Biotechnol. Bioprocess Eng. 2000, 5: 202-206

Enhancement in the Viability and Biosensing Activity of Freeze-Dried Recombinant Bioluminescent Bacteria

Sue Hyung Choi and Man Bock Gu*

Department of Environmental Science and Engineering, Kwangju Institute of Science and Technology (K-JIST), 1 Oryong-dong, Puk-gu, Kwangju 500-712, Korea

Abstract The genetically-engineered *Escherichia coli* strain, DPD2540, which contains a *fabA..luxCDABE* fusion gene, gives a bioluminescent output when membrane fatty acid synthesis is needed. For more practical application of this strain in the field as biosensor, freeze-drying was adopted. A 12% sucrose solution with Luria-Bertani (LB) broth, as determined by the viability after freeze-drying, was found to be the most effective composition for lyophilization solution among various compositions tested. Rapid freezing with liquid nitrogen also gave the best viability after freeze-drying as compared to samples frozen at -70°C and -20°C. The biosensing activities of the cells showed a greater sensitivity when the cells from the exponential phase were freeze-dried. Finally, the optimum temperature for use of the freeze-dried cells in the biosensor field was determined.

Keywords: recombinant bioluminescent bacteria, bioluminescence, cryoprotectant, optimum freezing and monitoring temperatures

INTRODUCTION

Freeze-drying is a method of preserving microorganism for extended periods. With this process, water is made unavailable to the cells, and the dehydrated cells are protected from water activity [1,2]. Many researchers have studied the parameters for optimum freeze-drying conditions to increase the viability of freeze-dried cells [3-9].

Bioluminescent bacteria having a fusion of a stress promoter and the *luxCDABE*-genes have been developed as toxicity biosensors [10,11]. Some previous works include characterization of these biosensing cells and fabrication of a miniature bioreactor for a continuous monitoring system [12-14]. For more practical usage of bioluminescent bacteria in field applications, the freeze-drying method was adopted for possible ease in storage and transportation of the bioluminescent cells.

The freeze-drying conditions that were previously studied include the influence of various cryoprotectants, including sucrose, trehalose, sorbitol and mannitol, the drying time, and the effects of the cell concentration [15]. The effects of the storage conditions and the influence of the rehydrating solution on the biosensing activity of freeze-dried cells has been also investigated [15]

In this study, we have studied some parameters more in depth, such as the concentration of sucrose, the freezing temperature, and the effect of the culture's growth stage. Various sucrose concentrations were introduced to optimize the concentration effects to obtain the best freeze-drying efficiency, and the optimum freezing temperature was examined to increase the viability during freeze-drying. The influences of the cell growth stage on freeze-drying were also studied via toxicity monitoring. In addition, the optimum experimental temperature for the use of the freeze-dried DPD2540 cells was also determined.

* Corresponding author
Tel: +82-62-970-2440 Fax: +82-62-970-2434
e-mail. mbgu@kjist.ac kr

MATERIALS AND METHODS

Cell Culture and Sample Preparation

The recombinant bioluminescent *E. coli* strain DPD2540, RFM443 harboring the plasmid p*FabALux6*, was used in this study. Plasmid p*FabALux6*, constructed at the DuPont Co , contains a transcriptional fusion of the *E. coli* stress promoter for *fabA* to the *Vibrio fisheri luxCDABE* genes and confers resistance to kanamycin and ampicillin [10,11]. The *fabA* gene encodes for 3-hydroxydecanoyl-ACP dehydrase, an enzyme responsible for the key reaction in the biosynthesis of membrane fatty acids [16]. Therefore, this strain gives a bioluminescent output when membrane fatty acid synthesis is needed. The medium used in this study was Luria-Bertani (LB) broth, pH7, supplemented with 25 mg/mL kanamycin monosulfate to maintain the plasmid. LB agar with kanamycin (Km) was used for plating and CFU (colony forming unit) enumeration. Seed cultures (100 mL) were grown with agitation at 250 rpm in a

Table 1. The effect of the temperature used for freezing on the viability of freeze-dried DPD2540

Freezing temperature	Freezing time	CFU(#/mL) after freeze-drying [$\times 10^5$]
Liquid nitrogen	30 min	23.70 ± 1 41
-70°C	2 days	9 65 ± 0.21
-20°C	2 days	0.25 ± 0.07
-20°C → liquid nitrogen	1 day → 30 min	0.05 ± 0 01
-20°C → -70°C	1day → 1day	0.015 ± 0.007
-20°C → -70°C → liquid nitrogen	1 day → 1 day → 30 min	0 016 ± 0.005

shaking incubator in 250 mL culture flasks at 30°C A new flask, containing 100 mL LB with kanamycin, was inoculated with 2 mL of the seed culture. When the optical density (OD_{600}) reached 0.08 (the early exponential phase), the cells were then centrifuged at 4000 rpm for 30 min. A spectrophotometer was used for off-line optical density (600 nm) measurements. After centrifugation, the pellet was resuspended in 10 mL of fresh LB medium and then mixed with 10 mL of a 24% sucrose solution. One mL aliquots were then pipetted into sterile 1.5 mL glass vials and freeze-dried.

Sucrose Concentration Effects and Growth Stage Effects

To determine the optimum composition for lyophilization medium, various concentrations of sucrose, with final concentrations of between 2 and 12% after being mixed with LB or LBG (LB medium+10% glucose), were tested. For the growth stage experiments, four different samples were prepared using cultures at different optical densities (OD_{600}), the early exponential (OD_{600} 0.08), late exponential (OD_{600} 0.37), early stationary (OD_{600} 0.8) and late stationary (OD_{600} 1.1) phases. After centrifugation, the cultures were diluted with fresh media to the same cell concentration as the early exponential phase sample before being mixed with the sucrose solution.

Freezing, Freeze-Drying and Viability

To measure the damage done to the cells dependent on the temperature used for freezing, the prepared samples were frozen using the six different conditions described in Table 1. The optimum temperature for freezing was determined by comparing the viability of the samples for each of the conditions. The frozen samples were dried at -50°C and under a pressure of less than 20 milli-torrs for 2 days using a freeze-dryer system/Freezone®4.5 (Labconco Co., USA) After reconstitution in 1 mL sterile distilled water, the number of colony forming units (CFU) for each sample was determined by serially diluting the sample and plating on LB agar

Biosensing Activity and Bioluminescence

The biosensing activity was determined on the basis of measuring the bioluminescent response ratio to 4-chlorophenol [DPD2540's bioluminescent response to the tested concentration of 4-chlorophenol (BLs)/DPD-2540's uninduced bioluminescence (BLc)], since 4-chlorophenol is known to induce transcription from the *fabA* promoter [12]. Emitted bioluminescence (arbitrary units, AU) was monitored using a highly sensitive luminometer (Model 20e, Turner Design, CA, USA). The luminometer was adjusted so that a single-photon tritium light source would give a reading of 5 arbitrary units (AU).

RESULTS AND DISCUSSION

A genetically-engineered *E. coli* strain, DPD2540, containing a *fabA::luxCDABE* fusion gene, gives a bioluminescent output when membrane fatty acid synthesis is needed [10,11]. Based on this principle, this strain should be able to detect many kinds of membrane fatty acid damaging agents [17]. 4-chlorophenol is known as a membrane damaging agent and a good triggering chemical of transcription from the *fabA* promoter of DPD2540 [12]. In this study, 4-chloro-phenol was used as a model toxicant to optimize the freeze-drying conditions for DPD2540 by comparing the biosensing activity of the samples after freeze-drying. Viability after freeze-drying was used to compare and optimize the freeze-drying conditions.

Sucrose as a Cryoprotectant

Sucrose was previously found to protect membranes and proteins of the cells during drying [5]. Various sucrose concentrations, mixed with either LB or LBG, were compared to determine the best composition of the lyophilization medium for freeze-drying. The six different compositions showed very different viabilities. When the cells were freeze-dried with various sucrose concentrations and LB medium, the viability of the cells in 12% sucrose solution was the greatest (Fig. 1(a)). However, when a sucrose and LBG (LB + glucose 10%) solution was used, 6% sucrose gave the highest viability (Fig. 1(b)). The cells may have been damaged during freeze-drying if the sugar, *i.e.* the cryoprotectant, concentration was not high enough to protect them, while an excess of sugar may cause osmotic damage. Therefore, an optimal sugar concentration is needed to increase the freeze-drying efficiency. Among the tested concentrations, 12% sucrose with LB medium showed the best efficiency, and this condition was selected for further experiments. Experiments with sucrose concentrations of greater than 12% were not tested because the primary concern was to test commonly used concentrations, which were generally below 12% for some cells and proteins [18-20]. As well, tests with a glucose and sucrose mixture showed that for higher final sugar

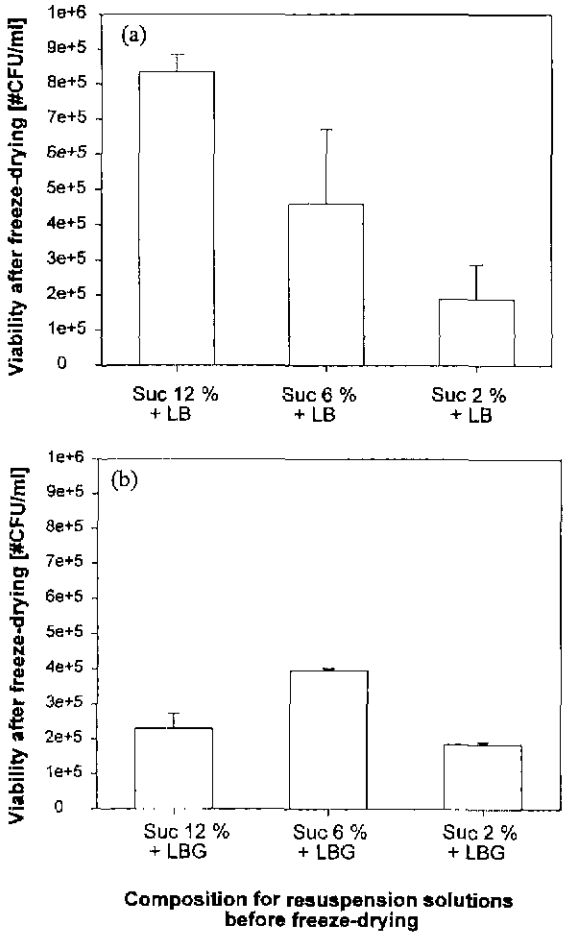

Fig. 1. Effect of the sucrose concentration in the lyophilization solution on the viability during freeze-drying; (a) Various sucrose concentrations with 50% LB medium, (b) Various sucrose concentrations with 50% LBG (LB + glucose 10%) medium

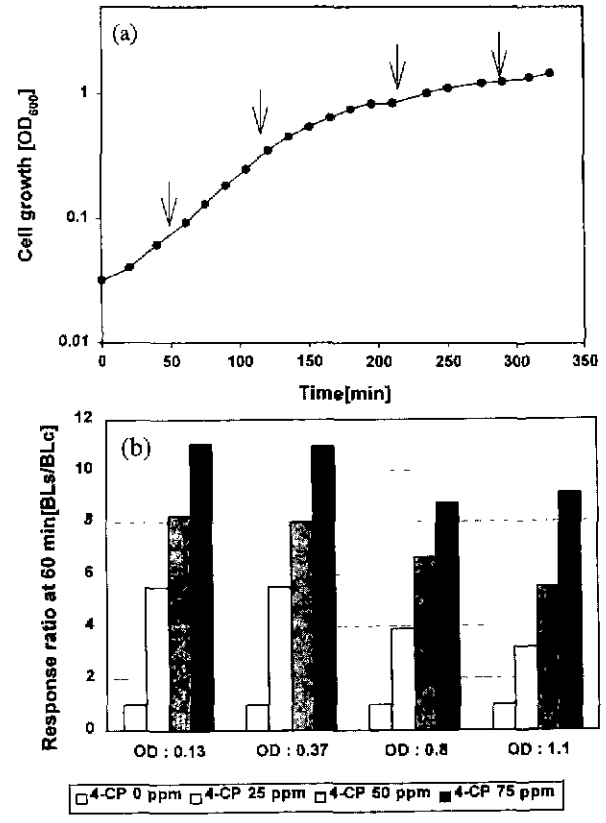

Fig. 2. Effect of the cell growth stage on the biosensing activity; (a) Fermentation profile of the cell growth, (b) Bioluminescent response of freeze-dried DPD2540 with various 4-chlorophenol concentrations depending on the cellular growth stage. Bioluminescent response ratio at 60 min: DPD2540's bioluminescent response at 60 min after induction by adding 4-chlorophenol to a tested concentration (BLs)/DPD2540's uninduced bioluminescent response at 60 min (BLc).

concentrations, i.e. the sample having 12% sucrose plus 5% glucose, a loss in viability was seen (see fig. 1(b) - the first bar). In both cases, sucrose alone (Fig. 1(a)) and sucrose and glucose (Fig. 1(b)), a final concentration of about 11-12% gave the greatest viabilities while addition of glucose had no significant effect with the lower sucrose concentrations.

Freezing Temperature Effects

To investigate the effect of the temperature used for freezing on the viability during freeze-drying, various temperatures were tested. As shown in Table 1, it was found that nearly all the cells were killed when frozen at -20°C regardless if another freezing step was added later. On the other hand, the freeze-dried cells frozen in liquid nitrogen showed the greatest viability. The viability depending on the freezing temperature seemed to correlate well with the rate of freezing at the lower temperatures, and therefore, a faster rate of freezing gives the highest viability.

It is known that a slow and steady removal of water by freezing increases the concentration of solutes in the remaining aqueous phase, causing the solution to become increasingly more concentrated [2]. For this reason, the viability of freeze-dried cells frozen rapidly at lower temperatures show a relatively higher value due to the prevention of these concentrated pockets from forming.

Cell Growth Stage Effect on the Toxicity Response

The status of the bacterial cell differs depending on the growth stage. Thus, the same concentration of cells at different growth stages were freeze-dried to measure the growth stage effect on biosensing activity. Fig. 2(b) shows the bioluminescent response ratio of the freeze-dried cells with various concentrations of 4-chlorophenol according to the growth stage and optical density as shown in Fig. 2(a). The initial cell concentrations were synchronized by diluting the centrifuged cells to

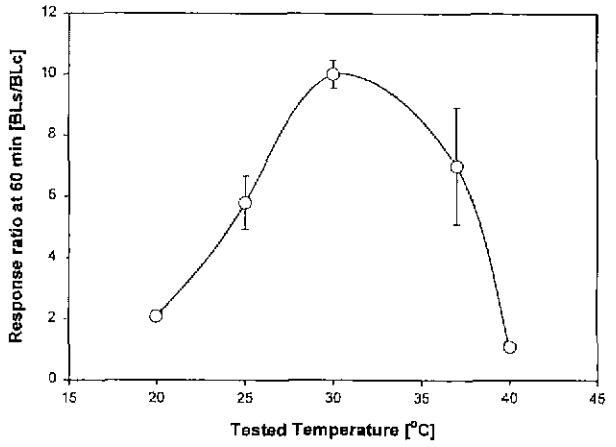

Fig. 3. Effect of the experimental temperature on the biosensing activity Bioluminescent response ratio at 60 min: DPD-540's bioluminescent response 60 min after induction by adding 4-chlorophenol to a concentration of 50 ppm (BLs)/DPD2540's uninduced bioluminescent response at 60 min (BLc)

the same optical density (OD_{600}) before mixing with sucrose. When the early and the late exponential cells were freeze-dried (see OD_{600} 0.08 and OD_{600} 0.37 in Fig. 2(b)), the bioluminescent response ratios were very similar for all the tested 4-chlorophenol concentrations. On the other hand, the cells freeze-dried during the early and the late stationary phase (see OD_{600} 0.8 and OD_{600} 1.1 in Fig. 2(b)) showed a comparative decrease in their biosensing sensitivity Therefore, the exponential phase cells would appear better in terms of their biosensing sensitivity.

Temperatures for Toxicity Monitoring

To determine the optimum temperature and response stability for biosensing, various temperatures were tested to determine their effect on the bioluminescent response when 50 ppm of 4-chlorophenol was added. As can be seen in Fig. 3, DPD2540 had a very distinct optimal temperature of 30°C, which is due to the fact that the *lux* genes are from *Vibrio fisheri* [21]. Accordingly, the optimal temperature for the best toxicity response should be 30°C.

Acknowledgements Authors wish to thank Dr Robert A LaRossa in DuPont Co. for his kindness in supplying the DPD2540 strain for this experiment. This work was supported by a grant from Minister of Education of Korea (Grant # 97-F-20).

REFERENCES

[1] Carstro, H. P., P. M. Teiseira, and R. Kirby (1996) Changes in the cell membrane of *Lactobacillus bulgaricus* during storage following freeze-drying. *Biotechnol Lett.* 18: 99-104

[2] Perry, S. F. (1995) *Methods in Molecular Biology: Cryopreservation and Freeze-drying Protocols.* pp. 21-30. Human Press, Totowa, NJ.

[3] Valdez, G. F., G Martos, M. P. Taranto, G. L. Lorca, G. Oliver, and A. P. Ruiz Holgado (1997) Influence of bile on β-galactosidase activity and cell viability of *Lactobacillus reuteri* when subjected to freeze-drying. *J. Dairy Sci.* 80: 1955-1958.

[4] Slade, C J. and E N. Vulfson (1998) Induction of catalytic activity in proteins by lyophilization in the presence of a transition state analogue *Biotechnol. Bioeng.* 57. 211-215.

[5] Leslie, S. B., E. Israeli, B. Lighthart, J. H Crowe, and L. M. Crowe (1995) Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Appl. Environ. Micorbiol.* 61: 3592-3597.

[6] Prestrelski, S. J., N. Tedeschi, T. Arakawa , and J. F. Carpenter (1993) Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophys. J.* 65: 661-671

[7] Gaiek, R. L., C. R Lange, A. S. Weber (1994) The effects of freeze-drying and storage on phenol degradation. *Wat. Environ Res.* 66: 698-706.

[8] Coutinho, C , E. Bernardes, D. Felix, and A. D. Panek (1988) Trehalose as cryoprotectant for preservation of yeast strains. *J. Biotechnol.* 7: 23-32.

[9] Norman, M. C., E. B. Franck, and R. V. Choate (1970) Preservation of *Mycoplasma* strains by freezing in liquid nitrogen and by lyophilization with sucrose. *Appl. Microbiol.* 20: 69-71

[10] Belkin, S. (1998) Stress-responsive luminous bacteria for toxicity and genotoxicity monitoring. pp 171-183. In: P. G. Wells, K Lee, and C. Blaise (eds). *Microscale Aquatic Toxicology – Advances, Techniques and Practice* CRC Press, Boca Raton, FL, USA.

[11] Belkin, S., D. R. Smulski, S. Dadon, A. C. Vollmer, T. K. Van Dyk, and R. A. LaRossa (1997) A panel of stress-responsive luminous bacteria for toxicity detection *Wat. Res.* 31: 3009-3016

[12] Choi, S. H. and M. B. Gu (2000). Monitoring and classification of toxicity using recombinant bioluminescent bacteria. *Wat. Sci. Tech.* In press.

[13] Gu, M. B , P. S. Dhurjati, T. K. Van Dyk, and R. A. LaRossa (1996) A Miniature Bioreactor for sensing toxicity using recombinant bioluminescent *Escherichia coli. Biotechnol. Prog.* 12: 393-397.

[14] Gu, M. B , G. C. Gil, and J. H. Kim (1999) A two-stage minibioreactor system for continuous toxicity monitoring *Biosensors Bioelectronics* 14: 355-361.

[15] Gu, M. B., S. H. Choi, and S W. Kim (2000) Freeze-drying of recombinant bioluminescent *Escherichia coli* for the development of portable biosensors. *Biotechnol. Bioeng.* Submitted for publication.

[16] Magnuson, K., S. Jackowski, C. O Rock, J. E. Jr. Cronan (1993) Regulation of fatty acid biosynthesis in *Escherichia coli. Microbiol. Rev.* 57: 522-542.

[17] Choi, S H. and M. B Gu (1999) A whole cell bioluminescent biosensor for the detection of membrane-damaging toxicity *Biotechnol. Bioprocess Eng* 4 59-62.

[18] Kurosawa, H., S Endo, T Hirano, K Nakamura, and Y. Amano (1997) Stabilization of freeze-dried *Thiobacillus thiooxidants* cells as a bacterial deodorant for removal of hydrogen sulfide. *J Ferment. Bioeng* 2 213-215

[19] Andrea, G D , M. L. Salucci, and L Avigliano (1996) Effect of lyoprotectants on ascorbate oxidase activity after freeze-drying and storage *Process Biochem.* 31: 173-178.

[20] Winona Wagner, L and T. K. Van Dyk (1998) Cryopreservation and Reawakening. pp. 123-127. In: R. A. LaRossa (ed). *Bioluminescence Methods and Protocols* Human Press Inc Totowa, NJ

[21] Tyulkova, N. A and T. P. Sandalova (1996) Comparative study of temperature effects on bacterial luciferases *Biochemistry* (Moscow) 61 205-214.

[Received April 15, 2000; accepted June 13, 2000]