# Preservation of Lactic Acid Bacteria by Freeze-Drying

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Lactic acid bacteria are widely used in the manufacture of fermented milk products, such as cheeses, sour cream butter, yoghurt and fermented milk beverages. These microorganisms may also be applied to the production of fermented meat products and silage.

We may easily cultivate lactic acid bacteria in milk. However, it is a laborious work to preserve the organisms in liquid state because viability of the conventional milk culture is often lost within one or two weeks even at 4 to  $7^{\circ}$ C.

Freezing is useful for extended storage of lactic acid bacteria. Recently, in the United States and some European countries, frozen concentrated cultures of lactic acid bacteria can be supplied in an active form which eliminates the need of regular transfer in plant. Frozen cultures are highly promising but their shipment is troublesome.

Freeze-drying is applied successfully to the preservation of microorganisms and it has been employed in the distribution of lactic acid bacteria.

This article gives an outline of the author's study intended to establish the preparation method of active freeze-dried culture of lactic acid bacteria.

### Nature of protective solutes

As the freeze-drying process kills part of the bacterial cells, we have to add some protective substances to the cell suspension to be freeze-dried. It has been recognized that the suspending medium is the most important factor both in increasing the survival rate of bacteria after freeze-drying and in minimizing the death rate of the dried organisms during storage. In Japan, sodium glutamate has been used practically as a protective solute in the production of freeze-dried BCG (Bacille de Calmette et Guérin) vaccine.

The author confirmed that 1% sodium glutamate prevented remarkably the death of lactic

| Suspending                                     | Survival (%)                      |                                      |                                    |  |
|--|-----------------------------------|--------------------------------------|------------------------------------|--|
| $\begin{pmatrix} 0.06M, \\ pH 7 \end{pmatrix}$ | Streptococcus<br>cremoris<br>H 61 | Streptococcus<br>thermophilus<br>510 | Lactobacillus<br>bulgaricus<br>B-1 |  |
| Distilled wat                                  | er                                |                                      | 8                                  |  |
| (control)                                      | 2                                 | 3                                    | <1                                 |  |
| Glutamate                                      | 46                                | 58                                   | 16                                 |  |
| Aspartate                                      | 32                                | 47                                   | 9                                  |  |
| Pyroglutama                                    | te 53                             | 72                                   | 9                                  |  |
| Acetylglycine                                  | 48                                | 44                                   | 23                                 |  |
| Malate   | 26                                | 63                                   | 15                                 |  |
| Arginine                                       | 57                                | 48                                   | 35                                 |  |
| DL-threonine                                   | e 39                              | 46                                   | 10                                 |  |
| Glucose  | 48                                | 29                                   | 15                                 |  |
| Lactose  | 47                                | 31                                   | 34                                 |  |
| Sucrose  | 51                                | 34                                   | 39                                 |  |
| Skim milk<br>(8% SNF)                          | 68                                | 71                                   | 50                                 |  |
| Skim milk<br>+glutama                          | te 89                             | 66                                   | 64                                 |  |
| Skim milk<br>+malate                           |                                   | 101                                  |                                    |  |
| Skim milk<br>+arginine                         |                                   |                                      | 84                                 |  |

#### Table 1. Effect of suspending medium on survival of lactic acid bacteria after freeze-drying

Concentration of viable cells was 1 to  $2 \times 10^9/\text{ml}$ before freeze-drying. Protective effect of test compounds was given in percentage of survivors immediately after freeze-drying. acid bacteria (16 species, 32 strains) subjected to freeze-drying<sup>5).7)</sup>. Systematic survey revealed that a variety of low molecular weight substances are also protective for lactic acid bacteria during freeze-drying<sup>7).8)</sup>. Representative results are presented in Table 1. The protective activity of solutes is closely related to their chemical structure.

Generally speaking, the essential structure of the effective compounds seems to be characterized by the presence of three or more hydrogen-bonding and/or ionizing functional groups in an appropriate conformation. The protectives are considered to stabilize, in place of bound water, the cellular constituents located at or near the cell membrane<sup>3)</sup>.

Skim milk has been used widely as a suspending medium in the freeze-drying of lactic acid bacteria. The present results may be helpful to improve the protective activity of skim milk. As shown in Table 1, 84 to 100% of survival was attained after freeze-drying when *Streptococcus cremoris*, *S. thermophilus* or *Lactobacillus bulgaricus* cells were suspended in skim milk enriched with 0.06M glutamate, malate or arginine, respectively.

It is noteworthy that some of the solutes, which protect bacterial cells from damage caused by freeze-drying, are also protective for the cells in the frozen state. For instance, when *S. lactis* and *S. cremoris* cells were frozen and stored at minus  $23.3^{\circ}$ C, malic acid (0.5%, pH 6.8) exerted a protective effect against the loss of viability during storage<sup>19</sup>.

# Cellular injury caused by freezedrying

Freeze-dried preparations of bacteria generally contain unharmed cells and dead cells as well as those sublethally injured. Subsequent viability of the latest depends on several environmental factors. Sublethal injury is evidenced by an increase in nutritional requirement, a greater sensitivity to potential inhibitors, a narrower temperature range permitting growth, or a prolonged lag, etc.

As presented in Table 2, when S. faecalis

#### Table 2. Effect of plating medium on viability of freeze-dried Streptococcus faecalis

|       | 10 A. |      |
|-------|-------|------|
| Walte |       | <br> |

|                              | viable count per mi equivalent |                          |  |  |
|------------------------------|--------------------------------|--------------------------|--|--|
| Plating medium               | Freeze-dried in                |                          |  |  |
|                              | distilled water                | 1% Na glutamate          |  |  |
| TYGO                         | 4.4 × 107 (100)                | $3.7 \times 10^8 (100)$  |  |  |
| SF (0.05% NaN <sub>3</sub> ) | $3.1 \times 10^7 (70)$         | 3.4×10 <sup>8</sup> (92) |  |  |
| MA (no peptides)             | 1.9×107(43)                    | 3.4×10 <sup>8</sup> (92) |  |  |
| TYGS (6% NaCl)               | 4.9×10 <sup>6</sup> (11)       | $1.8 \times 10^{8}(49)$  |  |  |

 Medium: TYGO=tryptone 2%, yeast extract 0.5%, glucose 0.5% (pH 7.0). SF=SF medium (Difco). MA=a complete synthetic medium consisting of glucose, Na acetate, 19 amino acids, 7 vitamins, 4 purines and pyrimidines, and 5 inorganic salts. TYGS= TYGO containing 6% NaCl. Agar (1.5%) was added to all the media.

cells were freeze-dried with no additive, 89%, 30%, and 57% of the viable population exhibited NaCl-sensitive, azide-sensitive and peptide-requiring types of injury, respectively<sup>101</sup>.

It is remarkable that the addition of glutamate could prevent the induction of both azide-sensitive and peptide-requiring injured cells, although it was insufficient for maintaining a normal salt-tolerance of this organism after freeze-drying.

The NaCl-sensitive cells recovered their salt tolerance within 30 minutes when they were incubated in tryptone-yeast extract-glucose broth at 37°C. Neither protein synthesis nor cell wall synthesis was directly involved in the recovery process but the repair was found to be linked to RNA resynthesis<sup>49</sup>.

In order to obtain maximum recovery of viable cells, freeze-dried bacteria should be plated on a "rich medium" containing all the necessary nutrients, including amino acids and peptides but no potential inhibitor.

In addition, modification of a "rich medium" could sometimes provide freeze-dried cells an opportunity of overcoming severe injury. For instance, the recovery of viable cells of freezedried and stored *S. thermophilus* was greatly

Original cell suspension contained 5×10<sup>8</sup>/ml before freeze-drying.

increased by the addition of reducing agent, such as 0.1% cysteine, dithiothreitol or glutathione, to the plating medium<sup>4)</sup>.

Rehydration of freeze-dried bacteria is a critical process which sometimes largely affects their viability. Freeze-dried L. bulgaricus cells were highly sensitive to the rehydration temperature. As shown in Fig. 1, maximum



Fig. 1. Effect of rehydration temperature on the recovery of viable cells of freeze-dried *Lactobacillus bulgaricus* 

recovery of viable cells was observed when they were rehydrated at 20 to  $25^{\circ}$ C. It is remarkable that a large number of freeze-dried cells failed to grow when the temperature of rehydrating fluid was 37 to  $50^{\circ}$ C<sup>9)</sup>.

The present results indicate that a majority of freeze-dried bacterial cells, which survive on the strength of the protective additives, is more or less injured and liable to lose the viability under unfavorable conditions of rehydration and/or cultivation.

# Change in acid producing activity

From the practical point of view, effect of freeze-drying and subsequent storage on the activity of lactic acid bacteria is of great importance. Rate of acid production by these organisms is decisively influenced by the inoculum size, i.e., the initial concentration of viable cells which are inoculated into standardized skim milk (10% SNF).

In the present study, the activity was determined by the time elapsed until the test culture attained to a definite acidity. Result obtained in intact cells of *S. lactis* at 30°C is presented in Fig. 2. Below  $10^{7.5}$ /ml of inoculum



Fig. 2. Effect of freeze-drying and subsequent storage on acid producing activity of *Streptococcus lactis* in skim milk at 30°C

size, the incubation time required to attain 0.45% of acidity was proportional to logarithm of the inoculum size.<sup>6)</sup>

Similar relationship was also found in cases of S. cremoris and L. bulgaricus cultures. Slope of the regression line shown in Fig. 2 was proven to be determined principally by the mean generation time of test organism. The above-mentioned relationship is helpful to estimate the acid producing activity of freeze-dried lactic acid bacteria. In the present study, *S. lactis* cells were washed and suspended in 1% solution of glutamate to make final cell concentration to  $10^{10}$ /ml. One ml of the cell suspension was transferred into a series of ampoules.

Freeze-drying was carried out in a chambertype equipment, with initial freezing at minus 30°C for 1 hour, and desiccation under vacuum of 30  $\mu$ m Hg for 8 to 10 hours followed by the last heating up to 30°C for 2 hours. Seventy to 85% of the cells survived immediately after freeze-drying. Effect of the freezedrying on acid producing activity of test organism is shown in Fig. 2. Compared at the same level of inoculum size, the acid production of freeze-dried cells was 0.3 to 1 hour slower than that of intact ones. However, the slope of regression line did not significantly change after freeze-drying. These results indicate that the freeze-drying treatment prolongs the lag period of test organism by about 1 hour, but has little or no influence on the growth rate in exponential phase.

After storage of the freeze-dried preparations under vacuum at 38°C for 5 weeks, 30 to 50% of the initial population were found to be still viable. As presented in Fig. 2, the lag period of freeze-dried and stored organisms extended 3 to 4 more hours in skim milk compared with the intact cells. No significant change in growth rate was observed even after the storage and acid development returned to normal on subculturing.

As was stated previously, the freeze-dried cells have to repair several types of sublethal injury before they start to multiply normally. The prolonged lag observed here may correspond to the recovery process.

# Factors affecting preservability

Based on the present findings, brief discussions on a variety of factors affecting preservability of freeze-dried lactic acid bacteria could be given as follows: Nature of organisms: Thermoduric streptococci, such as S. faecalis and S. bovis, were relatively resistant to freeze-drying. In contrast, L. bulgaricus was the most sensitive species among a number of lactic acid bacteria tested<sup>79</sup>.

Harvesting medium: S. lactis was grown in broth containing polypeptone, yeast extract, lactose and sodium succinate (a buffer). Carbon source in the broth should be lactose, if a normal acid development in milk by the harvested cells is to be expected. However, the contents of polypeptone and of yeast extract in the harvesting medium did not influence significantly the cellular resistance to freeze-drying<sup>6)</sup>.

*Culture age*: Cells harvested at early exponential phase showed generally a much higher sensitivity to freeze-drying. The resistance of *S. cremoris* or *L. bulgaricus* cells was confirmed to be the greatest in the early stationary phase.

*Cell concentration*: In general, high cell concentrations were favorable for a higher survival. Concentration of more than 10<sup>10</sup> cells per ml is to be recommended.

Suspending medium: Details were described previously.

Moisture content: Several workers revealed that 1 to 3% of residual moisture was usually favorable to ensure the best survival of freezedried organisms during storage. Less than 3% of moisture content could be obtained in the freeze-dried preparations stabilized by 0.06M glutamate, arginine or lactose.

Temperature of storage: Freeze-dried organisms should be kept at as low a temperature as possible. As presented in Fig. 3, the death of freeze-dried lactic acid bacteria proceeded logarithmically during storage, at least in a period of observation. Temperature coefficient  $(Q_{10})$  of the death rate was found to be 2.2 to 3.5 in temperature range from 10 to 38°C.

Effect of oxygen: It is well known that the freeze-dried bacteria are extremely sensitive to oxygen. Accordingly, the freeze-dried preparations should be stored under vacuum in general. In the present study, however,



Fig. 3. Change in viability of freeze-dried Streptococcus thermophilus stored at 38°C

S. cremoris cells were suspended in 1% glutamate solution, freeze-dried and then stored at 38°C for 16 weeks in dry air or in vacuum. There was no significant difference in survival under the two conditions of storage. The result indicates that sodium glutamate could protect the freeze-dried cells against the lethal effect of oxygen.

Amino-carbonyl reaction: Reactive carbonyl compounds, such as ribose, diacetyl or pyruvate, should be eliminated from cell suspension to be freeze-dried because the death of freeze-dried organisms is accelerated by the presence of these compounds which may react with amino groups in essential components of bacterial cells. According to Marshall & Scott<sup>2</sup>, long term preservation of freeze-dried bacteria may be assisted by suspending the cells in mixtures containing non-reducing sugars (0.1 to 0.3M), amino acids including glutamate (0.1 to 0.3M), and semicarbazide (0.01 to 0.03M).

Rehydration: In the present study, the composition of rehydrating fluid had little or no influence on the recovery of viable cells from freeze-dried lactic acid bacteria<sup>59</sup>. As already described, freeze-dried *L. bulgaricus* should be rehydrated at 20 to  $25^{\circ}$ C. However,

freeze-dried preparations of S. cremoris, S. thermophilus or L. arabinosus did not show any marked response to rehydration temperature. Therefore, the susceptibility to rehydration temperature may differ among species of microorganisms.

# Conclusion

Freeze-drying procedure of lactic acid bacteria was established. Freeze-dried cells could be preserved for years as the seed for active starter. And further, freeze-dried preparations may be directly applicable to the production of fermented dairy products, etc. In connection with this point, a practical problem is to be solved, that is to develop an economical large-scale cultivation method of lactic acid bacteria.

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