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Long-term preservation of high initial bioluminescence of lyophilized *Photobacterium phosphoreum*: Effect of skim milk and saccharose at various temperatures

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Abstract−A lyophilization method for long-term preservation of the initial bioluminescence of *Photobacterium phosphoreum* was investigated. The initial bioluminescence of lyophilized *P. phosphoreum* at different temperatures, –20 °C, 4 °C, room temperature (16-25 °C) and 45 °C, was monitored for 6 months with different additives. Saccharose, skim milk, and a mixture of saccharose and skim milk were tested. Skim milk showed the best protection for bioluminescence among the additives used. The initial bioluminescence remained high during 6 months at a wide range of storage temperatures, i.e., –20 °C, 4 °C, and room temperature, with skim milk as an additive. The average bioluminescence of lyophilized *P. phosphoreum* using skim milk as additive reached 33392 RLU within 30 min of regeneration. Bioluminescence increased slightly after 10 h of incubation.

Key words: *Photobacterium phosphoreum*, Initial Bioluminescence, Lyophilization, Long-Term Preservation

INTRODUCTION

Bioluminescent bacteria have been widely used for toxicity monitoring in water resources with high sensitivity and rapid response against toxicants [1]. Bioluminescent bacteria have been used to detect many kinds of toxicants including heavy metals [2], chromated copper arsenate [3], mycotoxins [4], substituted benzenes [5], tetramethyl ammonium hydroxide [6], etc. An *in situ* intermittent testing method using continuously cultured bioluminescent bacteria was developed for long-term monitoring. Recently, in order to develop continuous real time monitoring of pollutants, the immobilization of *P. phosphoreum* has been studied [7]. In addition, immobilization of *P. phosphoreum* can restrict the appearance of mutant variants and can remove contaminant microorganisms growing outside of the beads during cultivation [8,9].

Lyophilization is one of the most effective methods for the long-term preservation of cells without causing significant changes in their morphology and properties [10]. Protectants are needed to increase viability during lyophilization and storage. Many kinds of compounds have been investigated as protective additives that provide cryoprotection. Abrashev et al. [10] used a mixture of 10% sucrose, 1.5% gelatin, and 0.1% agar or 5.5% dried skim-milk as an additive for long-term preservation of luminescent bacteria. Costa et al. [11] found that saccharose and skim milk are effective for lyophilization and rehydration of *Pantoea agglomerans* CPA-2, respectively.

The research was conducted to identify the best additive for long-term preservation of high bioluminescence of *P. phosphoreum* by using a lyophilization method, with a special focus on attaining high initial bioluminescence after preservation. The effects of commonly used additives in the lyophilization procedure including 3% saccharose (w/w), 10% skim milk (w/w), and a mixture of 3% saccharose (w/w) and 10% skim milk (w/w) were studied. Lyophilized cells were preserved for 6 months at various temperatures including –20 °C, 4 °C, room temperature (16-25 °C), and 45 °C and initial bioluminescence of regenerated cells was monitored.

MATERIALS AND METHODS

1. Preparation of *P. phosphoreum* for Cryopreservation

A modified LB medium consisting of 0.3% yeast extract (w/w) (Difco Co., USA) and 2% NaCl (w/w) in distilled water was used for cultivation of *P. phosphoreum*. Firstly, *P. phosphoreum* colonies were grown on agar plates, which were stored at 4 °C. The colonies were then reactivated in an Erlenmeyer flask using a shaking incubator at 200 rpm and 18 °C for 12 h. Afterwards, the activated strain was cultured in 1 L modified LB medium using the shaking incubator at 200 rpm and 18 °C. After 15 h of incubation, the cell broth was centrifuged at 9,000 g for 20 min to recover the cells. The cell pellet was resuspended and diluted with 10 ml of 2% NaCl solution (w/w). The concentration of cells was measured by a spectrophotometer (Spectronic GENESYS 5, Thermo Fisher Scientific Inc., USA). The OD_{600} was 0.39 when the cell pellet was diluted 100 times.

2. Preparation of Cell Solutions with Additives for Lyophilization

The cell solution (prepared above) was divided into 4 test tubes. Saccharose, skim milk, and a mixture of saccharose and skim milk were added to each test tube. The final concentrations of saccha-

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Table 1. Components of cell solution and additives

Additive (s)	Components	
Without additives	Cell solution	1 ml
	2% NaCl (w/w)	11 ml
	Medium solution	18 ml
Saccharose (3%)	Cell solution	1 ml
	2% NaCl (w/w)	9 ml
	45% saccharose (w/w)	2 ml
	Medium solution	18 ml
Skim milk (10%)	Cell solution	1 ml
	2% NaCl (w/w)	2 ml
	33% skim milk (w/w)	9 ml
	Medium solution	18 ml
Saccharose (3%) and skim milk (10%)	Cell solution	1 ml
	45% saccharose (w/w)	2 ml
	33% skim milk (w/w)	9 ml
	Medium solution	18 ml

*The total volume of each solution was 30 ml.

rose and skim milk after mixing with cell solutions were 10% and 3%, respectively. The components and volume to make each solution are shown in Table 1. The test tubes (8×50 POLYPRO, Turner Designs Inc., USA), which were covered tightly by aluminum foil cap and solutions, were sterilized in an autoclave at 121 °C for 20 min. The mixtures of cells and additive solutions were mixed thoroughly and 100 µl of mixtures was distributed into each test tube. The test tubes were capped again by aluminum foil and lyophilized.

3. Procedure of Lyophilization

All samples in test tubes were frozen at −70 °C for 12 h. The samples were then lyophilized for 3 days to completely remove water from the tightly capped test tubes. Finally, they were placed in aluminum pouches with silica-gel beads to remove the remaining moisture and for storage. The pouches were sealed by a heat sealer to protect the samples from air and moisture and then stored at different temperatures including, i.e., −20 °C, 4 °C, room temperature (16-25 °C), and 45 °C, for 6 months. A refrigerator or convection oven was used for the preservation according to the temperature.

4. Measurement of Bioluminescence

The cryopreserved *P. phosphoreum* were regenerated by addition of 200 µl of modified LB medium. After 30 min, the first measurement of each sample was carried out. The bioluminescence was measured as relative light units (RLU) by using a Turner TD-20e luminometer (Turner Designs Inc., USA) at 2 h of intervals for 26 h. In case of higher bioluminescence, a detector system with a photomultiplier tube (type H5784, Hamamastsu Co., Japan) and a data acquisition system (NI Co., USA) was used. We defined a light intensity lower than 100 RLU as insufficient bioluminescence to be used for the monitoring of toxicants. The data acquisition system including a SCXI-1600 chassis, a SCXI-1124 digital-to-analogue module, and a SCXI-1120/D signal conditioning module was controlled by Labview 7.1 software. The data measurement by the detector system was converted into RLU following the pre-determined correlation ratio with the Turner TD-20e luminometer.

RESULTS AND DISCUSSION

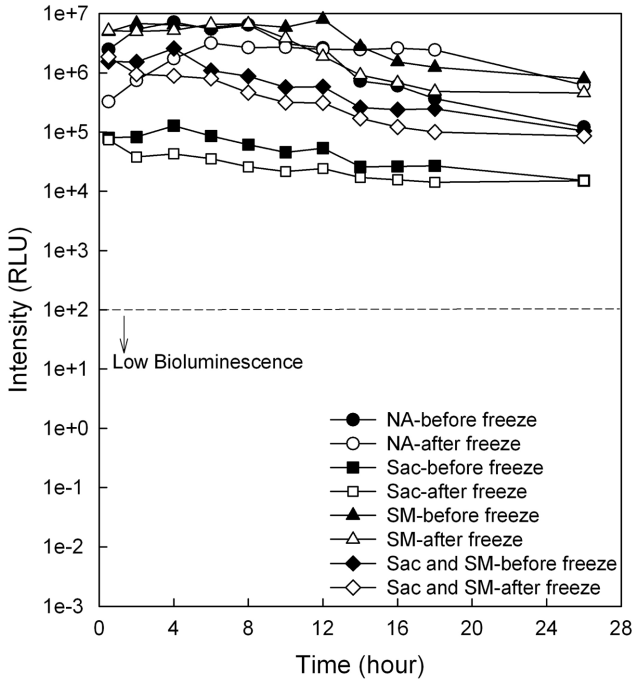

Fig. 1. Bioluminescence of *P. phosphoreum* before and after deep-freezing (−70 °C). NA-No additive; Sac-Saccharose; SM-Skim milk.

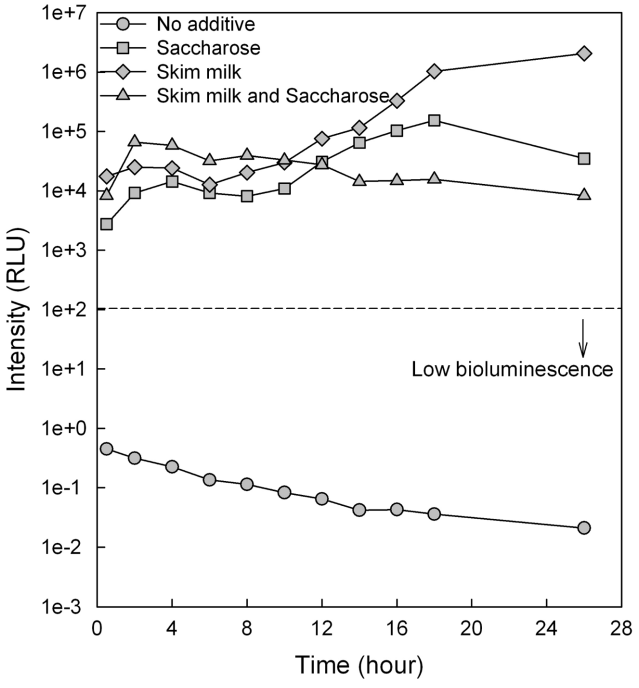

Fig. 2. Effect of additives on lyophilization before and after freeze drying.

1. Effect of Additives on Bioluminescence during Freezing Step

In the principle of lyophilization, the samples should be deep-frozen before freeze drying. The effect of additives on bioluminescence at each step should be investigated. Firstly, the effect of the additives during a freezing step was determined. The samples were

frozen in a deep-freezer at −70 ºC for 12 h before being lyophilized. The bioluminescence of the samples before and after deep-freezing was compared. The results are shown in Fig. 1. The bioluminescence was slightly lowered by deep-freezing in all cases. The additives do not have much of a role during the freezing step, because the bioluminescence with and without additive does not show much difference. Comparing the no-additive and skim milk added samples, the initial bioluminescence was lowered by the addition of saccharose by around 1 order. Half of the bioluminescence of the no-additive and skim milk added samples was obtained when skim milk was used with saccharose together (shown in Fig. 1). Consequently, it was determined that saccharose damaged the bioluminescence during the freezing of *P. phosphoreum*.

2. Effect of Additives on Bioluminescence at Lyophilization Step

The bioluminescence of the samples was measured immediately after performing lyophilization. The lyophilized cells were thawed and rehydrated by addition of modified LB media at room temperature. The results are shown in Fig. 2. The initial bioluminescence was decreased by lyophilization relative to the initial bioluminescence after the freezing step. The effect of additives on the viability of *P. phosphoreum* is clear. *P. phosphoreum* cannot survive without additives. Clearly, skim milk and saccharose have a protective effect on the cells and bioluminescence at the extreme conditions of lyophilization, considering that an unoptimized thawing and rehydration procedure was used in this experiment. The initial bioluminescence was relatively higher than other cases when the mixture of saccharose and skim milk was added. However, bioluminescence was gradually decreased during incubation, reflecting insufficient revival of cells. The bioluminescence was increased when saccharose was added even when the initial bioluminescence was less than 10000 RLU. Again, skim milk showed the highest bioluminescence after 12 h of incubation and a relatively higher initial bioluminescence than the mixture of skim milk and saccharose.

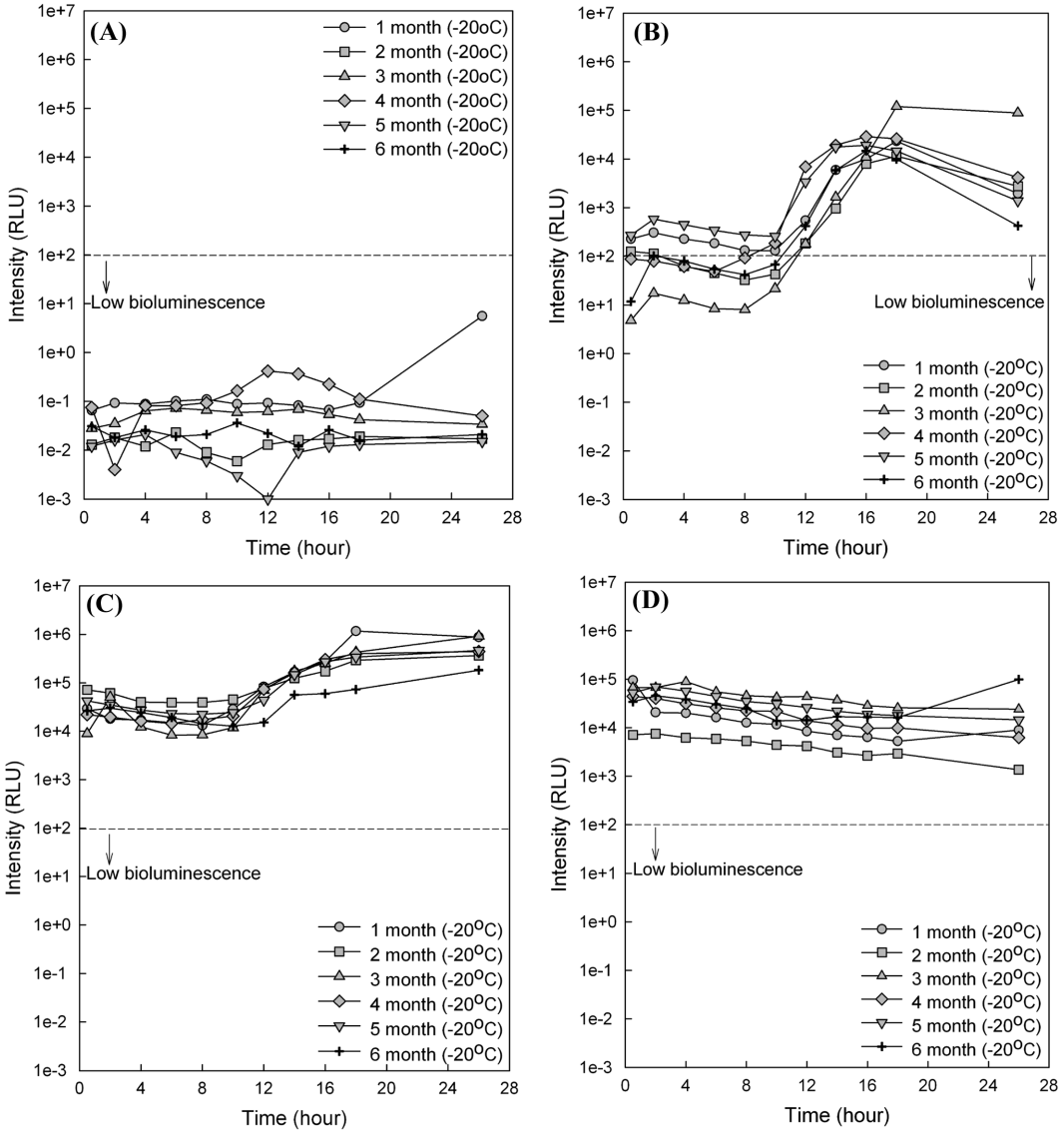

Fig. 3. Effect of additives on the recovery of bioluminescence during 6 months of storage at −20 ºC. (A) No additive as control, (B) Saccharose, (C) Skim milk, (D) Mixture of saccharose and skim milk.

3. Long-Term Preservation

Fig. 3 shows the effect of additives on the recovery of bioluminescence during 6 months of storage at −20 ºC. Bioluminescence was not observed in the control sample without additives (Fig. 3A). When saccharose was used as an additive, the initial bioluminescence was low; however it increased after 10 h of incubation and decreased slightly after 26 h (Fig. 3B). When skim milk was used as an additive, *P. phosphoreum* emitted high bioluminescence within 30 min. In addition, the intensity of bioluminescence remained stable for approximately 10 h and then slightly increased (Fig. 3C). When the mixture of saccharose and skim milk was used as an additive, the high intensity of bioluminescence was maintained very stable for a long period of time. However, the intensity of bioluminescence decreased slightly during 26 h of incubation and was lower than that of the skim milk added sample after 12 h (Fig. 3D).

During 6 months of storage at −20 ºC, *P. phosphoreum* still produced bioluminescence with high intensity when skim milk or a mixture of saccharose and skim milk was used. It is clear that skim milk and the mixture of saccharose and skim milk are better than saccharose alone for the purpose of long-term preservation of *P. phosphoreum* for high bioluminescence. The skim milk plays an important role in the protection of cells in the extreme conditions of lyophilization. With the presence of skim milk (that is, both cases of skim milk only and the mixture of skim milk and saccharose), *P. phosphoreum* responded to the medium rapidly with high bioluminescence. The rapid response of *P. phosphoreum* within 30 min illustrates its usefulness for rapid toxicant detection. Moreover, the lyophilized samples using skim milk as an additive can be applied to a wide range of storage temperatures, from −20 ºC to room temperature (16-25 ºC) (Fig. 4). The bioluminescence was similarly high when the samples were stored at −20 ºC and 4 ºC (Fig. 4A and 4B). However, when the samples were stored at room temperature, which varied from 16 to 25 ºC, the bioluminescence was slightly lower than that at −20 ºC and 4 ºC (Fig. 4C). The bioluminescence

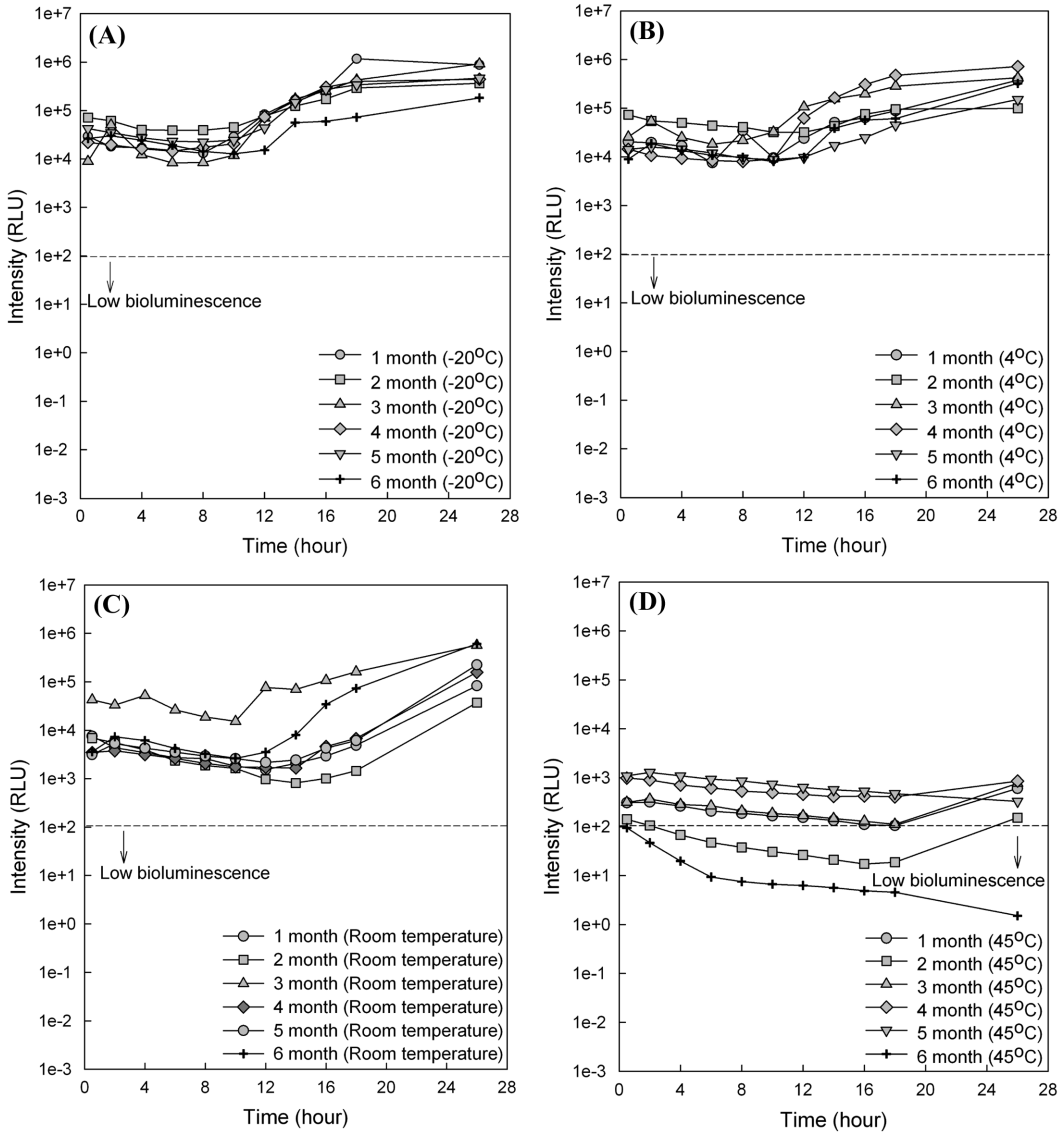

Fig. 4. Bioluminescence of lyophilized samples stored at different temperatures using skim milk as an additive. (A) −20 ºC, (B) 4 ºC, (C) room temperature (16-25 ºC), (D) 45 ºC.

remained at 45 ºC but was lower than other cases and gradually decreased even with skim milk (Fig. 4D). Thus, storage at temperatures lower than room temperature is recommended for long-term preservation of bioluminescence where skim milk is employed as an additive.

CONCLUSION

Lyophilization is an efficient method for long-term preservation of *P. phosphoreum*. Skim milk was the best additive among the compounds we tested. With the simple procedure of lyophilization using skim milk as an additive, initial bioluminescence and viability of *P. phosphoreum* were kept high for more than 6 months at a wide range of storage temperatures. The good initial bioluminescence of lyophilized *P. phosphoreum* at different storage temperatures will make the lyophilized samples easy to use for portable and rapid toxicant monitoring applications.

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