and 3.5% NaCl yielded a better result than the mixed solution. Moreover, reduction in viable cells was lowest ($6.00 \times 10^7$ CFU/mL in beads, compared with $1.83 \times 10^7$ CFU/mL in solution) in Experimental group 1 using an unsterile DW solution containing 3.5% NaCl. This result indicates that unsterile 3.5% NaCl played some role in the maintenance of PSB populations inside beads.

This is because NaCl can reduce microbial community richness and diversity, thereby having a preservative effect to some extent (Pang et al., 2020). In addition, NaCl can affect antimicrobial activity both directly and indirectly, depending on the amount added and the function it performs (Ravishankar and Juneja, 2014). The mechanism of inhibition of microorganisms by NaCl mainly involves lowering the water activity of the substrate. It is also reported that NaCl can influence substrate utilisation in microorganisms.

During storage of beads at 30 ℃ for 14 days, the biggest change in pH occurred in all experimental groups within 3 days (Fig. 5). After 3 days, pH dropped from 6.85 (control and Experimental group 3), 7.04 (Experimental groups 1 and 4), 6.38 (Experiment groups 2 and 5) to 4.82 (control), 4.89 (Experimental group 3), 5.94 (Experimental group 1), 5.28 (Experimental group 4), 3.84 (Experimental group 2) and 3.83 (Experimental group 5). Hereafter, changes in pH were not significant. Thus, the biggest drop in pH was observed in both Experimental groups 2 and 5, in which the mixed solution contained 1% PEG#200, 1.02% sucrose and 0.03% NaCl as preservatives. The pH drop was relatively slow in Experimental groups 1 and 4 in which 3.5% NaCl was added. Considering pH changes together with cell viability during storage of beads, the pH drop affected the viable cell number. When the pH was dropped, the viable cell number in beads decreased in all experimental groups, but as pH was maintained or slightly increased, this declining tendency almost disappeared. It has been reported that pH influences the properties of alginate beads, such as surface

structures of droplets, mechanical properties, and storage stability (Lin et al., 2022). Thus, 3.5% NaCl caused a lower pH drop and was deemed to most effective preservative.

During storage of PSB beads for 14 days, there were changes in the appearance of beads stored in plastic containers (Fig. 6). Beads floated on the surface of preservative solution, then sank to the bottom repeatedly. The earliest floating beads were observed in the control group, followed by Experimental group 3, Experimental groups 1 and 4, and Experimental groups 2 and 5, as time progressed. The red colour of beads in Experimental groups 2 and 5 gradually faded due to cell leakage. This phenomenon was stimulated by gas production by PSB, since PSB undergoing denitrification under anaerobic conditions produce $N_2$ gas (Cho et al., 2006). Therefore, beads floated when the produced gas remained in beads, but sank when gas escaped beads. This change (expansion by gas and shrinking following release of gas) in beads could weaken the strength of beads, resulting in more PSB leakage from beads as storage was prolonged. In this respect, alginate beads with high elasticity are more appropriate for longer storage. After 14 days, a globular bead shape was maintained in Experimental groups 1 and 4, and Experimental groups 2 and 5, but not in controls or Experimental group 3. Thus, cell viability was favourably maintained in 3.5% NaCl with preservation of both the external appearance and colour of beads, enhancing their commercial value.

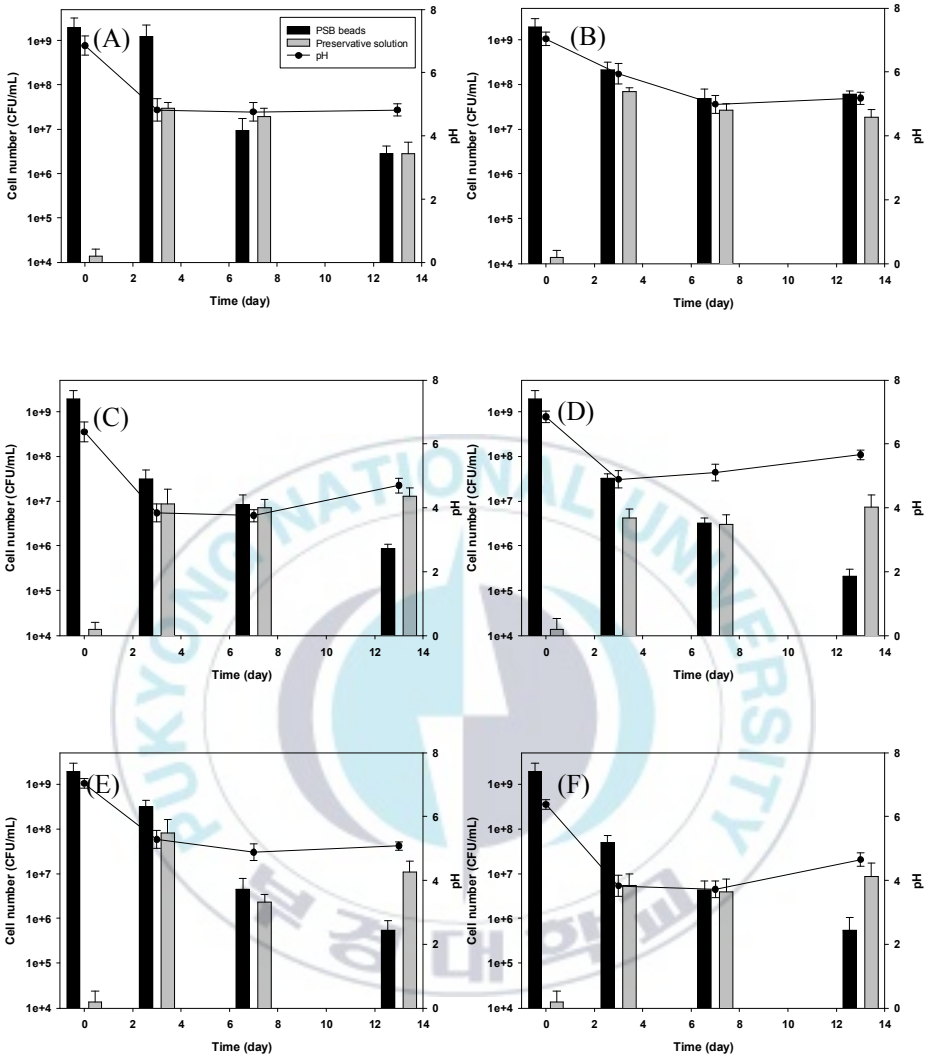


**Fig. 5.** pH and viable cell number for *R. capsulatus* in beads and preservative solution after 0, 3, 7 and 13 days. Beads were stored in various preservative solutions. (A) Controls, (B) Experimental group 1, (C) Experimental group 2, (D) Experimental group 3, (E) Experimental group 4, (F) Experimental group 5.

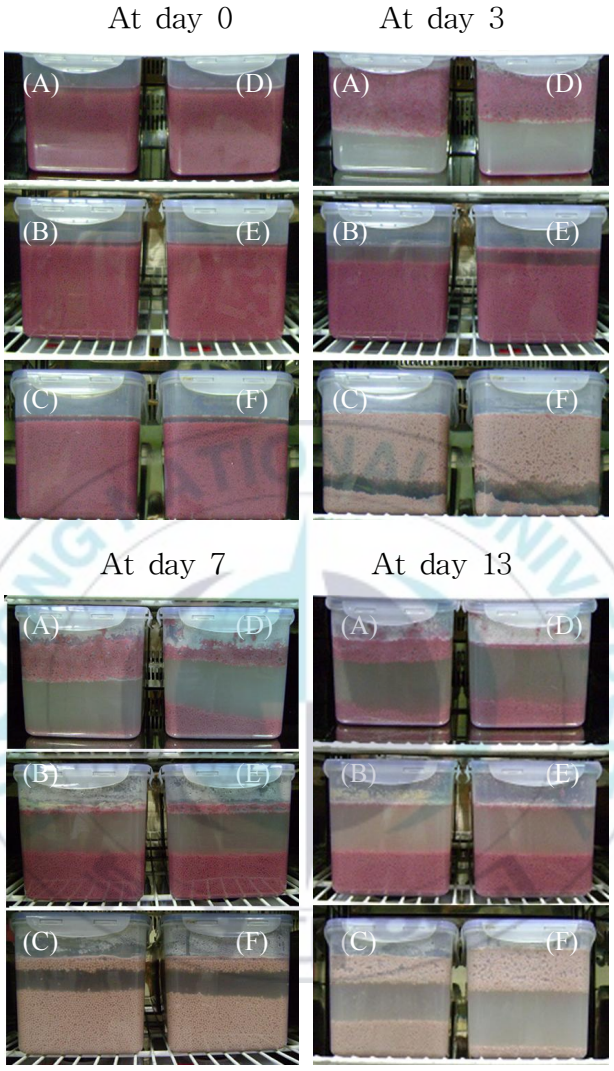


**Fig. 6.** Appearances of PSB beads at days 0, 3, 7 and 13 of storage (A) Controls, (B) Experimental group 1, (C) Experimental group 2, (D) Experimental group 3, (E) Experimental group 4, (F) Experimental group 5.

## 3.3. Cell viability after lyophilisation with cryoprotective additives

Although lyophilised products are considerably easier to stabilise than their liquid counterparts, lyophilisation can provoke sublethal damage to microbes, requiring appropriate cryoprotective additives to protect microbes when lyophilised. Therefore, in this study, the effects of various cryoprotective additives on cell viability were investigated to increase the storage period of PSB and maintain cell viability. The results for viable cell number before and after lyophilisation are shown in Fig. 7. Prior to lyophilisation, the viable cell number in each experimental group together with controls was almost the same (ranging from $5.75 \times 10^9$ to $8.50 \times 10^9$ CFU/mL). After lyophilisation, the highest survival for PSB ($2.61 \times 10^9$ CFU/mL) was observed in Experimental group 1, followed by Experimental group 3 ($2.47 \times 10^9$ CFU/mL), Experimental group 4 ($2.31 \times 10^9$ CFU/mL), Experimental group 2 ($1.60 \times 10^9$ CFU/mL) and controls ($1.40 \times 10^8$ CFU/mL) in descending order. The survival rates were calculated to be 32.6% (Experimental group 1), 29.9% (Experimental group 3), 27.1% (Experimental group 4), 26.7% (Experimental group 2) and 2.4% (controls). All experimental groups revealed considerably higher survivability than controls. In conclusion, Experimental group 1 achieved the best results in terms of both the number and survivability of PSB cells. In Experimental group 1, cryoprotective additives skim milk (9 %, w/v) and dextrose (2 %, w/v) were added

before lyophilisation. These cryoprotective additives have been used to enhance the stability of cell viability during the storage of lactic acid bacteria (Kim et al., 2019; Yeo et al., 2018). Cell survivability of *Lactobacillus fermentum* SK152 was best when dextran (5.6 %, w/v) was added, the performance of which was superior to that of skim milk (2.2%, w/v), glucose (1.7%, w/v) and trehalose (1.5%, w/v). As a cryoprotectant, addition of skim milk was best, achieving a cell survivability of 90% at 4 ℃ and 12% at 20 ℃ for *L. fermentum* SK152 stored for 8 weeks (Kim et al., 2019). In the case of *Lactobacillus salivarius* W13, cell survivability was measured after lyophilised bacterial powders were stored at －80 ℃ for 2 weeks, and the best cell survivability (59.5%) was obtained when a combination of skim milk (10%, w/v), sucrose (10%, w/v) and sodium glutamate (2.5%, w/v) was used as a cryoprotectant, which was significantly higher than the rate (37%) obtained from the use of skim milk (10%, w/v) alone (Yeo et al., 2018). The effects of cryoprotective additives on PSB have been reported in previous studies. As protective agents for lyophilisation, dimethyl sulphoxide (5% v/v) and glycerin (15% v/v) were the most promising cryoprotectants for preservation of the cyanobacterial strain *Trichocoleus sociatus* (Witthohn et al., 2020). Dimethyl sulphoxide and glycerin displayed cell viability rates of 80－90% and 60－70% after 4 weeks of cryopreservation, respectively. Moreover, a combined cryoprotectant of raffinose (5% w/v) and skim milk (20% w/v) was reported to maintain cell viability of *Rhodospirillaceae* sp. during 2－3 years of storage at 9 ℃, with no

loss of functionality for photoautotrophy, diazotrophy, hydrogen production and pigmentation (Malik, 1988). Similarly, in the present study, a combination of skim milk (9%, w/v) and dextrose (2%, w/v) yielded the best results for cell viability, although the effects of cryoprotective additives may differ between bacterial strains.

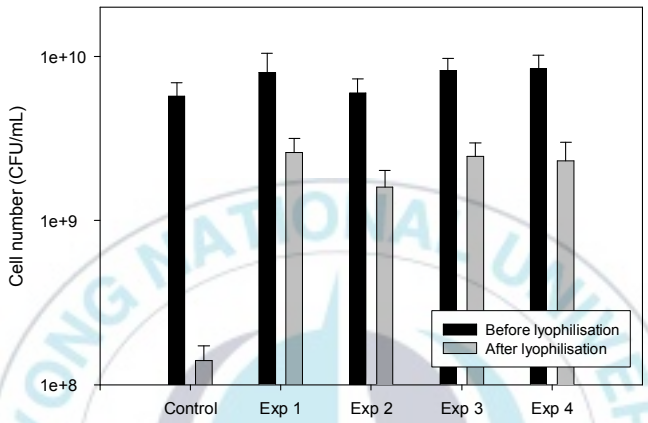


**Fig. 7.** Measurement of viable cell number before lyophilisation and after lyophilisation (A) Controls, (B) Experimental group 1, (C) Experimental group 2, (D) Experimental group 3, (E) Experimental group 4.

## 3.4. Field application of PSB powder and beads

To explore practical use, PSB with enhanced microbial stability through cell immobilisation and lyophilisation were applied to *in situ* shrimp aquaculture to reduce the concentrations of $NH_4^+$, $NO_3^-$ and $NO_2^-$ present in the breeding water. Application of PSB powder and beads to shrimp aquaculture revealed that the quality of shrimp breeding water was favourably enhanced in comparison with the control group. In the control group, the concentration of $NH_4^+$ was initially 0.11 mg/L, and this peaked at 0.45 mg/L after 11 weeks, then decreased and finished at 0.22 mg/L; $NO_3^-$ was initially 0 mg/L, and it peaked at 25 mg/L after 11 weeks, then decreased and finished at 9.8 mg/L; $NO_2^-$ was initially 0 mg/L, and it peaked at 0.3 mg/L after 1 weeks, then decreased and finished at 0.04 mg/L (Fig. 8A). In the experimental group, the initial concentration (0.10 mg/L) of $NH_4^+$ was slightly increased to 0.12 mg/L after 4 weeks, and it decreased to 0.07 mg/L after 11 weeks, then returned to the initial concentration after 18 weeks. For $NO_3^-$ and $NO_2^-$, the concentrations were maintained at 0 mg/L throughout the 18 week shrimp breeding duration (Fig. 8B). Compared with controls, the removal percentage for $NH_4^+$ was 55% in the experimental group using PSB powder and beads, whereas values for $NO_3^-$ and $NO_2^-$ were 100%. This indicates that PSB powder and beads resulted in less loss of cell viability, and this improved the quality of shrimp breeding water (i.e. they enhanced the treatment of residual uneaten feed and faeces). Lyophilised *R. sphaeroides* and *R.*

*capsulatus* entrapped in beads are known to be effective pollutant removers in wastewater (Liu et al., 2015) and bioremediation agents for denitrification and nitrification of municipal wastewater (Costa et al., 2017), respectively. Therefore, the use of both PSB powder and beads was effective for removing nitrous substances present in shrimp aquaculture water.

PSB have been applied to shrimp aquaculture as a protein source, a probiotic, and a bioremediation agent for water purification. A mixture of shrimp feed and lyophilised *R. sphaeroides* SS15 as a single-cell protein at ratios of 1-5% was used for the cultivation of white shrimp (*Litopenaeus vannamei*) (Chumpol et al., 2018). In a 60-day shrimp breeding study, growth of shrimps was enhanced maximally by 19.2% with a 5.7% higher survival rate, but water quality (concentrations of $NH_4^+$, $NO_2^-$ and $NO_3^-$) was not significantly affected. As a probiotic, 0.01% fresh weight of *Rhodovulum sulfidophilum* was added to shrimp feed, and the combined feed was used for 70 day breeding of *Marsupenaeus japonicus* (kuruma shrimp) (Koga et al., 2021). The survival rate of shrimps was significantly enhanced by 14.5%, with a reduction in feed conversion rate, but the average body weight of shrimps was not significantly altered. On the other hand, bioremediation using PSB contributes not only to enhancing water quality, but also to maintaining the health and stability of shrimp aquaculture systems, stimulating shrimp production through elimination of excess nitrogen resulting from residual feed and faeces (Antony and Philip, 2006). When a bacterial consortium

containing *Rhodopseudomonas palustris* was used in white shrimp (*Penaeus vannamei*) farming, a better survival rate (by 22.1%) and increased body weight (by 19.7% in maximum) were reported, compared with controls (Barman et al., 2017).

Additionally, various effects of PSB on water quality in aquaculture have been reported. When *R. palustris* was used to remove nitrogen from aquaculture water, levels of $NH_4^+$, $NO_3^-$ and $NO_2^-$ in the treatment group were significantly lower ($p$ <0.05) than those in controls, accompanied by changes in microbial community structure (Zhang et al., 2014). Polyvinyl alcohol-gel beads containing PSB (*R. capsulatus*) have also been employed for purification of water in goldfish rearing (Jeong et al., 2009). During a 6 month treatment, the use of PSB beads enhanced the removal of $NH_4^+$, $NO_3^-$ and $NO_2^-$ by 62.5%, 27.1% and 32.5%, respectively, relative to controls. The role of PSB in water purification has been thoroughly investigated. Since water purification can contribute to disease control and the health of breeding organisms, the use of PSB powder and beads that can maintain high cell viability is desirable for sustainable aquaculture.

In this study, removal rates of $NH_4^+$, $NO_3^-$ and $NO_2^-$ by PBS beads and powder in shrimp aquaculture water were enhanced by 55%, 100% and 100%, respectively over an 18-week breeding, compared with controls. Since maintenance of stable water quality in aquaculture is critical to health care of breeding organisms, various products have been used for water purification: 65.3% of ammonia removal was obtained from the treatment with 20 g of granular activated carbon in

0.1 $m^3$ tank breeding three spotted tilapia over 9 h (Sichula et al., 2011); and 76.6% of ammonia removal was obtained from the treatment with 10 ppt of zeolite in 0.05 $m^3$ tank breeding seabass over 35 days (Aly et al., 2016). For good selection of water purifying agents, removal efficiency of pollutants, cost effectiveness, fish species, etc. have to be considered together. The use of water-purifying agents recently attract a lot of attention, extending to aquarium industry.

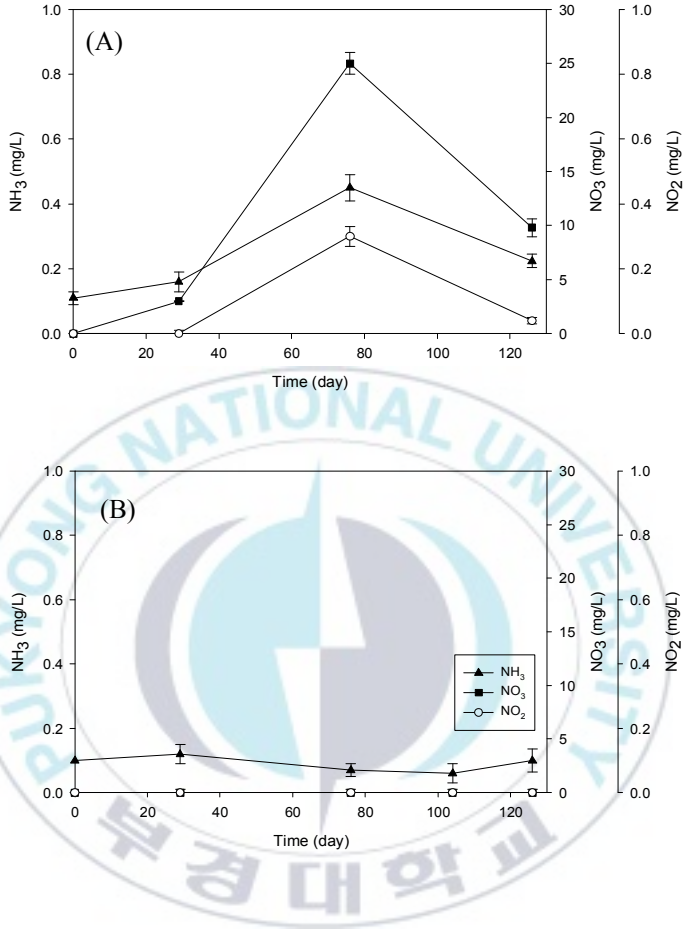


**Fig. 8.** Changes in the concentrations of $NH_3$, $NO_3$ and $NO_2$ during shrimp breeding in the presence of PSB powder and beads as water-purifying agents. (A) Control group. (B) Experimental group.

# 4. Conclusions

Photosynthetic bacteria (PSB) have great potential for water purification. However, PSB can struggle to maintain microbial stability from sub-culturing to preservation. To increase field applications, enhancement of microbial stability of PSB is critical. In this study, optimised conditions for cell immobilisation and lyophilisation were investigated. PSB beads (4 mm diameter) solidified in 0.1 M $CaCl_2$ were the most cost-effective and displayed the highest loading ($1.91 \times 10^9$ CFU/mL) of viable cells. The external appearance and colour of PSB beads was best in 3.5% NaCl, accompanied by relatively high maintenance of cell viability during storage. When lyophilised, addition of skim milk (9%) and dextrose (2%) were the best additives for reducing loss of cell viability. Under optimal conditions, PBS beads and lyophilised powder exhibited good removal ability for $NH_4^+$, $NO_3^-$ and $NO_2^-$ (enhanced by 55%, 100% and 100%, respectively, relative to controls) in shrimp aquaculture water over an 18-week breeding experiment. Therefore, the wide dissemination of microbially stable PSB powder and beads for water purification would be beneficial for sustainable aquaculture.

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# Isolation, cultivation and cell-viability maintenance of useful bacteria suitable for treatment of aquaculture wastewater

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## Abstract

As a source of healthy protein, seafood is a favorite food for many people, and its demand has increased every year. As a result, it is difficult for the catch to meet this demand, and the production of aquatic products through aquaculture is greatly increasing, and the importance of the aquaculture industry as the 4th industry responsible for future food is drawing attention. However, the development of the aquaculture industry has led to an increase in consumption of aquatic food, and consequently, an increase in the amount of waste and wastewater, resulting in the need for efficient waste/wastewater treatment to preserve the environment and increase production of aquaculture products.

For efficient and environmentally friendly aquaculture waste/wastewater treatment, many wastewater treatment technologies using microorganisms have been developed. Microorganisms living in the viscera of rockworms and photosynthetic bacteria are known to be very useful microorganisms in treating wastewater discharged from seawater farm. Therefore, in this study, useful microorganisms suitable for aquaculture wastewater treatment are isolated, mass culture and simple culture technology increase the ease of

wastewater treatment of these microorganisms, and microbial stability maintenance technology is used to treat wastewater from actual shrimp farms. In order to increase the efficiency, the possibility of practical use of aquaculture wastewater treatment using useful microorganisms was studied.

In Chapter 1, useful microorganisms were isolated from the viscera of the rockworm(*Marphysa sanguinea*), which inhabits the bottom of the shrimp farm and has the ability to purify sediment, and investigated the wastewater treatment ability of the microorganisms. Samples obtained from the viscera of rockworms were cultured in 6 different nutrient media, and a total of 87 isolates with different characteristics were isolated through colony shape and microscopic observation. Among these isolates, isolates with excellent growth and protein and lipid degradation. After searching, 8 isolates without mutual antagonism were finally obtained.

In order to match the characteristics of farm wastewater containing various components, the finally obtained 8 isolates were designed as 12 different microbial combinations with various compositions and then applied to similar farm wastewater. As a result, the microbial combination with excellent protein degrading ability and high viable cell number($4.2 \times 10^6$ CFU/mL) was a combination of 6 isolates including *Bacillus paramycoides, B. pumilus, Stenotrophomonas sp.,* 2 *B. paranthracis, and Micrococcus luteus.* As a result of wastewater treatment of shrimp farming using this complex microorganism, COD and TN were removed by 51.0% and 44.6%, respectively, after 12 hours of treatment, and the C/N ratio was maintained almost constant at 10, so that this complex microorganism group was stably, it has been shown that wastewater can be treated. Therefore, it was confirmed that useful microorganisms isolated from rockworm intestines are suitable for wastewater treatment of shrimp farm.

In Chapter 2, photosynthetic bacteria, which have been reported as useful

microorganisms that have excellent water purification ability and play an excellent role in bioremediation in the agricultural and livestock sector, are high value-added microorganisms, but are difficult to cultivate simply due to the difficulty of pure culture, reducing their utilization. Therefore, since there is a great need for development and dissemination of development technology related to this, in this study, a culture method that can be used more easily for field input and treatment efficiency of easily cultured photosynthetic bacteria in actual shrimp farm wastewater treatment were investigated.

Among the photosynthetic bacteria used, the most suitable strain for scale-up production was *Rhodobacter azotoformans* EBN-7, which showed the highest specific growth rate (0.20/h) in Basic medium. *R. azotoformans* EBN-7 strain showed $4.50 \times 10^{10}$ CFU/mL, dry cell weight of 26.8 g/L, and specific growth rate (0.15/h) in a 500 L pilot-scale culture. As a result of culturing in a 15 L simple incubator using the final culture medium obtained here as a seed, it was confirmed through microscopic observation that photosynthetic bacteria dominate after 3 days of culture. The maximum permissible storage period of *R. azotoformans* EBN-7 for the production of active seed for on-site wastewater treatment was investigated as 3 months at 4 ℃. When *R. azotoformans* EBN-7 cultured in a 250 L simple bioreactor was applied to shrimp farm wastewater, $NH_4^+$-N was significantly reduced to 60.7% compared to the control group. Therefore, it was found that the *R. azotoformans* EBN-7 strain cultured by a simple culture technique can be effectively used in shrimp farm wastewater treatment.

In Chapter 3, methods to maintain the microbial stability for a long time when useful microorganisms are introduced into the field for wastewater treatment were investigated. The useful microorganisms used in this study were photosynthetic bacteria, and the microbial stability of the photosynthetic bacteria was maintained for a long time using cell immobilisation and

lyophilisation technology, and the wastewater treatment efficiency was investigated through field application. Among the divalent cation solutions used for photosynthetic bacteria immobilisation, 0.1 M $CaCl_2$ is the most effective divalent cation solution in terms of no environmental pollution, substrate diffusivity, size (4 mm) favorable for cell loading, and cost effectiveness. Beads prepared using this have a relatively high viable cell number($1.91 \times 10^9$ CFU/mL). As a storage method for the photosynthetic bacteria bead produced, the highest microbial stability was shown in a 3.5% NaCl solution at 4 ℃. In the lyophilisation method, the highest microbial stability was confirmed when skim milk (9%) and glucose (2%) were added as cryoprotective additives. When photosynthetic bacteria beads for aquaculture bottom improvement and lyophilisation powder for aquaculture water treatment manufactured under optimal conditions were applied to shrimp farms for 18 weeks, 55% of $NH_4^+$ was removed compared to the control group, and $NO_3^-$ and $NO_2^-$ 100% removed. Therefore, cell immobilisation and lyophilisation under optimal conditions were able to maintain the microbial stability of photosynthetic bacteria longer, and as a result, it was shown that effective *in situ* shrimp aquaculture water treatment was possible.

Through the studies in Chapters 1-3 above, it was confirmed that the appropriate complex microbial combination of microorganisms living in the viscera of rockworms can be usefully used for wastewater treatment in seawater farms, and the spawning of photosynthetic bacteria difficult to culture through simple culture technology. The production method made it easier to use photosynthetic bacteria when inputting them to the farm wastewater treatment site. It was confirmed that it is very useful for *in situ* farm water treatment. Therefore, from the results obtained in this study, the importance of developing and commercializing useful microorganisms and

related technologies in sustainable aquaculture was confirmed.