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Effect of Trehalose on Bioluminescence and Viability of Freeze-Dried Bacterial Cells

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Abstract Two recombinant bacteria containing *luxAB* showed an increased tolerance to stresses associated with lyophilization, when the cells were freeze-dried in the presence of trehalose. In the case of a recombinant, UV2, only 2.5% of the original bioluminescence and 2.7% of the cell viability were restored after 4 h of freeze-drying without trehalose, which implies that the cells were heavily damaged during the dehydration. To improve these losses, trehalose was added before freeze-drying using different modes. Trehalose increased the bioluminescence and the viability of freeze-dried UV2 under all conditions tested, and it was also observed that the addition of trehalose to the cultures (final concentration of 0.08 M) for 15 min before the freeze-drying resulted in the restoration of 45% of the original bioluminescence and 50% of the cell viability. Trehalose also showed a similar efficacy with the other luminescent recombinant, YH9. Therefore, it was tentatively concluded that trehalose played a role as a protective agent in the freeze-drying of bacterial cells.

Key words: Trehalose, freeze-drying, bioluminescence, viability

Freeze-drying is often used for the preservation and long-term storage of cells, drugs, and foods [6]. However, the stresses associated with freeze-drying cause damages leading to decrease in the viability and physiological functions of cells [4]. To prevent such damages, stabilizers such as glycerol or skim milk are added to cell suspensions before freeze-drying. In addition to these stabilizers, it has also been reported that nonreducing disaccharides can protect biological materials from damage originating from freeze-drying [10, 15]. Among disaccharides, trehalose is known to have an excellent efficacy against high temperatures, osmosis, and drying [8]. Trehalose is a nonreducing sugar of two glucose residues with an α-1,1-linkage. It has been reported that trehalose can also protect cells from various physical and chemical attacks [2, 5, 6]. Two hypotheses have been proposed to explain the protective function of trehalose during freeze-drying. The first is based on the theory that the removal of hydrogen-bonded water from phospholipid headgroups in cell membranes causes structural changes in the cells in a dry state. As such, trehalose forms hydrogen bonds with phospholipid headgroups when the water is removed, thereby preventing structural changes in the cells [1, 8, 14]. In the second mechanism, trehalose protects the cells by forming a glassy matrix against ice formation during freezing [9, 13]. Thus, trehalose flows into the cells according to its concentration gradient and protects the cytosol against freeze-drying. Using scintillation counter earlier, trehalose was found to be transported into the cells [8]. Accordingly, the current study attempted to investigate the effect of trehalose on the bioluminescence and viability of freeze-dried *luxAB*-containing recombinant bacteria.

In order to obtain luminescent recombinants, bacterial strains, identified as *Pantoea agglomerans* and *Janthionobacterium lividum* [7], were isolated [3] from a river and a deep groundwater, respectively. These two natural isolates were genetically modified to provide bioluminescent phenotype by conjugating the plasmid pUT containing *luxAB* genes and were named as UV2 (*P. agglomerans*) and YH9 (*J. lividum*). The culture media for the recombinant UV2 and YH9 were antibiotic-supplemented (100 μg/ml of ampicillin and 7.5 μg/ml of tetracycline) LB and R2A, respectively [11]. Seed cultures (50 ml) were inoculated with frozen glycerol stocks, grown to exponential phase in a shaking incubator at 200 rpm and 30°C, and then transferred (5 vol. %) into fresh media. When the cells reached the exponential phase (A_{600} 1.5 for UV2 and 0.6 for YH9), trehalose was added to the flask. In another set of experiments, trehalose was added at the beginning of the second culture, so that the cells continuously grew in the presence of this sugar until harvest or added to the cell

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suspension right before freeze-drying. Thirty μl of the grown cell suspensions were loaded into the holes in a 384-well plate and frozen by immersing the plate in liquid nitrogen for 5 min, followed by 4 h of freeze-drying (DW 1.0-110, Heto, Denmark). The freeze-dried cells were then resuspended in 30 μl of fresh media to measure their bioluminescence as a relative light unit (RLU) (TD-20/20, Turner, U.S.A.) and viability as a colony-forming unit (CFU).

Although freeze-drying is known to be a reliable method for the preservation of biological materials, the resuscitation efficiency of freeze-dried cells is usually extremely low. As shown in Fig. 1, the CFU of the freeze-dried cells was only 3.5% and 12% of the untreated (freeze-drying of cells without any protective agents) UV2 and YH9 cells, respectively. Similar to the cell viability, the bioluminescence also decreased to 2.7% (UV2) and 9.8% (YH9) of the untreated cells. When trehalose was added to the cultures immediately before freeze-drying, UV2 retained 39.0% of its original bioluminescence and 40.4% of its cell viability at 0.33 M trehalose concentration. In the case of YH9, 26.9% of its original bioluminescence and 27.7% of its cell viability were restored with 0.16 M trehalose concentration (Fig. 1). Interestingly, the protective efficacy of trehalose on the freeze-drying of UV2 cells linearly increased as the trehalose concentration increased within the experimental range, whereas for YH9, no further increase was observed beyond 0.16 M trehalose.

Based on the assumption that trehalose needs to be transported into the cells to exert its protective activity, UV2 and YH9 were grown for 6-7 hrs in media containing various concentrations of trehalose and then freeze-dried. For both strains, the CFU and luminescence of the freeze-dried cells increased as the trehalose concentration increased (Fig. 2) up to 0.1 M (UV2) and 0.16 M (YH9); however, they did not grow at higher concentrations of trehalose, probably due to osmosis. Since no decrease of trehalose was observed during the cultivations (data not shown), it would seem that neither strain was able to assimilate trehalose, as only trace amounts were transported into the cells. Nonetheless, the incubation of the cells with trehalose

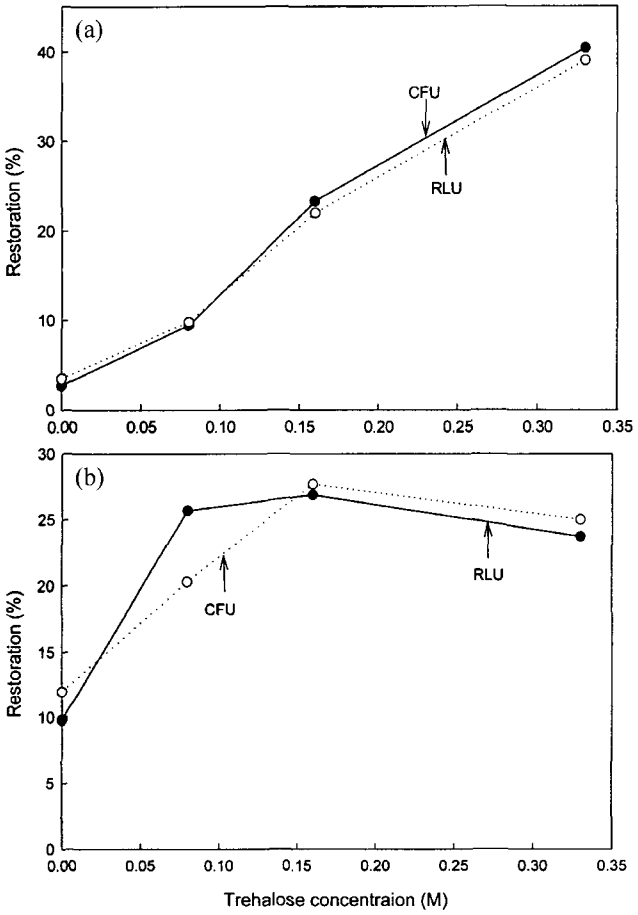

Fig. 1. Effect of trehalose on cell viability and luminescence of recombinant bacteria containing *luxAB* genes.
Trehalose was added to the cell suspension right before freeze-drying the cells. (a) UV2, (b) YH9.

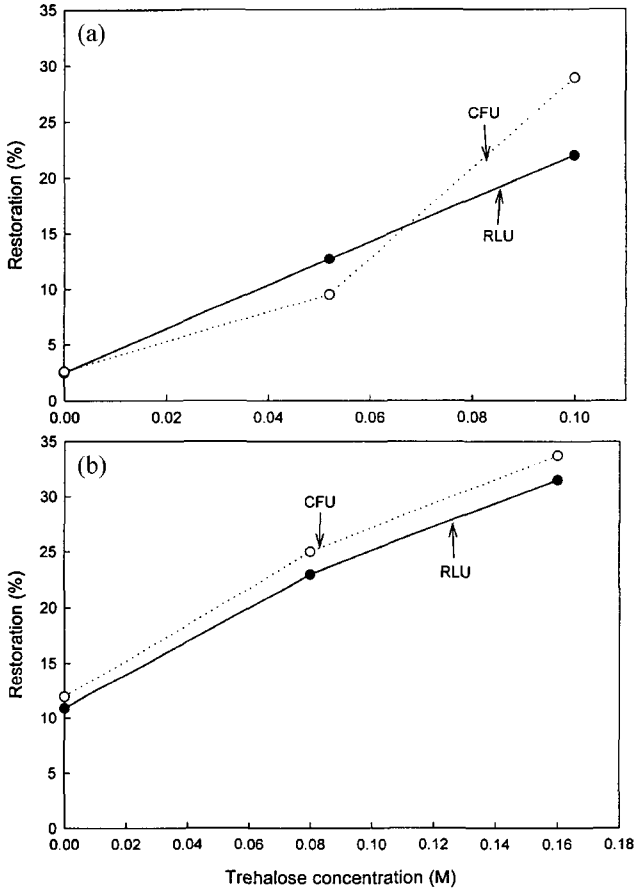

Fig. 2. Effect of trehalose concentration on cell viability and luminescence of UV2 (a) and YH9 (b).
The cells were grown in the presence of trehalose for 6-7 hours before freeze-drying.

was more effective, since higher CFU and luminescence, particularly for UV2, were obtained than with identical trehalose concentrations added immediately before freeze-drying. For instance, restoration efficiencies of 22% and 28.9% for the luminescence and viability of UV2, respectively, were obtained at trehalose concentration of 0.1 M in the case of 6 h incubation, whereas, as seen in Fig. 1, the efficiencies were only around 10% when the cells were freeze-dried immediately after the addition of trehalose.

In order to overcome the no-growth problem due to osmosis at high concentrations of trehalose and to estimate time required for the sugar to diffuse across cell membranes into cytoplasm, UV2 and YH9 cells were grown first in trehalose-free media, then trehalose was added to the grown cell suspensions and incubated for 15 min before the cell harvest for freeze-drying. As shown in Fig. 3, the CFU and luminescence of the freeze-dried cells increased as the trehalose concentration increased up to 0.16 M for both strains. The range of linear increase in Fig. 3 was essentially the same as that in Fig. 2, yet further improvements were obtained with 0.16 M trehalose. At this concentration, the restoration efficiencies of viability and luminescence for UV2 were 51.9% and 45.5%, respectively, while those for YH9 were 54.1% and 48.7%, respectively. To confirm the possibility of further increase in cell viability and luminescence with an optimized incubation time, the grown cells were incubated with 0.16 M trehalose for 5–30 min prior to freeze-drying (Fig. 4). However, no significant improvement was observed with incubation times for 5 to 30 min. Therefore, it was concluded that 5 min was long enough for the trehalose to diffuse across the cell membranes.

During the present experiments, it was found that bioluminescence and cell viability were linearly dependent on each other (Fig. 5). Bioluminescence is a reaction product of luciferase (*luxAB*) acting on an appropriate substrate (e.g., *n*-decyl aldehyde). The reducing power or energy source of this light-yielding reaction is $FMNH_2$

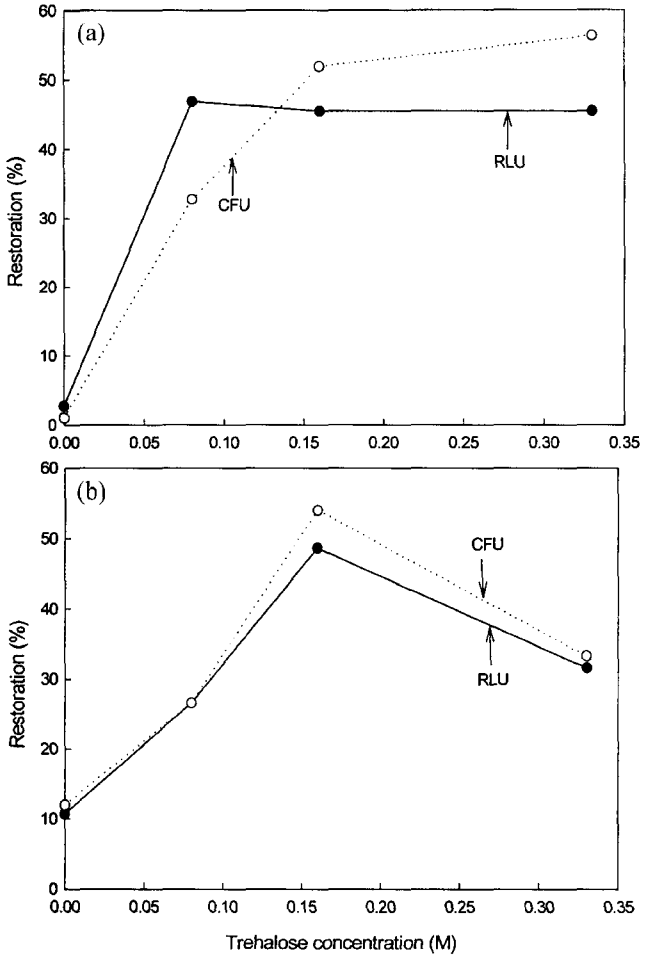

Fig. 3. Effect of trehalose concentration on cell viability and luminescence of UV2 (a) and YH9 (b).
The cells were incubated with trehalose for 15 min before freeze-drying.

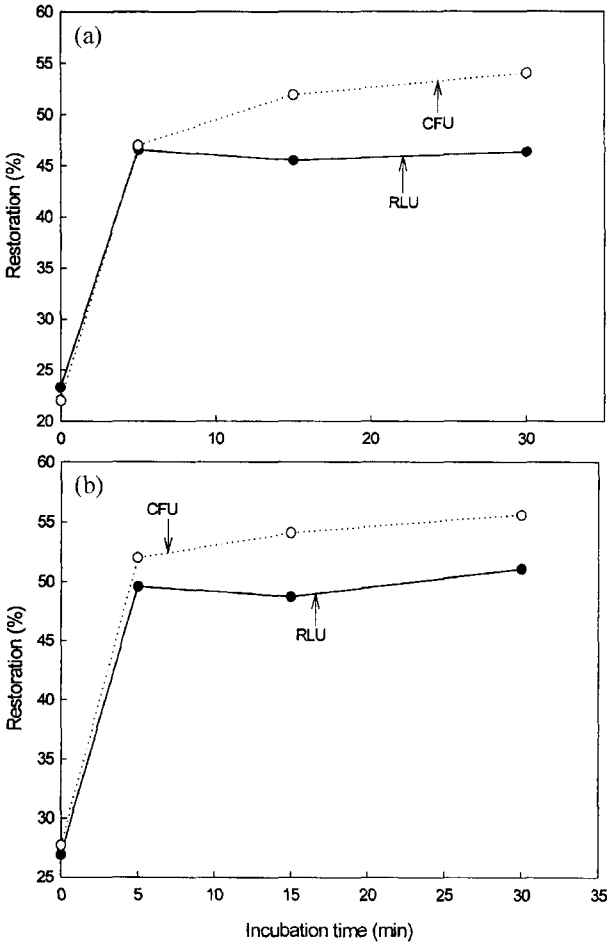

Fig. 4. Effect of incubation time of UV2 (a) and YH9 (b) with 0.16 M of trehalose.
The cells were freeze-dried after the incubation.

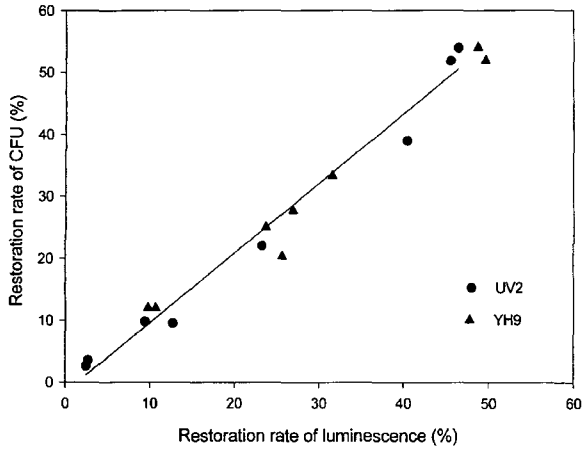

Fig. 5. Correlation between cell viability and luminescence of freeze-dried UV2 (●) and YH9 (▲).

(RCHO+FMNH$_2$+O$_2$+luciferase→RCOOH+FMN+H$_2$O+hν) [12]. For the luminescent recombinants used in this study, aldehyde was exogenously added to the suspensions of the freeze-dried cells, whereas the cellular FADH$_2$ present in the cells prior to freeze-drying was supposedly consumed in the reaction. Therefore, light was produced, not necessarily accompanied by the whole life function. However, according to experimental results, the luminescence and viability of the freeze-dried cells were strongly correlated. This implied that the luciferase and reducing power of the cells were severely damaged in the course of cell death. Hence, cell viability must be preserved in order to protect cell functions.

In addition to protecting cell viability and luminescence against freeze-drying, trehalose was also found to be effective in the long-term preservation of freeze-dried cells. As shown in Fig. 6, the cell viability and luminescence of

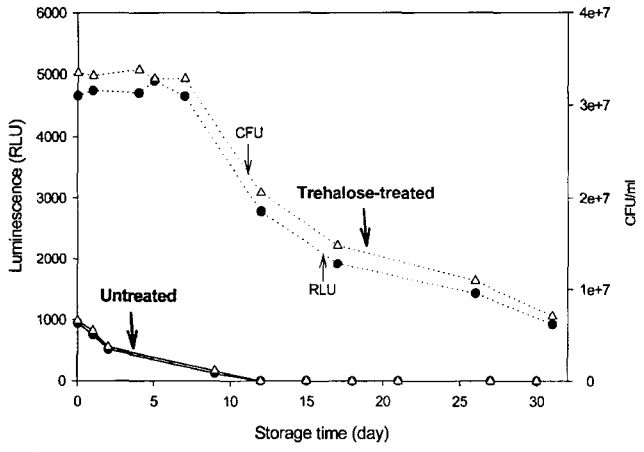

Fig. 6. Long-term storage of freeze-dried luminescent cells.
The trehalose (0.16 M)-treated (15 min of incubation) cells showed higher recovery rates of viability and luminescence after freeze-drying and during storage.

YH9 decreased immediately after freeze-drying when no trehalose was used, whereas trehalose-treated cells exhibited higher cell viability and luminescence after freeze-drying, together with much longer (up to 7 days) stable maintenance of life functions. If freeze-dried cells had been contained in a sealed glass vial, this storage time would be even longer, because aluminum tape used to seal the freeze-dried 384-well plate in the current study did not prevent the infiltration of oxygen and moisture. In conclusion, trehalose was found to be effective in preserving viability and luminescent function in both freeze-drying and long-term storage of freeze-dried cells.

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