Short Communication

SURVIVAL OF FREEZE-DRIED *THIOBACILLUS FERROOXIDANS* NCIB 8455 AFTER LONG-TERM STORAGE

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Thiobacillus ferrooxidans is a chemoautotrophic and acidophilic bacterium utilizing the oxidation energy of ferrous iron to ferric iron for growth. Because of such properties, the bacterial stock cultures are usually maintained by subculturing the growing cells in fresh liquid medium containing ferrous iron at regular short intervals, about once a week. This is very time-consuming and troublesome. Therefore, it would be useful to investigate a method for long-term storage of the microorganism. Freeze-drying (lyophilization) has been a widespread method for the preservation of many types of microorganisms. However, there is little information about the condition suitable for freeze-drying of iron-oxidizing bacteria and the viability during long-term storage. This paper describes the effects of various conditions of freeze-drying and long-term storage on the viability of the iron-oxidizing bacteria.

T. ferrooxidans strain NCIB 8455, obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, U. K., was cultivated on 9K basal salts medium (4) (pH 2.0) supplemented with 4% w/v $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. To prepare dense cell suspensions, 3-l cultures were made in 5-l flasks under forced aeration. Viable cells were counted by spreading the diluted cell suspension (0.1 ml) on silica gel plates of the ferrous iron 9K medium prepared as described previously (5). All the cultivation was made at 30°C.

Cells were harvested by centrifugation at the late-exponential phase and washed four times with sterilized water to remove iron precipitates and acid. The cell pellets were widely dispersed in a small volume of various freeze-drying suspending fluids

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and used as original cell suspensions (10^{11} – 10^{12} cells/ml).

The suspending media used (2,3) were: Mist. desiccans, modified Mist. desiccans, 20% skim milk (Difco Laboratories, dehydrated), 10% skim milk + 1% Na-glutamate, and 2% Na-glutamate in 0.1 M phosphate buffer (pH 7). Mist. desiccans was prepared by mixing 100 ml of distilled water containing 30 g of glucose and 1.3 g of nutrient broth (Difco) and 300 ml of horse serum (Research Foundation of Microbial Disease, Osaka Univ., Osaka). Modified Mist. desiccans was prepared by replacing the glucose with lactose, sucrose, or *myo*-inositol.

The dense cell suspensions (0.1 ml) were dispensed into sterile cotton-plugged ampoules which were 5 mm i.d. $\times$ 120 mm and globular (10 mm inside diam.) at one end and then frozen quickly at about -70°C in a dry ice/ethanol bath. The ampoules were immediately attached to the manifold unit of a freeze-dryer (Yamato Model DC-31) and continued to dry at room temperature for about 5 h. The freeze-dried ampoules were sealed under vacuum by a coal-gas cross-fire burner. After checking for leaks using a high frequency tester, the ampoules were stored in the dark at 4°C .

The sealed ampoules were opened aseptically and rehydrated first by injecting with 0.1 ml of ferrous iron 9K medium. The rehydrated cells were added to 9.9 ml of 9K basal salts medium and mixed thoroughly, and further decimal dilutions were made using the same medium for viable cell counts on silica gel plates.

Horse serum is an important component of Mist. desiccans. When horse serum in a bottle repeatedly frozen and thawed many times was used as a suspending fluid, the survival value was only 1% of that when fresh serum was used. So the use of fresh horse serum is recommended as much as possible.

The effect of pH of Mist. desiccans on the viability of *T. ferrooxidans* was examined. The percentage survival after freeze-drying using acid Mist. desiccans (pH 2.3–3.0) decreased greatly, two or three figures as compared with that obtained using neutral Mist. desiccans. Apparently the protective action of the suspending fluid deteriorates at low pH values. Therefore, it is important to wash the cultures of *T. ferrooxidans* thoroughly with sterile water to remove residual acid.

The recovery of freeze-dried cultures of microorganisms may be affected by the rehydrating medium. For *T. ferrooxidans* NCIB 8455 freeze-dried using Mist. desiccans with glucose, ferrous iron 9K medium (pH 2) gave better recovery of viable cells than 9K basal salts medium and distilled water adjusted to pH 2 by H_2SO_4 .

The effects of various suspending fluids on survival of *T. ferrooxidans* NCIB 8455 are summarized in Table 1. As a whole, viable counts decreased in three figures just after freeze-drying. Among the suspending fluids used here, Mist. desiccans and modified Mist. desiccans gave almost the same protective effect, and a little better effect than the other suspending fluids tested. The percent survival using distilled water was extremely low, indicating the effective protection of these suspending fluids.

Table 2 shows the survival values of *T. ferrooxidans* NCIB 8455 after

Table 1. Survival of freeze-dried *T. ferrooxidans* NCIB 8455 in various suspending fluids.^a

Suspending fluids	Viable count (cells/ml)		Percentage survival (%) ^b	Percentage ratio (%) ^c
	Before freeze-drying	Just after freeze-drying		
Mist. desiccans ^d	4.5×10^{11}	3.5×10^8	0.08	100
(lactose)	4.4×10^{11}	3.1×10^8	0.07	88
(sucrose)	5.6×10^{11}	3.2×10^8	0.06	75
(<i>myo</i> -inositol)	7.8×10^{11}	4.5×10^8	0.06	75
Mist. desiccans	4.4×10^{12}	4.0×10^9	0.09	100
Skim milk	5.4×10^{12}	1.4×10^9	0.03	33
Skim milk + Na-glutamate	5.4×10^{12}	1.2×10^9	0.02	22
Na-glutamate in phosphate buffer (pH 7)	4.4×10^{12}	1.4×10^9	0.03	33
Distilled water	4.0×10^{12}	1.0×10^5	2.5×10^{-6}	28×10^{-6}

^a Two groups of experiments are described. See text for details of suspending fluids.

^b Viable count in each suspending fluid before freeze-drying was taken as a control (100%).

^c Percentage survival (%) of Mist. desiccans was taken as a control (100%).

^d Glucose in Mist. desiccans was replaced by lactose, sucrose, or *myo*-inositol.

Table 2. Survival of freeze-dried *T. ferrooxidans* NCIB 8455 after long-term storage.

	Before freeze-drying ^a	Just after freeze-drying	After storage at 4°C for			
			1 (m) ^b	1 (y) ^b	2 (y)	10 (y)
Viable count (cells/ml)	2.9×10^{12}	2.7×10^9	2.3×10^9	1.1×10^9	3.0×10^6	2.0×10^6
Percentage survival (%) ^c		100	85	41	0.11	0.07

^a Original cell suspension prepared with Mist. desiccans (*I*).

^b (m) and (y) indicate month and year, respectively.

^c Viable count just after freeze-drying was taken as a control (100%).

freeze-drying and during storage at 4°C. Approximately half of the cells counted just after freeze-drying remained alive after storage for 1 year. However, viable counts decreased greatly during storage from 1 to 2 years, and then was almost constant from 2 to 10 years. The reason for such unusually rapid decrease in viable cells at 2 years is not explained. The decrease in viable counts showed a nearly linear decline on a logarithmic scale, excepting the 2 years datum. Apparently the specimens of freeze-dried cells of *T. ferrooxidans* NCIB 8455 could be viable for about 30 years. Freeze-dried cultures of the bacterium stored for 10 years were

cultivated in fresh ferrous iron 9K medium on a shaker for one week. Satisfactory active cultures were recovered.

It is concluded that *T. ferrooxidans* is particularly sensitive to injury during freeze-drying and exhibits low survival during long-term storage, but the desiccated cultures retain their viability for 10 years or more if the initial cell suspension has adequate numbers to sustain the loss in viability. It is worth testing continuously to show the viability of freeze-dried specimens of *T. ferrooxidans* NCIB 8455 after preservation for longer periods. Furthermore, considering the variable survival values of the chemolithotrophic bacteria preserved by L-drying (1), it is necessary to examine the preservation of many different strains of *T. ferrooxidans* by freeze-drying.

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