# Cell Membrane Injury in the Freezing of *Lactobacilli*

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A large number of studies have been done on the freezing of microorganisms in connection with their deaths.

During the study on the actions of surface active agents upon microorganisms[1,2] it was observed that there are remarkable parallelisms between the death of the cells and the degree of the injury in the permeability of the cells. The degree of the injury in the permeability could be checked by comparing the measured leakage of intracellular substances, such as 260 m$\mu$ absorbing materials including nicotineamide adenine dinucleotide (NAD), and the measured increase in the activity of lactic dehydrogenase of the cells, as the increase in the enzyme activity could be regared as the increase in the penetration of substrate, lactate, into the cells[3].

It was ascertained that the injury of the cells of *Lactobacilli* in the freezing is affected by the suspending fluid. The present report deals with some results obtained.

## Materials and Methods

*Lactobacillus saké* 012 and *Lactobacillus plantarum* 11, supplied by the Institute of Applied Microbiology, University of Tokyo, were used for this work.

Organisms were grown statically in the medium containing 1% polypeptone, 0.5% yeast extract (Difco), 0.01% $MgSO_4 \cdot 7H_2O$, 1% sodium acetate (anhydrous) and 3% glucose for 40 hours at 30°, unless otherwise stated. The cells were harvested by centrifugation, and washed three times with distilled water.

The dry-weight of the cells were determined, following a previously prepared standard-curve of dry-weight relating to optical density at 660 m$\mu$.

NAD was determined fluorometrically, as stated in the preceding paper[4].

Activity of lactic dehydrogenase of the cells was assayed, using 2,6-dichlorophenol indophenol as a hydrogen acceptor. To cell suspension (about $5 \times 10^8$ cells) 0.1 ml of $5 \times 10^{-2}$ M of substrate (L-sodium lactate), 0.4 ml of 0.1 M phosphate buffer (pH 6.5) and 0.1 ml of $5 \times 10^{-4}$ M of 2,6-dichlorophenol indophenol were added, and then the reaction mixture was brought to 1.2 ml with water. Activity of the enzyme was indicated by the length of time needed for decolorizing the pigment at 30°. Increasing rate of the enzyme activity was expressed as the reciprocal ratio of the decolorizing time.

The visible cells were counted by plate-count method.

The capacity of the cells for producing lactate from sugars was determined by the following procedure. Cell suspension, 0.4 ml of 0.05 M phosphate buffer (pH 6.5) and 10 mg of each sugar were mixed, total volume being brought to 1.0 ml with distilled water. After incubating the mixture for 2 hours at 35°, lactate produced was determined by the

method of Barker and Summerson[5].

The experimental treatments of freezing and thawing were performed in the following procedure. Suspension of cells in distilled water was mixed with the same volume of 2% aqueous solution of the additive, and then 2 ml of the mixture in test tube was cooled rapidly in acetone cooled in deep-freezer at -20°. After being kept at -20°, it was thawed at 10°. The cells were harvested from the suspension by centrifugation. The supernatant was used for measuring the substances which were being released. On the other hand, the cells were washed two times with distilled water, and the activities of lactic dehydrogenase and viable cells were measured. For the sake of contrast, a set of tube containing cell suspension together with additive was maintained at 4° throughout the course of the experiment.

The procedures were carried out in duplicate, and the results were expressed as the means.

When the cells were lyophilized, the dried cells were suspended in distilled water, and the resultant suspension was separated into the cells and supernatant by centrifugation.

## Experimentals and Results

### Effect of the suspending medium on the freezing of the cells of *L. saké*

The suspending medium must be a most important factor in determining the survival of an organism in freezing. The effects of the various additives added to the cell suspension on the freezing injury of the cells were studied. These results are summarized in Table 1. The remarkable parallelisms between the rate of cellular death and the injury of the cells were observed. When lactose, sucrose, maltose, raffinose and glycerol were used as the additive, the injury of the cells was relatively small. Less injury was done in the freezing without additive. It is worth noting that D-calcium lactate is less injurious than L- or DL-aolcium lactate to the cells, which produce L-lactate from glucose under the cultural conditions used for obtaining the cells[6].

Concerning sugars, it seems that superior survival of the cells was observed when non-fermentable sugars of the cells were used as the additive, with the exception of xylose and arabinose. When the cells were suspended in 5% glucose, less injurious action was observed than when suspended in 1% glucose. Among the additives used, sodium chloride and potassium chloride were most injurious to the cells.

The cells grown in the medium that substituted sucrose for glucose were used for the freezing-experiment in the same way. In this case sucrose, which is fermented by the cells, was injurious in almost equal degree with glucose.

### Effect of the time of exposure to the low temperature.

Cell suspension was freezed at -20°, and maintained at the temperature for 2 or 24 hours. As shown in Table 2, an increase in survival was brought, by shortening the time. Even in only 2 hours maintainance, however, significant injury was observed and the injurious action of each additive showed a similar tendency regardless of the length of time.

Table 1. Effect of Suspending Medium in the Freezing of *L. saké* cells (freezing of 5 hr.)

| Suspending medium 1% | Cells grown in glucose-medium | | | | Cells grown in sucrose-medium | |
|---|---|---|---|---|---|---|
| | Increasing rate of L-lactic dehydrogenase of the cells | Leakage of 260mμ-absorbing materials* % | Percentage of death-cells | Fermenting capacity of the cells for each additive (lactate formation) μg/mg cells /4 hr. | Leakage of 260mμ absorbing materials* % | Fermenting capacity of the cells for each additive (lactate formation) μg/mg cells/4hr. |
| Glucose | 2.3 | 41 | 38 | 530 | 60 | 700 |
| Glucose (5%) | — | 21 | 29 | 530 | 50 | 700 |
| Fructose | 2.5 | 56 | 47 | 570 | 67 | 680 |
| Galactose | 1.5 | 31 | 33 | 260 | 52 | 490 |
| Mannose | 1.8 | 38 | 52 | 305 | 38 | 640 |
| Xylose | 1.8 | 49 | 80 | 0 | — | 0 |
| Arabinose | 2.0 | 52 | 85 | 0 | — | 0 |
| Maltose | 1.2 | 19 | 30 | 0 | 35 | 0 |
| Sucrose | 1.2 | 19 | 23 | 0 | 65 | 590 |
| Lactose | 1.2 | 11 | 15 | 0 | 34 | 0 |
| Raffinose | 1.2 | 14 | — | 0 | — | — |
| Glycerol | 1.3 | 24 | — | 0 | — | — |
| D-Lactate (Ca) | 1.0 | 18 | 25 | — | 22 | — |
| L-Lactate (Ca) | 2.5 | 30 | 70 | — | 39 | — |
| DL-Lactate (Ca) | 2.5 | 35 | 68 | — | 39 | — |
| NaCl | 3.4 | 99 | 100 | — | 104 | — |
| KCl | 2.9 | 96 | 100 | — | 98 | — |
| N/30 Phosphate buffer (Na) (pH 5.5) | 2.5 | 58 | 85 | — | — | — |
| Distilled water | 0.8 | 12 | 20 | — | 26 | — |
| As contrast, maintained at 40° for 5 hr. | | | | | | |
| Glucose | 0.6 | 12 | — | — | — | — |
| Lactose | 0.8 | 9 | — | | — | — |
| NaCl | 1.3 | 26 | — | — | — | — |
| Distilled water | 1.0 | 15 | 20 | — | — | — |

* Leakage of 260 mμ absorbing materials was expressed as relative 260 mμ absorbancy assuming the 260mμ abtorbancy of the boiling water extract of the cells to be 100.

Table 2. Effect of the Time of Exposure to the Temperature at −20°. (*L. saké*)

| Time of exposure | Suspending media (1%) | Leakage of 260 mμ-absorbing materials (%) | Death-cells (%) |
|---|---|---|---|
| 2 hr. | glucose | 36 | 62 |
| | lactose | 17 | 38 |
| | NaCl | 68 | 84 |
| | distilled water | 15 | 46 |
| 24 hr. | glucose | 50 | 82 |
| | lactose | 16 | 60 |
| | NaCl | 75 | 98 |
| | distilled water | 20 | 65 |
| Boiling water extract | | 100 | |

Cell concentration of suspending medium: $4.8\times10^9$ cells/ml

**Comparison of the freezing injury with other treatments of the cells of *L. saké*.**

Injurious action of the freezing in 0.5% sodium chloride was compared with lyophilization, and cetyltrimethyl ammonium bromide (CTAB)-treatment. CTAB-treatment was carried out by adding CTAB to the cell suspension at the rate of 52$\mu$g per g dried cells. After being maintained for 20 minutes at 4°, the suspension was centrifuged. For a contrast, cell suspension in distilled water was boiled for 5 minutes. As shown in Table 3, all the treatments leaked 260 m$\mu$ absorbing materials including NAD derived from the cells. Therefore, such freezing of cells in sodium chloride solution may be adopted as a method for extracting NAD, which is similar to CTAB-treatment[2].

Table 3. Comparison of the Freezing Injury with other Treatment of *L. saké* Cells

| Treatment | Leakage of 260m$\mu$ absorbing material | |
|---|---|---|
| | 260m$\mu$-absorbancy (10 mg dry cells/ml) | NAD mg/g cells |
| None | 0 | 0 |
| CTAB-tratment | 7.15 | 6.5 |
| Lyophilization | 5.50 | 5.1 |
| Freezed in 0.5% NaCl for 20 hr. | 4.70 | 5.0 |
| Boiling water extract | 10.10 | 6.2 |

**Effect of the freezing on the injury of the cells of *Lactobacillus plantarum*.**

In the same way, injurious action of the freezing was studied using the cells of *L. plantarum*. Judging from the amount of 260 m$\mu$ absorbing materials leaked, it seems that sodium chloride is of the most injurious action to *L. plantarum*, and lactose is less injurious as is to be seen in the case of *L. saké*.

Table 4. Freezing Injury of *Lactobacillus plantarum* Cells Suspended in Various Media

| Suspending media | Leakage of 260 m$\mu$ absorbing materials % | Increaring rate of the activity of D-lactic dehydrogenase |
|---|---|---|
| 2% glucose | 47.5 | 1.6 |
| 〃 lactose | 23.7 | 1.1 |
| 〃 NaCl | 89.8 | 1.8 |
| Distilled water | 13.3 | 1.1 |
| Boiling water extract | 100 % | |

## Discussion

Mazur et al[7] and many other observers reported that sugar, such as lactose and glucose, protects various types of cells in the freezing. The protective effect of sugars was assumed to be caused by the prevention of intracellular freezing. And there is a hypothesis that ice-formation within the cells is lessened by the penetration of the additive into

cells. Luyet[8] suggested that the hypertonic solutions dehydrate the cells, and the dehydration reduces the extent of intracellular freezing.

The cells used for the present experiment were exceptional in the fact that less freezing injury occurred when the cells were suspended in distilled water. Therefore, the present experiment had better not be discussed on the protective effect of the additive, but rather on the injurious effect. From this viewpoint, the results obtained seem to agree with the result of Mazur et al[7], who observed that gelatine-saline solution which contains 0.1% gelatine and 0.8% sodium chloride is more injurious to the cells of *Pasteurella tularensis* than distilled water.

The degree of the injurious action of each sugar added to the medium may be affected by the fermentability of the cells, as shown from the result that sucrose was injurious to the cells grown in sucrose.

It was suggested that the survival of the bacterial cells exposed to low temperature depended, in a complex way, on the experimental conditions, including the rate of cooling, the temperature to which the cells were cooled, the rate of subsequent warming, the concentration of the cells and the nature of the suspending medium.

The fact that the high concentration of glucose is less injurious than the diluted solution, may be caused by another mechanism, for example the effect of the hypertonic solution, as Luyet suggested[8].

Since the freezing affects cells in such a complex way, further studies will be necessary to clarify the mechanisms for protective and injurious effects of the additive.

## Summary

The effect of the suspending medium on the freezing injury of *Lactobacillus saké* and *Lactobacillus plantarum* was studied. The cells were cooled to $-20°$ suspending in various media, and then thawed at 10°. The injury of the cells was determined by the following items; the leakage of 260 mμ absorbing materials, the increase in the activity of lactic dehydrogenase of the cells and the ratio of death-cells. Some remarkable parallelisms were observed between these phenomena.

When the cells were frozen in the distilled water without any additive, the least injury was observed. Among the additives added to the suspending media, lactose, sucrose, maltose, raffinose and glycerol were injurious comparatively small to the cells of *L. saké* which were grown in glucose medium. Monosaccharides, such as glucose, fructose, mannose, galactose, xylose and arabinose were significantly injurious. On the other hand, to the cells grown in sucrose medium, sucrose is injurious in almost equal degree in case of monosaccharides. Inorganic salts, such as sodium chloride and potassium chloride, were most strictly injurious, and the cells were completely reduced to be dead. About 80% of nicotineamide adenine dinucleotide of the cells of *L. saké* was leaked by freezing the cells in 0.5% sodium chloride solution.

In addition, the similar results were obtained in the case of *L. plantarum*.

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