J. Mar. Biosci. Biotechnol. 2022, p. 112 - 123 Vol. 14, No. 2 [Research Paper]

https://doi.org/10.15433/ksmb.2022.14.2.112 ISSN 2383-5400 (Online)

Improvement in microbial stability of photosynthetic bacteria via optimized cell immobilization and lyophilization: Application to the treatment of shrimp aquaculture water

Kyoung Sook Cho[1,2], Joong Kyun Kim[1,*]

[1]Department of Biotechnology, Pukyong National University, Busan 48513, Republic of Korea
[2]Busan Customs Laboratory, Korea Customs Service, Busan 48940, Republic of Korea

(Received 18 Nov 2022, Revised 13 Dec 2022, Accepted 21 Dec 2022)

Abstract Photosynthetic bacteria (PSB) play an important role in water purification, and their application is beneficial for sustainable aquaculture. However, maintaining the microbial stability of PSB from subculturing to preservation is a challenging task. Since improvement in the microbial stability of PSB is a crucial parameter, optimized conditions for cell immobilization and lyophilization were investigated. In PSB immobilization, 0.1-M $CaCl_2$ was found to be the most effective divalent metal ion solution in terms of cost-effectiveness, resulting in beads with a 4-mm diameter and high loading (1.91×10^9 CFU/mL) of viable cells. Maintenance of cell viability, external appearance, and color of PSB beads was best in 3.5% NaCl during storage. In lyophilization, the addition of skim milk (9%) and dextrose (2%) as cryoprotective additives allowed the highest cell viability. Over an 18-week shrimp breeding period, when optimally manufactured beads and lyophilized powder of PSB were applied to shrimp aquaculture water, NH^{4+}, NO_3^-, and NO_2^- were more effectively removed by 55%, 100%, and 100%, respectively, compared to controls. Thus, microbial stability of PSB through optimized cell immobilization and lyophilization was successfully enhanced, enabling a wide application.

Keywords : Photosynthetic bacteria; Microbial stability; Cell immobilization; Lyophilization; Shrimp aquaculture water

1. Introduction

Biological treatment of wastewater is an eco-friendly method for degrading pollutants dissolved in diverse types of effluents. 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. 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.

* Corresponding author
Phone: +82-51-629-5866 Fax: +82-51-629-5863
E-mail: junekim@pknu.ac.kr

Increasing the cell density of useful microorganisms in treatment tanks can reduce treatment time in biological treatments. Microbial immobilization can be efficient in this regard, since it can enhance the performance and economics of various fermentation processes [1], Microbial immobilization can generate not only a higher cell density, but also a reduction in adverse interactions between microorganisms [2]. Other advantages include enhancement of microbial stability and reduction in reactor volume, compared with suspended culture systems [3]. Moreover, easy separation of the biomass from the liquid, facile product recovery, and specific metabolic enhancements can be achieved using cell immobilization [1]. To immobilize 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 [3−6]. 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 immobilization, cells entrapped in alginate beads are widely used in water purification processes due to their elastically porous characteristics [7].

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 [8]. 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 immobilized within suitable supports, improving their treatment efficiency [9−12]. Prior to practical use, the general preservation method for immobilized 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 immobilized cells, shelf life of PSB can be extended by lyophilization 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°C, 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 [13]. 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 lyophilization of PSB were also explored. Finally, optimally manufactured beads and lyophilized 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 immobilization and *Rhodobacter sphaeroides* for powdering by lyophilization; they were obtained from Ecobiznet Company (Chuncheon, Gangwon-do, Korea). *R. capsulatus* and *R. sphaeroides* were selected due to potential for water purification [10] and high maintenance of cell viability [14], respectively, and these characteristics were expected to help remove pollutants generated mainly from uneaten feed and feces 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°C 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°C until used.

2.2. Cell immobilization

To develop a stable format for photosynthetic cells (*R. capsulatus*) with maintenance of cell viability during storage, PSB were immobilized in Na-alginate. Cells used in the immobilization 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 immobilization was then conducted according to the method performed by Chen et al. (1998) [15]. 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°C 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 immobilized 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 sol-

ution (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°C 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. Lyophilization of PSB

To extend the storage period of PSB with maintenance of cell viability, 3 L of culture broth cultivated for 3 days was lyophilized using a freeze-dryer (PVTF20R, Ilshinbiobase, Gyeonggi-do, Korea) for 48 h to generate a powder. Prior to lyophilization, 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 lyophiliszation was stored at 4°C, and the powder weight and number of cells present in the powder were measured to calculate the survivability of PSB using the following equation:

$$\text{Survivability (\%)} = \frac{CNP \times PW}{TV \times CNL \times 10^3} \times 100$$

where CNP, PW, TV and CNL indicate cell numbers in powder (CFU/g), powder weight (g), total volume of initial liquid culture broth with preservative (L) and cell numbers in the liquid sample (CFU/mL), respectively.

2.5. Application of PSB in the field

To explore the microbial stability of the preserved PSB, both immobilized and lyophilized 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 lyophilized 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 immobilization

Beads of *R. capsulatus* immobilized in 1.5% Na-alginate solution were globular in shape, ~4 mm in diameter, and tinged with red (Figure 1). The viable cell number entrapped in beads was 1.91×10^9 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 (Figure 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.

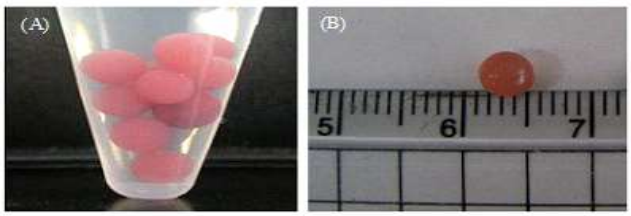

Figure 1. Na-alginate beads of *Rhodobacter capsulatus*. (A) Beads stored in DW. (B) Bead size in millimetres.

Figure 2. Beads solidified in 0.1 M divalent cation solutions. (A) $CaCl_2$. (B) $MgCl_2$. (C) $SrCl_2\cdot6H_2O$. (D) $SnCl_2\cdot2H_2O$. (E) $FeCl_3\cdot6H_2O$. (F) $CuCl_2\cdot2H_2O$. (G) $CoCl_2\cdot6H_2O$. (H) $NiCl_2\cdot6H_2O$.

In the experiment testing bead elasticity (shrinking and swelling), beads (loaded with red pigment) shrunk to different sizes when dried (Figure 3A). Beads also exhibited differences in the degree of restoration in DW (Figure 3B). In particular, beads solidified in 0.1 M $SnCl_2\cdot2H_2O$ 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 Figure 2.

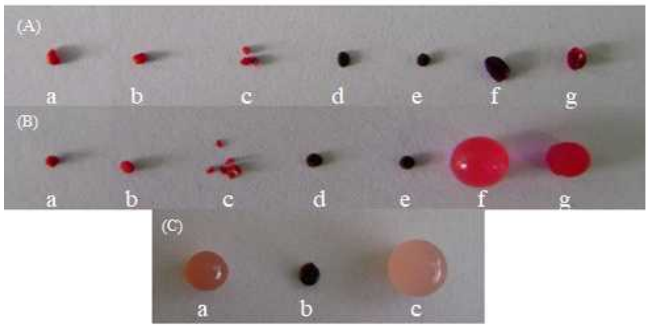

Figure 3. Results of elasticity tests for beads solidified in 0.1 M divalent cation solutions (a) $CaCl_2$, (b) $SrCl_2\cdot6H_2O$, (c) $SnCl_2\cdot2H_2O$, (d) $FeCl_3\cdot6H_2O$, (e) $CuCl_2\cdot2H_2O$, (f) $CoCl_2\cdot6H_2O$ and (g) $NiCl_2\cdot6H_2O$. (A) Beads dried for 24 h. (B) Beads restored in DW after drying. (C) Beads formed in 0.1 M $CoCl_2$ with loading of PSB: (a) original; (b) completely dried; (c) completely restored.

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 (Figure 3C). To utilize 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 (Figure 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 [16]. 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.

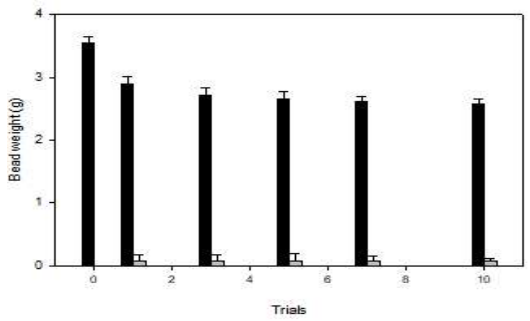

Figure 4. Results of elasticity testing of beads formed in 0.1 M $CoCl_2$ with loading of a red pigment after 10 cycles of shrinking and swelling. Grey bars= weight of dried beads; black bars= weight of restored beads.

Moreover, the size of beads can affect diffusivity of substrates as well as cell loading. Doherty Speirs et al. (1995) [17] 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) [18] 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 immobilized. 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 $CaCl_2$.

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 (Figure 5). There was no significant difference (in the viable cell number in beads and leaked viable cells) between the control group (2.83×10^6 and 2.83×10^6 CFU/mL) and Experimental group 3 (2.13×10^5 and 7.25×10^6 CFU/mL), Experimental group 1 (6.00×10^7 and 1.83×10^7 CFU/mL) and Experimental group 4 (5.50×10^5 and 1.08×10^7 CFU/mL), as well as Experimental group 2 (8.63×10^5 and 1.28×10^7 CFU/mL) and Experimental group 5 (5.38×10^5 and 8.75×10^6 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 (6.00×10^7 CFU/mL in beads, compared with 1.83×10^7 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

[19]. In addition, NaCl can affect antimicrobial activity both directly and indirectly, depending on the amount added and the function it performs [20]. 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.

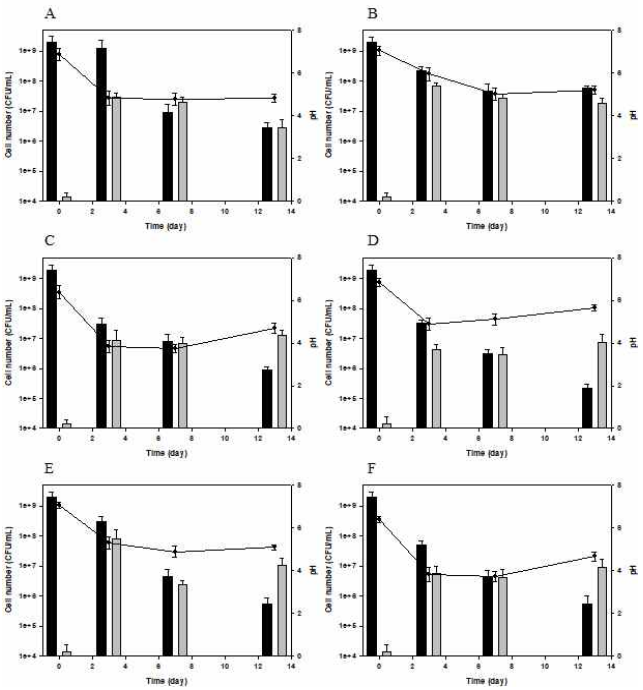

Figure 5. pH (—○—) and viable cell number for *R. capsulatus* in beads (black bars) and preservative solution (grey bars) after 0, 3, 7 and 13 days. Beads were stored in various preservative solutions. (A) Controls. (B) Experimental group 1. (C) Experimental group 2. (D) Experimental group 3. (E) Experimental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

During storage of beads at 30°C for 14 days, the biggest change in pH occurred in all experimental groups within 3 days (Figure 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 [21]. 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 (Figure 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 [22]. 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.

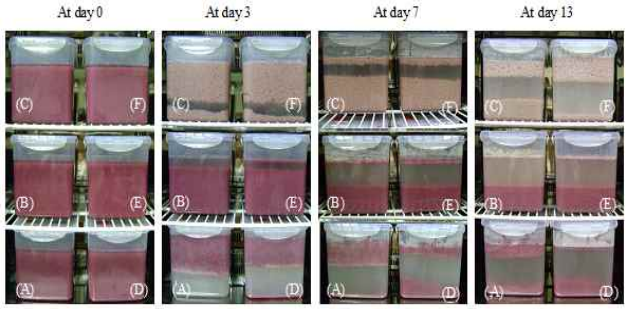

Figure 6. Appearances of PSB beads at days 0, 3, 7 and 13 of storage. (A) Controls. (B) Experimental group 1. (C) Experimental group 2. (D) Experimental group 3. (E) Experimental group 4. (F) Experimental group 5.

3.3. Cell viability after lyophilization with cryoprotective additives

Although lyophilized products are considerably easier to stabilize than their liquid counterparts, lyophilization can provoke sublethal damage to microbes, requiring appropriate cryoprotective additives to protect microbes when lyophilized. 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 lyophilization are shown in Figure 7. Prior to lyophilization, the viable cell number in each experimental group together with controls was almost the same (ranging from 5.75×10^9 to 8.50×10^9 CFU/mL). After lyophilization, the highest survival for PSB (2.61×10^9 CFU/mL) was observed in Experimental group 1, followed by Experimental group 3 (2.47×10^9 CFU/mL), Experimental group 4 (2.31×10^9 CFU/mL), Experimental group 2 (1.60×10^9 CFU/mL) and controls (1.40×10^8 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.

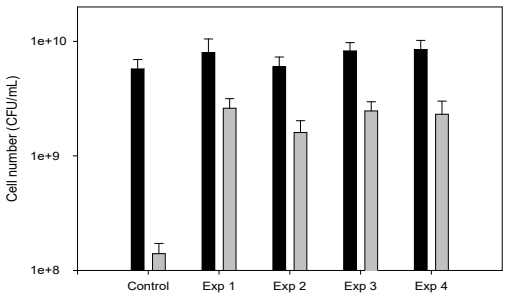

Figure 7. Measurement of viable cell number before lyophilization (black bars) and after lyophilization (grey bars). (A) Controls. (B) Experimental group 1. (C) Experimental group 2. (D) Experimental group 3. (E) Experimental group 4.

In Experimental group 1, cryoprotective additives skim milk (9%, w/v) and dextrose (2%, w/v) were added before lyophilization. These cryoprotective additives have been used to enhance the stability of cell viability during the storage of lactic acid bacteria [23,24]. 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°C and 12% at 20°C for *L. fermentum* SK152 stored for 8 weeks [23]. In the case of *Lactobacillus salivarius* W13, cell survivability was measured after lyophilized bacterial powders were stored at -80°C 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 [24]. The effects of cryoprotective additives on PSB have been reported in previous studies. As protective agents for lyophilization, dimethyl sulfoxide (5% v/v) and glycerin (15% v/v) were

the most promising cryoprotectants for preservation of the cyanobacterial strain *Trichocoleus sociatus* [25]. Dimethyl sulfoxide 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°C, with no loss of functionality for photoautotrophy, diazotrophy, hydrogen production and pigmentation [13]. 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.

3.4. Field application of PSB powder and beads

To explore practical use, PSB with enhanced microbial stability through cell immobilization and lyophilization 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 favorably 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 11 weeks, then decreased and finished at 0.04 mg/L (Figure 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 (Figure 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 feces). Lyophilized *R. sphaeroides* and *R. capsulatus* entrapped in beads are known to be effective pollutant removers in wastewater [14] and bioremediation agents for denitrification and nitrification of municipal wastewater [26], respectively. Therefore, the use of both PSB powder and beads was effective for removing nitrous substances present in shrimp aquaculture water.

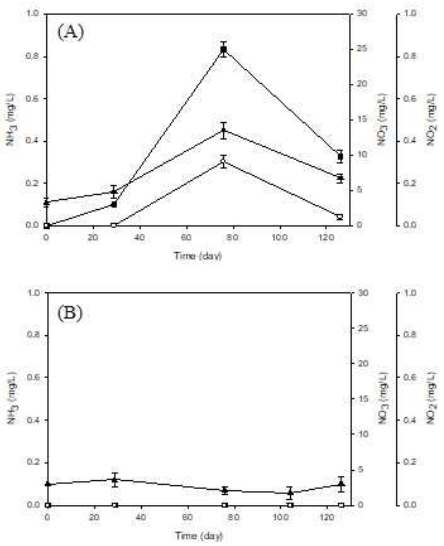

Figure 8. Changes in the concentrations of NH_4^+ (closed triangles), NO_3^- (closed rectangulars) and NO_2^- (open circles) during shrimp breeding in the presence of PSB powder and beads as water-purifying agents. (A) Control group. (B) Experimental group.

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*) [27]. 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 sulfidophi-*

lum was added to shrimp feed, and the combined feed was used for 70-day breeding of *Marsupenaeus japonicus* (kuruma shrimp) [28]. 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 [29]. 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 [30].

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 [31]. Polyvinyl alcohol-gel beads containing PSB (*R. capsulatus*) have also been employed for purification of water in goldfish rearing [10]. 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 [32]; 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 [33]. 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.

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, optimized conditions for cell immobilization and lyophilization were investigated. PSB beads (4 mm diameter) solidified in 0.1 M $CaCl_2$ were the most cost-effective and displayed the highest loading (1.91×10^9 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 lyophilized, addition of skim milk (9%) and dextrose (2%) were the best additives for reducing loss of cell viability. Under optimal conditions, PBS beads and lyophilized 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.

Acknowledgements

We are grateful to Ecobiznet Company (Chuncheon,

Gangwon-do, Korea) for invaluable assistance in providing photosynthetic bacterial species, culture media and reactors for the present study.

References

1. Zhu, Y. 2007. Immobilized cell fermentation for production of chemicals and fuels. In: Yang S.-T. (ed.), Bioprocessing for Value-Added Products from Renewable Resources. Elsevier Science, pp. 373-396.
2. Lu, J., Peng, W., Lv, Y., Jiang, Y., Xu, B., Zhang, W., Zhou, J., Dong, W., Xin, F. and Jiang, M. 2020. Application of cell immobilization technology in microbial cocultivation systems for biochemicals production. *Ind. Eng. Chem. Res*. 59, 17026-17034.
3. Elkahlout, K., Alipour, S., Eroglu, I., Gunduz, U. and Yucel, M. 2016. Long-term biological hydrogen production by agar immobilized *Rhodobacter capsulatus* in a sequential batch photobioreactor. *Bioproc. Biosyst. Eng.* 40, 589-599.
4. Lopez, A. Lazaro, N. and Marques, A. M. 1997. The interphase technique; a simple method of cell immobilization in gel-beads, *J. Microbiol. Methods* 30, 231-234.
5. Katzbauer, B., Narodoslawsky, M. and Moser, A. 1995. Classification system for immobilization techniques, *Bioprocess Eng.* 12, 173-179.
6. Tian, X., Liao, Q., Liu, W., Wang, Y. Z., Zhu, X., Li, J. and Wang, H. 2009. Photo-hydrogen production rate of a PVA-boric acid gel granule containing immobilized photosynthetic bacteria cells. *Int. J. Hydrogen Energy* 34, 4708-4717.
7. Rocher, V., Siaugue, J. M., Cabuil, V. and Bee, A. 2008. Removal of organic dyes by magnetic alginate beads. *Water Res.* 42, 1290-1298.
8. Idi, A., Nor, M. H. M., Wahab, M. F. A. and Ibrahim, Z. 2015. Photosynthetic bacteria: an eco-friendly and cheap tool for bioremediation. *Rev. Environ. Sci. Biotechnol.* 14, 271-285.
9. Xu, Q., Ju, Y. and Ge, H. 2012. Immobilization and ammonia removal of photosynthetic bacteria. *Adv. Mat. Res*. 610-613, 311-314.
10. Jeong, S. K., Cho, J. S., Kong, I. S., Jeong, H. D. and Kim, J. K. 2009. Purification of aquarium water by PVA gel-immobilized photosynthetic bacteria during goldfish rearing. *Biotechnol. Bioprocess Eng.* 14, 238-247.
11. Chen, J., Wei, J., Ma, C., Yanf, Z., Li, Z., Yanf, X., Wang, M., Zhang, H., Hu, J. and Zhang, C. 2020. Photosynthetic bacteria-based technology is a potential alternative to meet sustainable wastewater treatment requirement? *Environ. Int.* 137, 105417.
12. Peng, M., Xu, H., Yang, G. and Zhang, G. 2021. Purifying heavily polluted river water using immobilized native photosynthetic bacteria. *J. Environ. Eng.* 147, 04021021.
13. Malik, K. A. 1988. Long-term preservation of some *Rhodospirillaceae* by freeze-drying. *J. Microbiol. Methods* 8, 273-280.
14. Liu, S., Li, X., Zhang, G. and Zhang, J. 2015. Optimization of influencing factors on biomass accumulation and 5-aminolevulinic acid (ALA) yield in *Rhodobacter sphaeroides* wastewater treatment. *J. Microbiol. Biotechnol*. 25, 1920-1927.
15. Chen, K. C., Lee, S. C., Chin, S. C. and Houng, J. Y. 1998. Simultaneous carbon-nitrogen removal in waste-water using phosphorylated PVA-immobilized microorganisms. *Enzyme Microb. Technol*. 23, 311-320.
16. Mahey, S., Kumar, R., Sharma, M., Kumar, V. and Bhardwaj, R. 2020. A critical review on toxicity of cobalt and its bioremediation strategies. *SN Appl. Sci*. 2, 1279.
17. Doherty Speirs, E., Halling, P. J. and McNeil, B. 1995. The importance of bead size measurement in mass-transfer modelling with immobilised cells. *Appl. Microbiol. Biotechnol.* 43, 440-444.
18. Lee, H. K., Jeong, D. W. Im, S. J. and Jang, A. 2020. Optimization of alginate bead size immobilized with *Chlorella vulgaris* and *Chlamydomonas reinhardtii* for nutrient removal. *Bioresour. Technol*. 302, 122891.
19. Pang, H., Xin, X., He, J., Cui, B., Guo, D., Liu, S., Yan, Z., Liu, C., Wang, X. and Nan, J. 2020. Effect of NaCl concentration on microbiological properties in NaCl assistant anaerobic fermentation: hydrolase activity and microbial community distribution. *Front. Microbiol*. 11, 589222.
20. Ravishankar, S. and Juneja, V. K. 2014. Preservatives: traditional preservatives – sodium chloride. In: Batt C. and Patel P. (eds.), Encyclopedia of Food Microbiology. 2nd ed., Elsevier Inc., pp. 131-136.
21. Lin, D. Kelly, A. L. and Miao, S. 2020. The impact of pH on mechanical properties, storage stability and digestion of alginate-based and soy protein iso-

late-stabilized emulsion gel beads with encapsulated lycopene. *Food Chem.* 372, 131262.

22. Cho, K. S., Park, K. J., Jeong, H. D., Nam, S. W., Lee, S. J., Park, T. J. and Kim, J. K. 2006. Characteristics of immobilized PVA beads in nitrate removal. *J. Microbiol. Biotechnol.* 16, 414-422.
23. Kim, S. H., Gye, H., Oh, J. K., Hwang, I. C. and Kang, D. K. 2019. Protective effect of cryoprotectants on the viability of freeze-dried *Lactobacillus fermentum* SK152. *J. Milk Sci. Biotechnol*. 37, 206-212.
24. Yeo, S., Shin, H. S., Lee, H. W., Hong, D., Park, H. and Holzapfel, W. 2018. Determination of optimized growth medium and cryoprotective additives to enhance the growth and survival of *Lactobacillus salivarius*. *J. Microbiol. Biotechnol*. 28, 718-731.
25. Witthohn, M., Schwarz, A., Walther, J., Strieth, D., Ulbe r, R. and Muffler, K. 2020. Novel method enabling a rapid vitality determination of cyanobacteria. *Eng. Life Sci.* 20, 580-584.
26. Costa, S., Ganzerli, S., Rugiero, I., Pellizzari, S., Pedrini, P. and Tamburini, E. 2017. Potential of *Rhodobacter capsulatus* grown in anaerobic-light or aerobic-dark conditions as bioremediation agent for biological wastewater treatments, *Water* 9, 108.
27. Chumpol, S., Kantachote, D., Nitoda, T. and Kanzaki, H. 2018. Administration of purple nonsulfur bacteria as single cell protein by mixing with shrimp feed to enhance growth, immune response and survival in white shrimp (*Litopenaeus vannamei*) cultivation. *Aquaculture* 489, 85-95.
28. Koga, A., Tani, Y., Ozaki, K. I., Maki, T., Hayashi, S., Yamamoto, S. and Miyasaka, H. 2021. The effects of a marine photosynthetic bacteria *Rhodovulum sulfidophilum* on the growth and survival rate of *Marsupenaeus japonicus* (kuruma shrimp). *Journal of Aquaculture, Fisheries & Fish Science (JAFFS)* 3, 245-249.
29. Antony, S. P. and Philip, R. 2006. Bioremediation in shrimp culture systems. NAGA, World Fish Center Quarterly 29, 62-66.
30. Barman, P., Raut, S., Sen, S. K., Shaikh, U., Bandyopadhyay, P. and Mohapatra, P. K. D. 2017. Effect of a three-component bacterial consortium in white shrimp farming for growth, survival and water quality management. *Acta Biol Szeged* 61, 35-44.
31. Zhang, X., Shu, M., Wang, Y., Fu, L., Li, W., Deng, B., Liang, Q. and Shen, W. 2014. Effect of photosynthetic bacteria on water quality and microbiota in grass carp culture. *World J. Microbiol. Biotechnol.* 30, 2523-2531.
32. Sichula, J., Makasa, M. L., Nkonde, G. K., Kefi, A. S., and□Katongo, C. 2011. Removal of ammonia from aquaculture water using maize cob activated carbon. *Malawi J. Aquac. Fish.* 1, 10-15.
33. Aly, H. A., Abdel Rahim, M. M., Lotfy, A. M., Abdelaty, B. S. and Sallam, G. R. 2016. The applicability of activated carbon, natural zeolites, and probiotics (EM®) and its effects on ammonia removal efficiency and fry performance of european seabass *Dicentrarchus labrax*. *J. Aquac. Res. Development* 7, 1000459.