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Liquid Nitrogen Preservation of *Saccharomyces carlsbergensis* and Its Use in a Rapid Biological Assay of Vitamin B_6 (Pyridoxine)

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ABSTRACT

TSUJI, KIYOSHI (The Upjohn Co., Kalamazoo, Mich.). Liquid nitrogen preservation of *Saccharomyces carlsbergensis* and its use in a rapid biological assay of vitamin B_6 (pyridoxine). Appl. Microbiol. **14**:456–461. 1966.—Growth medium as well as freezing menstruum greatly influenced the recovery of *Saccharomyces carlsbergensis* when it was quickly frozen in liquid nitrogen at −196 C and quickly thawed at 40 C. Nearly 90% recovery in viability was obtained when *S. carlsbergensis* was grown in Trypticase Soy Broth and frozen in vitamin B_6 basal assay medium. The growth phase of *S. carlsbergensis* also influenced recovery after freezing. When *S. carlsbergensis* was grown in Trypticase Soy Broth and frozen in the broth at the logarithmic-growth phase, only 7% viability was retained; the recovery rate increased to 81% when the culture was frozen in the maximal stationary phase. To have the least possible lag period of growth after thawing, a technique called growth-phase conditioning was introduced. After 1 hr of growth-phase conditioning, *S. carlsbergensis* was clearly out of lag phase, and budding was observed. A vitamin B_6 microbiological assay with a 6-hr incubation period and with the use of liquid nitrogen-frozen *S. carlsbergensis* is described.

Assay organisms, one of the major causes of variability in assays of both vitamins and antibiotics, have been successfully frozen in liquid nitrogen, thereby increasing the reliability of assays by enabling use of the same batch of inoculum for a long period of time (4, 6, 7). A frozen assay organism, *Lactobacillus leichmannii*, has also been reported by Sokolski et al. (6) to be stable for more than 1 year.

A system for using a frozen organism in the assay of vitamin B_6 (pyridoxine) was described by Sokolski and Stapert (5). They tested three freezing menstrua for *Saccharomyces carlsbergensis*, and reported that the assay of vitamin B_6 could be performed by use of an organism frozen in water.

The standard microbiological assay method for vitamin B_6, as developed by Atkin et al. (1), requires 16 to 18 hr of incubation. Kojima and Matsuya (2) conducted the vitamin B_6 bioassay with 8-hr incubation by use of a paper-disc method. The purpose of this report is to show that a frozen organism can be used to advantage to shorten the incubation time, thus making it possible to conduct the vitamin B_6 microbiological assay within one working day.

MATERIALS AND METHODS

Test organism. The test organism used throughout the study was *S. carlsbergensis* Fleischmann 4228.

Vitamin B_6 assay medium. The basal assay medium was that of Atkin et al. (1) as modified by Sokolski and Stapert (5).

Selection of growth media and freezing menstrua. Growth media studied were: Sabouraud liquid medium (BBL), Trypticase Soy Broth (BBL), Malt Extract Agar (Difco), and vitamin B_6 assay medium with 0.005 μg of pyridoxine-HCl per ml (1). Freezing menstrua used were: 10% glucose, 10% sucrose, 10% glycerol, 10% glycerol with 5 mmoles of $MgSO_4$, Trypticase diluent (3), Sabouraud liquid medium (BBL), Trypticase Soy Broth (BBL), and vitamin B_6 assay basal medium (no pyridoxine-HCl added).

The test organism, *S. carlsbergensis*, was grown in the various growth media described above for 24 hr in a water-bath shaker at 30 C. Cells were harvested and washed once with one of the freezing menstrua, and then were resuspended in the same menstrua and frozen. The freezing and thawing techniques were as described below. Viable counts were made before and after freezing and thawing by use of the Trypticase diluent of Masurovsky et al. (3) and by plating in Sabouraud glucose agar (Difco). The plates were incubated for 7 days at 25 C.

Growth-rate study. The growth rate of *S. carlsber-*

gensis after freezing and thawing was studied by inoculating the basal assay medium with 9×10^6 viable cells per milliliter. A 5-ml amount of this inoculated basal assay medium was then added to 5 ml of a standard vitamin B_6 solution containing 0.005 μg/ml of pyridoxine-HCl in 22 by 175 mm tubes. All tubes were incubated in a water-bath shaker (34 C), and the growth rate was followed turbidimetrically with a Beckman model C colorimeter at 650 mμ.

The dose-response tests were made in the same manner as in the assay procedure described below, except that the standard pyrodoxine solution was at a concentration of 0.01 μg/ml.

Preparation of the test organism for assay. S. carlsbergensis was cultured for 48 hr at 30 C on Malt Agar (Difco). One loop of this culture was suspended in 10 ml of physiological saline, and 0.05 ml of this suspension was inoculated into three tubes (22 by 175 mm), each containing 10 ml of vitamin B_6 assay medium with 0.005 μg of pyridoxine-HCl per ml. These were incubated for 16 hr on a water-bath shaker (30 C). Then 1,500 ml of vitamin B_6 assay medium with 0.005 μg of pyridoxine-HCl per ml was inoculated with 7.5 ml of actively growing culture. Samples (10-ml) of inoculated medium were poured into 150 tubes (22 by 175 mm) and incubated on a water-bath shaker (30 C) for 24 hr. The tubes were then pooled and harvested by centrifugation at 3,000 rev/min for 15 min in an IEC centrifuge (model EXC). The harvested cells were washed three times with 10% glycerol. During centrifugation, care was taken to maintain cell suspensions at or near 0 C. They were then resuspended in 100 ml of 10% glycerol and portions of this concentrated cell suspension were pipetted into 1.2-ml cryogenic ampoules (T. C. Wheaton Co., Millville, N.J.).

The ampoules were flame-sealed, coated with a mist of glycerol and talc, quickly frozen by immersing in liquid nitrogen, and stored in liquid nitrogen until used.

Preparation of stock standard solution for the assay. A standard vitamin B_6 solution containing 0.005 μg of pyridoxine-HCl per ml was poured into 50-ml vials which were then capped with rubber vial stoppers (West Co., Phoenixville, Pa.; No. S96). These vials were frozen in the gas atmosphere of a liquid nitrogen storage tank and stored there until used.

Sample vitamin B_6 assay. The frozen organism suspension was thawed by shaking the ampoule vigorously in a water bath at 40 C. The vitamin B_6 basal assay medium was brought to temperature in a water bath at 35 C, and then inoculated to 9×10^6 viable cells per milliliter. Growth-phase conditioning was accomplished by continual agitation on a shaker in a 35 C room for 1 hr. The samples were prepared according to the method of Atkin et al. (1) so as to have approximately 0.005 μg of pyridoxine-HCl per ml in the final dilution. The stock standard vitamin B_6 solution was thawed, and assay standards were prepared by pipetting into tubes (22 by 175 mm) in graded amounts: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 ml in duplicate. Final product dilutions were also pipetted in duplicate in graded amounts: 1.0, 1.5, and 2.0 ml per tube. The volume in each tube was made to 5 ml with distilled water, and the tubes were pasteurized by steaming (100 C) for 10 min. After 1 hr of growth-phase conditioning at 30 C, 5 ml of medium was added to each tube. All tubes were incubated at 34 C in a water bath shaker for 6 hr. At the end of the incubation period, 0.5 ml of Mercresin (The Upjohn Co., Kalamazoo, Mich.) was added to each tube to prevent further growth of the organism. Growth responses were read with a spectrophotometer at 650 mμ after adjusting to 100% transmittance with the inoculated assay medium without vitamin B_6.

RESULTS AND DISCUSSION

Growth medium and freezing menstruum. The effects of growth medium and freezing menstruum on the recovery of *S. carlsbergensis* after freezing and thawing may be seen in Table 1. Apparently, the growth phase of the organism greatly influenced the recovery. When cells were frozen during the logarithmic growth phase, the recovery

TABLE 1. *Effect of growth medium and freezing menstruum on the recovery, after freezing and thawing, of Saccharomyces carlsbergensis*

Growth medium	Freezing menstruum	Per cent recovery
Sabouraud liquid	Glucose, 10%	17
	Glycerol, 10%	19
	Trypticase diluent	38
	Vitamin B_6 basal assay medium	34
	Sabouraud liquid (max stationary phase; 24 hr)	17
	Sabouraud liquid (logarithmic-growth phase; 8 hr)	0.2
Trypticase soy	Glucose, 10%	44
	Glycerol, 10%	69
	Trypticase diluent	84
	Vitamin B_6 basal assay medium	90
	Trypticase Soy (max stationary phase)	81
	Trypticase Soy (logarithmic-growth phase)	7
Vitamin B_6 assay medium	Glucose, 10%	35
	Sucrose, 10%	27
	Glycerol, 10%	49
	Glycerol, 10%, with 5 mM $MgSO_4$	27
	Trypticase diluent	71
	Vitamin B_6 basal assay medium	75
Malt extract-agar	Glycerol, 10%	11

was only 0.2% with Sabouraud liquid medium and 7% with Trypticase Soy Broth. However, when cells in the maximal stationary phase were frozen, recovery was increased to 17% with Sabouraud liquid medium and up to 81% with Trypticase Soy Broth. Therefore, it seems probable that cells in the logarithmic growth phase are more susceptible to injury by freezing, possibly because of an unfavorable cell electrolyte concentration or unfavorable cell wall permeability, and that the culture should be at the maximal stationary phase for effective preservation by freezing in liquid nitrogen.

The growth medium also influenced recovery after freezing. In general, Sabouraud liquid medium yielded the poorest recovery and Trypticase Soy Broth the highest recovery.

The selection of the freezing menstruum also played an important role in the recovery efficiency. In general, 10% glycerol gave higher recoveries than either 10% glucose or sucrose. Attempts to increase the viability by adding 5 mmole of $MgSO_4$ (8) were unsuccessful. When *S. carlsbergensis* was grown in Trypticase Soy Broth and frozen in Trypticase diluent, nearly 84% recovery was obtained. A remarkable 90% recovery was observed when vitamin B_6 basal assay medium was used as the freezing menstruum instead of Trypticase diluent.

However, increases in recovery rate above 70% bore little relation to subsequent growth rates of cells (Fig. 1). Moreover, cells grown in Trypticase diluent tended to grow profusely in the blank tubes (the assay medium without pyridoxine HCl). It was decided, therefore, to use vitamin B_6 assay medium as the growth medium in further studies. It may be seen from Fig. 1 that

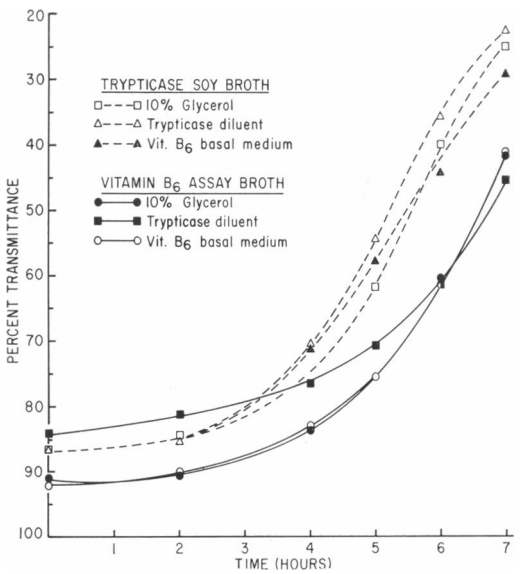

FIG. 1. *Effect of growth media and freezing menstrua on the growth of Saccharomyces carlsbergensis in vitamin B_6 assay medium at 34 C.*

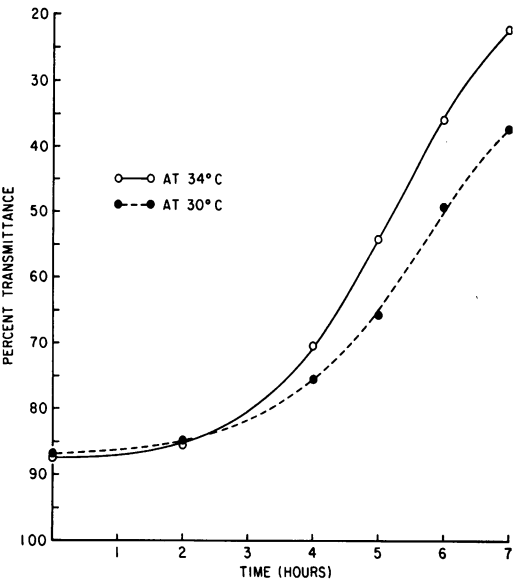

FIG. 2. *Effect of incubation temperature on the growth of Saccharomyces carlsbergensis in vitamin B_6 assay medium.*

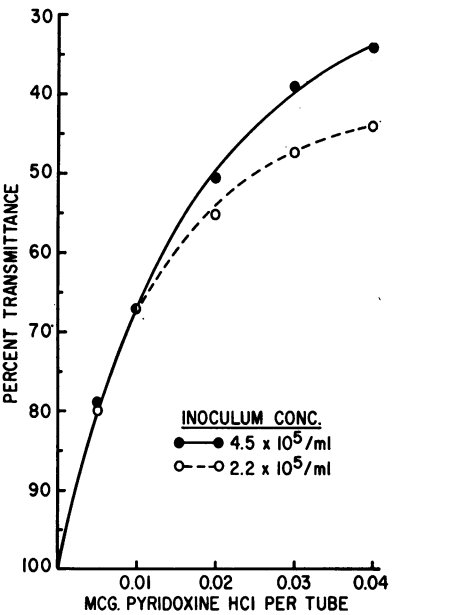

FIG. 3. *Effect of the concentration of inoculum on the slope of vitamin B_6 bioassay standard curve (6-hr assay).*

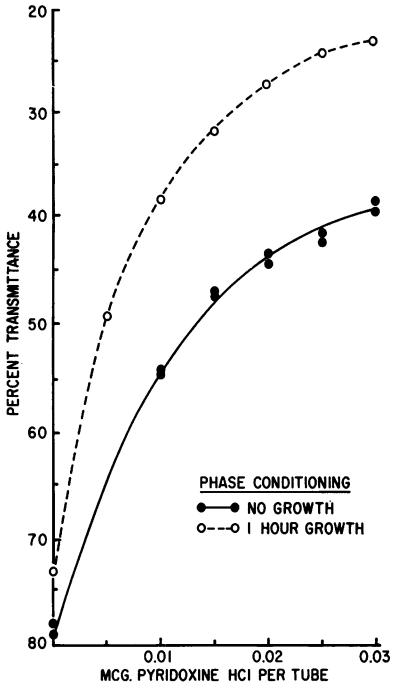

FIG. 4. *Effect of growth-phase conditioning on the vitamin B_6 bioassay standard curve (6-hr incubation).*

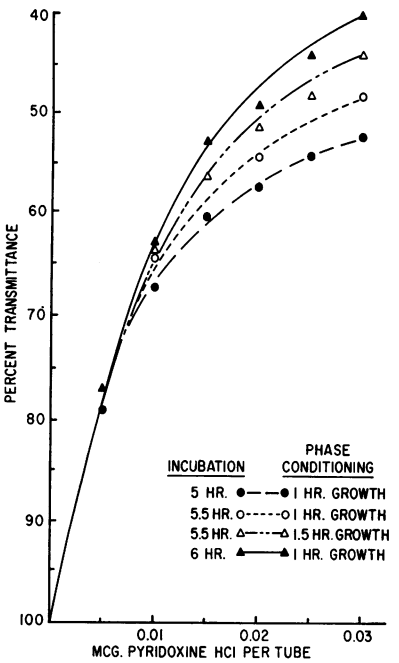

FIG. 5. *Effect of incubation time and growth-phase conditioning on the slope of vitamin B_6 bioassay standard curve.*

cells frozen in Trypticase diluent tended to have lower growth rates than those frozen in either 10% glycerol or vitamin B_6 basal assay medium. Growth rates of the latter two were nearly identical.

Incubation temperature and the amount of inoculum. Figure 2 shows the effects of incubation temperature on the growth of *S. carlsbergensis* during 7 hr of incubation. The growth rate at 34 C was superior to that at 30 C. However, an additional increase in the temperature to 37 C did not produce any further improvement in the growth rate. It is interesting to note that, when *S. carlsbergensis* was grown at 34 C rather than at 30 C before freezing, the growth rate after thawing was slower.

The amount of inoculum influenced the slope of the standard curve of the vitamin B_6 bioassay (Fig. 3). An increase in the slope of the standard curve was observed when the inoculum was 4.5×10^5 rather than 2.2×10^5 cells per milliliter.

Growth-phase conditioning and incubation time. Figure 4 suggests that the effective assay range would be extended if the slope of the standard curve beyond a pyridoxine-HCl concentration of 0.015 μg could be increased. It was thought that an increase in generation time might accomplish this, since *S. carlsbergensis* was found to nearly double in numbers during 6 hr of incubation in

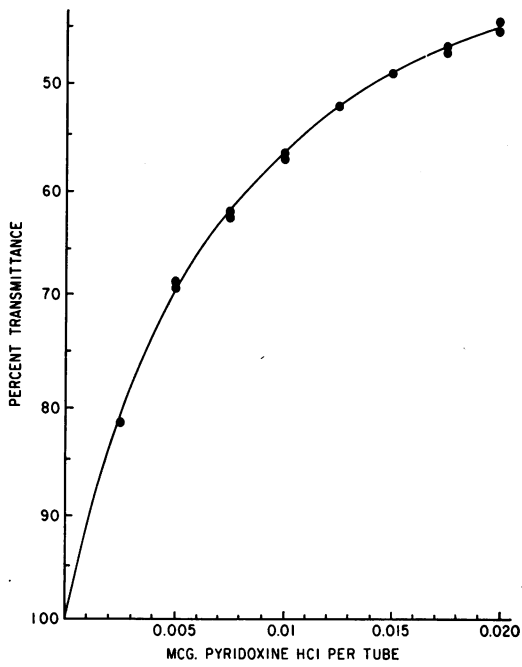

FIG. 6. *Vitamin B_6 bioassay standard curve (6-hr incubation with 1-hr growth-phase conditioning).*

TABLE 2. *Vitamin B_6 product assay by 6-hr assay method*

Determination	Vitamin B_6 concn (mg/unit volume)			
	Product A	Product B	Product C	Product D
Duplicate values at three levels	6.00	5.80	1.46	0.110
	5.70	5.70	1.40	0.114
	5.87	6.00	1.37	0.112
	5.60	5.87	1.44	0.116
	5.70	5.70	1.40	0.111
	5.70	6.00	1.40	0.111
Avg ($\overline{X}$)	5.76	5.87	1.41	0.112
SD	0.145	0.136	0.032	0.022
$\overline{X} \pm 2$ SD	5.76 ± 0.29	5.87 ± 0.27	1.41 ± 0.06	0.112 ± 0.044
Coefficient of Variation [s/$\overline{X}$]	2.52%	2.32%	2.27%	1.96%

TABLE 3. *Vitamin B_6 product assay by standard method*

Determination	Vitamin B_6 concn (mg/unit volume)			
	Product A	Product B	Product C	Product D
Duplicate values at three levels	5.75	6.00	1.30	0.113
	5.65	6.00	1.30	0.115
	5.67	5.67	1.30	0.113
	5.50	5.67	1.31	0.120
	5.75	5.75	1.30	0.115
	5.50	5.75	1.34	(0.095*)
Avg ($\overline{X}$)	5.64	5.81	1.31	0.115
SD	0.113	0.154	0.016	0.026
$\overline{X} \pm 2$ SD	5.64 ± 0.23	5.81 ± 0.31	1.31 ± 0.03	0.115 ± 0.051
Coefficient of Variation [s/$\overline{X}$]	2.00%	2.65%	1.22%	2.23%

* Discarded from calculation.

TABLE 4. *Stability of vitamin B_6 product final dilution at 4 C*

Product	Vitamin B_6 concn (mg/unit volume) after storage for		
	0 days	7 days	14 days
1	53.1	57.6	53.1
2	1.22	1.29	1.28
3	1.11	1.15	1.06
4	2.25	2.13	2.10

the basal medium. It would take at least 1 hr to set up the assay so that *S. carlsbergensis* could be incubated in the basal medium to extend the generation time without hindering the assay time schedule. Indeed, after incubating *S. carlsbergensis* in the basal assay medium for 1 hr, some budding was observed. This treatment, the incubation of the assay organism in the basal assay medium prior to the actual assay, has been termed growth-phase conditioning.

The effect of growth-phase conditioning on the slope of the standard curve after 6 hr of incubation may be seen in Fig. 4.

The effect of incubation time on the slope of the standard curve is shown in Fig. 5. The slope of the standard curve was found to increase according to the increase in the incubation time. Extending growth phase conditioning to 1.5 hr increased the slope of the 5.5-hr incubation standard curve, but did not increase the slope of the 6-hr incubation standard curve. The 6-hr assay is the one recommended for the vitamin B_6 bioassay, although a 5.5-hr incubation in conjunction with 1.5 hr of growth-phase conditioning might also be satisfactory. A typical standard curve for the 6-hr assay with 1 hr of growth-phase conditioning may be seen in Fig. 6.

Product assay. Four products were chosen at random from the Upjohn vitamin preparations. The 6-hr assay was performed as described previously, and the vitamin B_6 values obtained were compared with those of the standard assay method of Atkin et al. (1). Results are presented in Tables 2 and 3. The precision of the 6-hr

assay, expressed as the coefficient of variation, was comparable to that of the standard method (2.0 to 2.5% versus 1.2 to 2.7%). Also, there was no statistically significant difference between the product vitamin B_6 values obtained by the two methods. It was also found that the upper portion of the 6-hr assay standard curve, with transmittance as high as 46%, could be used to compute the result.

Stability of vitamin B_6 in the product final dilution. To make a one-working-day assay effective, the products should be prepared to the final dilution at least 1 day before the assay. Therefore, it was important to establish the stability of pyridoxine-HCl in the final dilution. Final dilutions of products were stored in amber-colored volumetric flasks in a refrigerator for 2 weeks. The pyridoxine-HCl concentration in these final product dilutions was approximately 0.005 μg/ml. At 1-week intervals, the final dilutions were assayed to determine the stability of the pyridoxine-HCl (Table 4). There was no significant decrease in pyridoxine-HCl concentration in the final product dilution after storage in a refrigerator for 2 weeks. Therefore, it is safe to assume that the final sample dilution could be stored at refrigeration temperature overnight or over the weekend without undue harm to its pyridoxine-HCl content. However, the diluted sample could be stored frozen in a freezer (−20 C) or in the gas phase of a commercial liquid nitrogen freezer (−150 to −170 C) for a considerable period, if this should prove more convenient.

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