Microbiological assay (MBA) has been routinely used for the quantification of vitamin B₆, inositol, biotin, niacin, pantothenic acid, folic acid, and vitamin B₁₂ in food. Saccharomyces cerevisiae, mainly ATCC 9080, is used in the assays for vitamin B₆ and inositol. Inositol was incorporated into the Japanese nutrition labeling standards of infant formulas categorized to Food for Special Dietary Uses in 2009 (1). The MBA of vitamin B₆ was introduced in 1943, and refined for food analysis. Studies by Parrish et al. showed that Saccharomyces cerevisiae responded unequally to the three vitamin B₆ vitamers (2). The differential growth response to the vitamers led to the development of chromatographic procedures. In particular, spectrofluorometric methods offer excellent sensitivity and selectivity (3). At present, MBA is an official AOAC method for the analysis of vitamins and has been commonly used for more than 50 y. The main disadvantage of the MBA procedure is that it involves time-consuming processes like cell precultivation and washing. If washed cells of the test strain can be stored at 20°C in physiological saline water and these were poured onto YM agar plate. The YM agar plates were incubated at 30°C for 18 h. The cells were harvested from the culture by centrifugation (2,000 × g, 10 min, 4°C), and washed twice with sterilized physiological saline (hereafter referred to as “intact cells”). The authors have reported the omission of cell precultivation and washing processes in MBAs for biotin, niacin, and pantothenic acid using lyophilized Lactobacillus plantarum ATCC 8014 with sucrose (4). However, the effects of protectants on yeast indicator have not been investigated in vitamin assays. Assessing the MBA using lyophilized Sac. cerevisiae ATCC 9080 for determining the concentrations of vitamin B₆ and inositol would be valuable. Various carbohydrates such as lactose, maltose, and trehalose, as well as proteinaceous substances such as skim milk, have been reported to have protective effects on the survival of Sac. cerevisiae during lyophilization and storage (5–7).

In this study, we investigated the effects of the four protectants, namely, skim milk, lactose, maltose, and trehalose, on vitamin assays to improve the MBA.

Materials and Methods

Bacterial strain and preparation of lyophilized cells. Saccharomyces cerevisiae ATCC 9080 was used in this study. Lyophilized cells were prepared as follows. Strain 9080 was incubated in 10 mL of YM broth (peptone, 5 g L⁻¹; yeast extract, 3 g L⁻¹; malt extract, 3 g L⁻¹; glucose, 10 g L⁻¹) at 30°C for 18 h. The cells were harvested from the culture by centrifugation (2,000 × g, 10 min, 4°C), and washed twice with sterilized physiological saline (hereafter referred to as “intact cells”). The cells were suspended in 1 mL of water, 10% reconstituted skim milk (SM) (Difco Laboratories, MD, USA), lactose, maltose, and trehalose solution, respectively. One milliliter samples were first frozen at −20°C and then desiccated under vacuum (50 mTorr) for 48 h at −20°C (Freeze dry system and stoppering tray dryer; Labconco, Kansas, USA). The lyophilized cells were stored at −20°C.

Viable cell counts of lyophilized cells. The lyophilized cells were resuspended in 1 mL of water. Serial dilutions were made of the 1 mL samples suspended in 9 mL physiological saline water and these were poured onto YM agar plate. The YM agar plates were incubated at
30˚C for 3 d. MBA for vitamin B₆ and inositol. Vitamin B₆ assay media were purchased from Nissui Pharmacy (Tokyo, Japan). Inositol assay medium was made according to Notification No. 0212001 of Department of Food Safety, Ministry of Health, Labour and Welfare, Japan (1). The lyophilized cells suspended in 100 mL of sterilized water (approximately 90% transmittance) were used as inocula, and the assay procedures were performed according to AOAC (8). The total volume was 5 mL. The concentrations of vitamin B₆ and inositol in the standard reference material (SRM) 1849 were measured. Concentrations of vitamin B₆ and inositol in SRM 1849 Infant/adult Nutritional Formula were 14.2 mg kg⁻¹ and 398 mg kg⁻¹, respectively (9). All assays were performed at least three times.

Statistical analysis. The optical densities of the cultures inoculated with intact and lyophilized cells were subjected to repeated measures analysis of variance by using Statview 5.0 (SAS Institute Inc., Cary, NC, USA).

Results and Discussion
Viable cell counts of lyophilized cells of strain 9080
Viable cell counts of lyophilized cells of strain 9080 were measured after storage at −20˚C for 3 mo. The viable cell counts of the lyophilized cells with four protectants were approximately 7 log CFU (colony forming unit) mL⁻¹ (Table 1).

Bolla et al. reported that survival of Sac. cerevisiae in lyophilized cells with milk was decreased until after 30-d storage and thereafter the survival was relatively stable (10). The lyophilized cells after 3-mo storage used in this paper was considered to be in the stable viable cell count phase.

Table 1. Viable cell counts in lyophilized cells after 3-mo-long storage at −20˚C.

| Protectant      | Viable cell count (log CFU mL⁻¹) |
|-----------------|----------------------------------|
| Before lyophilization | 8.2 (0.2)*                     |
| Skim milk       | 7.4 (0.2)                       |
| Lactose         | 7.0 (0.0)                       |
| Maltose         | 7.2 (0.1)                       |
| Trehalose       | 7.2 (0.2)                       |
| None            | 4.1 (0.2)                       |

*Values in parentheses represent one standard deviation.

Table 2. Standard curves for pyridoxine hydrochloride concentration and coefficients of correlation between the turbidity and pyridoxine hydrochloride concentration.

| Protectant      | Standard curve               | Coefficient of correlation | Measured value of SRM 1849* (mg kg⁻¹) |
|-----------------|------------------------------|----------------------------|---------------------------------------|
| Intact cell     | $y = 1.0533x + 0.1379$      | 0.9945                    | 15.3 (1.0)**                          |
| Skim milk       | $y = 0.8700x + 0.1054$      | 0.9914                    | 15.7 (0.1)                            |
| Lactose         | $y = 0.6873x + 0.0764$      | 0.9907                    | 14.5 (1.0)                            |
| Maltose         | $y = 0.5070x + 0.0820$      | 0.9929                    | 15.2 (1.3)                            |
| Trehalose       | $y = 0.7828x + 0.0817$      | 0.9917                    | 15.7 (0.5)                            |

*SRM 1849 contains 14.2 (1.5) mg-pyridoxine hydrochloride kg⁻¹.
**Values in parentheses represent one standard deviation.
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lyophilized cells and intact cells produced similar linear ranges (Fig. 2). The slope of the standard curve was the highest with the lyophilized cells with maltose, followed by the lyophilized cells with SM and the intact cells. The values of SRM 1849 measured using lyophilized cells were in the range of established value (Table 3). The optical densities of the cultures with the lyophilized cells with SM were higher than those with the intact cells at every inositol concentration (Fig. 2). Various nutrients in SM might have effect on cell growth in inositol assay medium. Therefore, lyophilized cells with maltose were considered alternative inocula for quantitative inositol assay. From these results, lyophilized cells with maltose can be alternative inocula for vitamin B₆ and inositol MBAs.

The activities of tested lyophilized cells, i.e., growth in vitamin B₆ and inositol assay media, were not equal, while the viabilities of lyophilized cells were similar for the four protectants in this study. SM and maltose preserved the activity to the maximum extent, whereas lactose appeared to have little protective effect for bacterial activity.

In this study, lyophilized cells were stored at −20°C for 3 mo. Therefore, the lyophilized cells with maltose should be used within at least 3-mo storage at −20°C. The time-consuming processes like cell precultivation and washing are unnecessary in MBAs for vitamin B₆ and inositol using lyophilized cells with maltose.

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