The Journal of Microbiology, September 2001, p.240-243 Vol. 39, No. 3

NOTE

Preservation of Marine Heterotrophic Bacteria by Using a Deep-freezing Method

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(Received July 25, 2001 / Accepted September 3, 2001)

The effect of cryoprotectants and suspending solutions on the preservation of marine heterotophic bacteria was investigated. Six halotolerant and four halophilic bacterial isolates suspended in either distilled water or artificial seawater were preserved in glycerol and dimethylsulfoxide at -70°C, respectively. After one year of preservation, the recovery rates on the appropriate agar plates were estimated. The survival rate was found to be dependent on the strain tested, regardless of the preservation conditions tested.

Key words: Deep freezing, marine bacteria, preservation

Because microbial cultures are extremely vulnerable, minimizing the loss of viability during processing and storage of a library of strains is as important as collecting the microorganisms. There are two methods, freeze-drying and freezing, for long-term preservation based on genetic stability. Microorganisms can be successfully maintained by freeze-drying through the removal of water under reduced pressure and storage at -70°C or at the temperature of liquid nitrogen in the presence of cryoprotectants. Freezing is usually the method of choice for both short- and long-term storage of microorganisms based on user requirements, such as the expense, number of cultures, frequency of use of cultures, etc (18). This method is very quick and easy and requires no subsequent manipulation during storage. It is ideally suited to the storage of large collections of isolates in screening programs. In general, cells from the stationary phase are less sensitive to damage by freezing and thawing than cells from other growth phases, and slow freezing and rapid thawing yield the highest recovery rate (5). The optimum freezing rate for maximum survival varies with the nature of the cell (13, 16). Yet freezing at a rate of 1°C/min is widely used because it is impractical to estimate the freezing rate for every microorganism (5). Although slow freezing prevents the formation of intracellular ice, cells suffer from solute concentration effects due to dehydration, identified as solution effects by Mazur (13). Cryoprotectants act to minimize such solution effects; glycerol and dimethlsulfoxide (DMSO) are the most commonly used cryoprotectants in the freezing storage of microorganisms (5, 18).

Marine microorganisms are important as sources of new bioactive compounds (1-3, 6, 7, 10, 15, 17, 19). Since the report on the storage of lyophilized and frozen cultures of marine bacteria (4), however, few reports have been made on methods for maintaining marine bacteria. Accordingly, this paper describes a freezing method for maintaining marine bacteria at -70°C, which was evaluated using halophiles and halotolerants. Distilled water (DW) and artificial seawater (ASW) were tested and compared as the cell suspension solutions, while glycerol and DMSO were examined and compared as the cryoprotective agents.

Among bacterial strains isolated from seawater, sediments and corals collected from the coastal areas of Cheju Island, Korea in 1996 (11, 12), 6 halotolerant and 4 halophilic strains were used for the long-term preservation test (Table 1). The DW, ASW, and 20% (v/v) glycerol were autoclaved for sterilization. The 20% (v/v) DMSO was sterilized by filtration through a 0.2 μm pore-size polytetrafluoroethylene filter pre-washed with DMSO. The components of 1 liter of ASW were as follows: NH_4NO_3 2 mg, H_3BO_3 27 mg, $CaCl_2$ 1.14 g, $FePO_4$ 1 mg, $MgCl_2$ 5.143 g, KBr 0.1 g, KCl 0.69 g, $NaHCO_3$ 0.2 g, NaF 3 mg, Na_2SiO_3 2 mg, Na_2SO_4 4.06 g, SrCl, 26 mg, and NaCl 23 g. $CaCl_2$ and $MgCl_2$ were dissolved sep-

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Table 1. Strains used for preservation test

Strains	Sources	Phylogenetic affiliations
96CJ10356[a]	Marine sediments	γ-Proteobacteria
SW10577	Sea water	α-Proteobacteria
SW10583	Sea water	α-Proteobacteria
SW10597	Sea water	Gram-positive, high G+C
SW10625	Sea water	Gram-positive, high G+C
Plex10635	*Plexauridae*	α-Proteobacteria
Plex10653	*Plexauridae*	α-Proteobacteria
Plex10669[a]	*Plexauridae*	N. D.[b]
Plex10670[a]	*Plexauridae*	N. D.[b]
Plex10683[a]	*Plexauridae*	Gram-positive, high G+C

The strains were isolated from the coastal area of Cheju Island, Korea in 1996. The phylogenetic affiliations of the strains were determined by using sequences of 16s rDNA produced by the PCR method (12).
[a]Halophilic bacteria.
[b]Sequence of 16S rDNA was not determined.

arately and mixed with the solution containing other compounds.

The cells grown on a ZoBell 2216e solid medium (peptone 5 g, yeast extract 1 g, $FePO_4$ 10 mg, agar 15 g, DW 250 ml, ASW 750 ml, pH 7.6) were suspended in sterile DW and ASW to 1-9 × 10^{10} cells/ml and estimated with a light microscope. The suspensions (0.25 ml) were mixed with an equal volume of glycerol or DMSO in sterile 1.5 ml microcentrifuge tubes, respectively. The final concentration of glycerol and DMSO was 10 percent. The prepared cells were then stored at -70°C and recovered after defined storage periods. To keep the thawing rate of the tested samples constant, the cells maintained at -70°C were thawed at 37°C for 0.5 to 1 min. The samples were then diluted with sterile DW or ASW and plated on the ZoBell 2216e medium. After incubation for 3 days at 25°C, colonies on the plates (CFUs) were counted as recovered cells.

In the case of the preservation of marine phytoplanktons using the deep-freezing method, the cell survival rates were higher with the use of DMSO compared with the other cryoprotectants such as glycerol and polyvinylpyrrolidone (8, 14). However, no significant difference in the recovery of halotolerant and halophilic bacteria was observed between the use of glycerol and DMSO as cryoprotectants, or DW and ASW as the suspension material (Table 2). These results indicate that among the conditions tested there was no superior method for the long-term preservation of the tested marine bacteria. The recovery rates were not found to be dependent on the suspension materials or cryoprotectants but rather on the strains themselves, thereby confirming the statement by Kirsop and Snell (9) that there is no universally successful preservation method for all bacteria.

Unfortunately, the recovery of the isolates in this study could not be compared quantitatively with that of other

Table 2. Comparison of recovery rates between cells stored in DMSO or glycerol as cryoprotectants and cells stored in DW or ASW as suspension solutions at -70°C

Strains	Initial cell number[a]	Storage periods (days)	Recovered cells[b]			
			DW/D[c]	DW/G[d]	ASW/D[e]	ASW/G[f]
96CJ10356	1.1 × 10^{10}	90	N. R.[g]	4.8 × 10^{8}	6.8 × 10^{7}	6.7 × 10^{8}
	1.1 × 10^{10}	180	N. R.[g]	N. R.[g]	N. R.[g]	N. R.[g]
SW10577	2.2 × 10^{10}	360	1.0 × 10^{8}	2.8 × 10^{8}		
	2.5 × 10^{10}	360			7.2 × 10^{7}	1.2 × 10^{9}
SW10583	1.6 × 10^{10}	360	1.0 × 10^{8}	6.0 × 10^{7}		
	1.8 × 10^{10}	360			2.6 × 10^{8}	3.2 × 10^{8}
SW10597	1.7 × 10^{10}	360	5.0 × 10^{8}	1.8 × 10^{8}		
	1.1 × 10^{10}	360			2.0 × 10^{7}	9.0 × 10^{7}
SW10625	6.1 × 10^{10}	360	4.3 × 10^{8}	3.9 × 10^{8}		
	4.7 × 10^{10}	360			4.0 × 10^{8}	3.0 × 10^{8}
Plex10635	1.1 × 10^{10}	360	2.0 × 10^{9}	2.0 × 10^{8}		
	1.1 × 10^{10}	360			2.0 × 10^{8}	1.0 × 10^{9}
Plex10653	1.4 × 10^{10}	360	7.2 × 10^{7}	6.4 × 10^{7}		
	2.3 × 10^{10}	360			2.0 × 10^{8}	3.0 × 10^{8}
Plex10669	3.1 × 10^{10}	360	6.8 × 10^{9}	6.2 × 10^{7}		
	4.2 × 10^{10}	360			6.0 × 10^{8}	1.8 × 10^{9}
Plex10670	2.2 × 10^{10}	360	2.0 × 10^{9}	1.4 × 10^{9}		
	3.8 × 10^{10}	360			8.0 × 10^{4}	4.0 × 10^{5}
Plex10683	3.4 × 10^{10}	360	1.5 × 10^{8}	1.0 × 10^{8}		
	3.1 × 10^{10}	360			2.2 × 10^{8}	3.0 × 10^{8}

[a]Cells were counted using a light microscope.
[b]CFUs were counted on ZoBell medium.
[c-f]DW, distilled water; ASW, artificial sea water; D, DMSO(final concentration, 10%); G, glycerol(final concentration, 10%).
[g]Not recovered. The cells were not recovered on the medium ZoBell.

Table 3. Comparison of recovery rates between cells continously stored at -70°C and cells stored with an interuption by thawing

Strains	Storage periods (days)	Number of recovered cells[a]			
		DW/D[b]	DW/G[c]	ASW/D[d]	ASW/G[e]
96CJ10356	180	N. R[f]	N. R[f]	N. R[f]	N. R[f]
	90 → 180[g]	N. R[f]	N. R[f]	N. R[f]	N. R[f]
SW10577	180	9.6×10^8	2.2×10^8	7.2×10^8	9.8×10^8
	90 → 180[g]	4.8×10^8	2.8×10^8	1.2×10^8	1.0×10^9
SW10583	180	1.4×10^8	1.4×10^8	3.4×10^8	2.8×10^8
	90 → 180[g]	1.2×10^8	1.2×10^8	2.6×10^8	3.2×10^8
SW10597	180	1.2×10^9	4.8×10^7	2.8×10^8	2.6×10^8
	90 → 180[g]	5.4×10^8	1.2×10^7	2.6×10^7	9.2×10^7
SW10625	180	5.2×10^8	4.6×10^8	4.8×10^8	3.2×10^9
	90 → 180[g]	5.0×10^8	4.3×10^8	2.4×10^8	3.0×10^9
Plex10635	180	2.8×10^8	8.2×10^8	1.8×10^9	1.2×10^9
	90 → 180[g]	2.4×10^8	1.4×10^8	1.8×10^8	1.0×10^9
Plex10653	180	3.2×10^9	4.2×10^9	2.6×10^9	8.2×10^8
	90 → 180[g]	3.8×10^8	3.0×10^8	6.8×10^8	3.2×10^8
Plex10669	180	8.8×10^9	6.8×10^8	1.8×10^9	2.2×10^9
	90 → 180[g]	8.2×10^9	7.8×10^7	6.2×10^9	1.4×10^9
Plex10683	180	1.2×10^9	5.4×10^8	1.4×10^9	1.3×10^9
	90 → 180[g]	1.7×10^8	1.4×10^8	1.6×10^8	5.4×10^8

[a]CFUs were estimated on ZoBell medium.
[b-e]DW, distilled water; ASW, artificial sea water; D, DMSO (final concentration, 10%); G, glycerol (final concentration, 10%).
[f]Not recovered. The cells were not recovered on the medium ZoBell.
[g]Cells were stored for 90 days at -70°C, thawed for 1 min at 37°C, and restored further for 90 days at -70°C.

marine bacteria after using the freezing method because no quantitative survival rates have yet been reported. *Vibrio* sp. strains preserved in 15% glycerol at -29°C were not recovered after 10 years (4). About 1% of the cells were recovered with the exception of strain 96CJ10356 after one year of storage at -70°C. The halophilic strain 96CJ10356 was not recovered after 6 months of storage. Accordingly, this strain should only be stored up to 3 months and then renewed with a fresh cell suspension. This strain did exhibit a higher recovery rate when suspended in ASW. In contrast, the recovery rates of strain Plex10670 were lower when it was suspended in ASW, even though the organism is halophilic. The different groups to which the strains belong should account for these differences in the recovery rates after the preservation periods and the suspension solutions between strain 96CJ10356 and the other strains. Strain 96CJ10356, a member of γ-Proteobacteria, showed much lower recovery rates compared with those of the tested strains from other phylogenetic types. However, this still remains questionable as 96CJ10356 was the only member of γ-Proteobacteria among the tested stains. Since there is also an optimal freezing rate for maximum survival that varies with the cells (13, 16), this could equally have been responsible for the low recovery rates of strain 96CJ10356.

Most of the damage leading to the loss of viability of microorganisms occurs during the freezing and thawing steps but not during the storage, according to Heckly (5). The cells stored for 3 months at -70°C were thawed, then immediately refrozen, and kept for another 3 months at -70°C. The recovery rates of the refrozen cells were compared with those of the cells stored for 6 months at -70°C. As shown in Table 3, there was no difference between the two storage procedures, which means that a thawing during storage does not appear to seriously affect the stability of the cultures. In the current study, repeated cooling and thawing did not reduce the survival of the tested microorganisms.

From the preservation test using specific marine isolates, no significant distinction was found between the cryoprotectants used on halophiles and halotolerants. It was also confirmed that the tested marine bacteria needed to be stored using a more efficient deep-freezing method because only around one percent of the preserved marine bacterial cells were recovered after one year of storage at -70°C. To identify the best preservation method for deep-freezing marine bacteria, more marine bacterial strains belonging to various microorganism types need to be examined along with diverse cryoprotectants.

Acknowledgments

This study was supported by the KORDI project Biodiversity in Cheju and by the Life Phenomena & Function Reseach Project of the Korean Ministry of Science and Technology.

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