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Improvement of the L-drying procedure to keep anaerobic conditions for long-term preservation of methanogens in a culture collection

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The L-drying (liquid-drying) method is currently one of the well-known methods for preserving microorganisms. We have optimized the method to make it applicable for the preservation of methanogens, a group of strict anaerobic archaea, by keeping anaerobic conditions in all procedures without using an anaerobic chamber. Three strains of methanogens, namely, *Methanobrevibacter arboriphilus* NBRC 101200, *Methanothermobacter thermautotrophicus* NBRC 100330^T (^{T}, type strain), and *Methanoculleus chikugoensis* NBRC 101202^T, were preserved stably by the L-drying method optimized in the present study, and recovered well even after an accelerated storage test at 37℃ for 2 weeks, which approximately corresponds to the normal storage at 4℃ for 20 years. The number of viable cells of these strains after L-drying was found to be between 10^4 and 10^6 cells/m*l* against 10^5 through 10^6 cells/m*l* of the original suspension. On the other hand, cells of *Methanosarcina mazei* NBRC 101201 grown on acetate were viable only when they were harvested before the stationary phase of growth. It was revealed that attention should be paid to the growth phase of the culture used for preservation. The L-drying method improved in this study is thus expected to be applicable for long-term preservation of various methanogens that can then be easily transported by postal or parcel delivery to other laboratories..

Key words: L-drying method, methanogens, anaerobes, preservation, culture collection

INTRODUCTION

Methane-producing archaea (methanogens) play important roles in the production of methane as an energy source as well as in anaerobic wastewater treatment. Technologies for methane fermentation and its proper regulation are expected to solve global energy and environmental problems.

Methanogens form a physiologically diverse group of prokaryotic organisms. They are strictly anaerobes, and are characterized by their ability to produce methane during the process of energy metabolism (Balch *et al*., 1979; Garrity & Holt, 2001; König & Stetter, 1989; Zeikus, 1977). Most methanogens grow autotrophically in the presence of H_2 and CO_2 or on acetate. In addition, some methanogens utilize formate, methanol, methylamines, etc. The optimum growth temperature for methanogens is remarkably broad, ranging from 20–25℃ up to 98℃. In Bergey's Manual of Systematic Bacteriology 2nd ed., 77 species belonging to 26 genera of methanogens are described (Garrity & Holt, 2001).

Methanogens have been generally preserved by freezing. In 1983, Winter reported that the preservation of 11 species (18 strains) of methanogens by freezing at −18℃ or by keeping them on an agar slant at 4℃ (Winter, 1983). Sixteen strains of methanogens among 15 species were preserved by freezing in liquid nitrogen (Koga & Ohga, 1992). Morinaga and Inoue (1990) preserved 5 strains of methanogens among 4 species by freeze-drying and/or L-drying (liquid-drying) with skim-milk plug by using an anaerobic chamber. Several methods for short- and long-term preservation of methanogens, such as subculturing, freezing in liquid nitrogen, and freeze-drying, have been described (Garrity & Holt, 2001; Hippe, 1984; König & Stetter, 1989). Drying methods are suitable for postal or parcel transportation of various kinds of microorganisms in culture collections. However, it is still difficult to employ drying methods for preserving methanogens because

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Table 1 Strains used in this study and cultural conditions

	Methanobrevibacter arboriphilus	*Methanoculleus chikugoensis*	*Methanosarcina mazei*	*Methanothermobacter thermautotrophicus*
Strain No.	NBRC 101200 = SA (Asakawa *et al.*)	NBRC 101202^T = MG62^T (Dianou *et al.*)	NBRC 101201 = TMA (Asakawa *et al.*)	NBRC 100330^T = ΔH^T (Zeikus)
Cell form	Coccobacillus	Irregular coccus	Pseudosarcina	Rod
Gram staining	Positive	Negative	Positive	Positive
Optimal temperature	30–37℃	25–30	30–40	65–70
Optimal initial pH	7.8–8.0	6.7–7.2	6.5–7.2	7.2–7.6
Substrate for growth[a]				
H_2/CO_2	+	+	−	+
Formate	+	+	−	w
2-Propanol	−	+	−	−
2-Butanol	−	+	−	−
Cyclopentanol	−	+	−	−
Acetate	−	−	+	−
Methanol	−	−	+	−
Methylamine	−	−	+	−
G+C content (mol%)	28–32	62.2	42	50
Source	cotton wood tree	paddy soil	sewage sludge	sewage sludge
Cultural conditions used in this study				
NBRC medium[b]	878	880	397	398
Temperature	37℃	30℃	37℃	65℃
Cultural period	1 week	1 week	1 week	2–3 days

[a] Symbols: +, grow; w, weakly grow; −, not grow.
[b] The media composition were described at the following website (http://www.nbrc.nite.go.jp/).

methanogens require strictly anaerobic conditions. Therefore, currently, methanogens are maintained mostly by freezing at −80℃ and/or in liquid nitrogen as well as subculturing, and shipped by active cultures.

The L-drying method, which involves vacuum-drying of samples directly from the liquid state without freezing, has been effectively used for long-term preservation of various aerobic and aerotolerant microorganisms (Annear, 1958; Malik, 1990; Sakane & Banno, 1980; Sakane & Kuroshima, 1997; Sakane *et al.*, 1992). In addition, ampoules of microorganisms prepared by the L-drying method can easily be shipped to other laboratories because they can be maintained at room temperature for a short time without suffering appreciable damage.

The present study undertook to improve the L-drying method for long-term preservation of methanogens in culture collections by maintaining not only their viability but also their biological activity such as methane production.

MATERIALS AND METHODS

Archaeal strains and cultivation

The four methanogens used in this study are listed in Table 1 with their cultural media and conditions. Strains were anaerobically cultivated in 20 m*l* of appropriate broth. The air in the vials and dissolved in media was replaced by anaerobic gas ($H_2 : CO_2 = 80 : 20$ or $N_2 : CO_2 = 80 : 20$) and the vials were sealed with tightly-fitting butyl rubber stoppers, and autoclaved at 121℃ for 15 min. The growth of organisms was monitored spectrophotometrically by reading the absorbance at 660 nm with a single-beam spectrophotometer (NOVASPEC II, GE Healthcare UK Ltd., Buckinghamshire, England).

Preparation of cells for L-drying

Cells were prepared by the method described in the appendix. All the preparative procedures were performed aseptically by using a deoxygenized gas injector (IP-8; Sanshin Industrial Co., Ltd., Tokyo, Japan) in a laminar flow without using an anaerobic chamber. The deoxygenized gas injector is an apparatus which removes oxygen completely from

anaerobic gas used for injection by passing the gas through a reduced copper column and sterilizes the gas through a filter with a pore size of 0.22 μm. An anaerobic chamber is not suitable for aseptic handling of a large number of anaerobic bacteria. The protective medium was composed of 0.1 M potassium phosphate buffer (pH 7.0), 3% sodium glutamate, 1.5% adonitol, and 0.05% L-cysteine monohydrate (Sakane & Imai, 1986).

L-drying procedure

L-drying of the cells was carried out as shown in detail in the appendix. All the procedures were operated under anaerobic gas current (H_2 : CO_2 = 80 : 20 or N_2 : CO_2 = 80 : 20) until the ampoules were closed by silicone tubes with stoppers. Glass ampoules (ϕ 9 mm×110 mm, 1 mm in thickness) were plugged with cotton wool and sterilized in an oven at 150℃ for 3 hours. After drying and sealing, the level of vacuum of ampoules was tested with a high-frequency tester (Edward High Vacuum Ltd., Model T2). Ampoules were stored at 4℃ for 1 week or at 37℃ for 2 weeks for an accelerated storage test (Greiff, 1967; Sakane *et al*., 1997).

Recovery from L-dried cells

The recovery of methanogens preserved by the L-drying method was examined immediately after drying, after storage at 4℃ for 1 week, and after storage at 37℃ for 2 weeks. Ampoules were opened in an anaerobic chamber, and 500 μl of liquid medium was added to the ampoules. The contents were mixed carefully to make a homogeneous cell suspension, which was then serially diluted with liquid medium for calculation of viability. Viability was defined as the maximum dilution numbers as growth was observed. All strains tested were incubated for one month under individual optimal growth conditions as shown in Table 1.

Determination of the amount of methane production

After cultivation, the gas of the head-space of culture bottles was collected by a glass syringe. The ratio of methane in the gas phase was analyzed by gas chromatography using a Shimadzu GC-14B (Shimadzu Corp., Kyoto, Japan) equipped with a thermal conductivity detector (TCD) and a glass column (3 mm diameter×1 m) packed with a Molecular Sieves 5A (Shinwa Chemical Industries, Ltd., Kyoto, Japan). Elution was performed with an argon carrier gas at 40 ml/min. The column temperature, injection temperature, and detector temperature were 60℃, 80℃ and 200℃, respectively.

RESULTS AND DISCUSSION

We optimized the L-drying method for methanogens, namely optimized it for strict anaerobes. For this L-drying method, we focused on establishing anaerobic conditions in all procedures by using a deoxygenized gas injector and not using an anaerobic chamber. As a result, we were able to prepare a large number of L-dried ampoules of methanogens simultaneously while avoiding cross contamination. This is advantageous especially for culture collections.

The viability of cells of *Methanobrevibacter arboriphilus* NBRC 101200 was found to decrease from 10^6 to 10^4 immediately after L-drying (Table 2). The cells were possibly damaged during the drying procedure. However, the number of cells remained at 10^4 after storage for 1 week at 4℃ or for 2 weeks at 37℃. The viability of cells of *Methanoculleus chikugoensis* NBRC 101202^T was quite high and the cell counts remained at 10^6 after the L-drying procedure, followed by storage at 4℃ for 1 week, or after storage at 37℃ for 2 weeks. The viability of cells of *Methanosarcina mazei* NBRC 101201 remained at 10^5 after the L-drying procedure, and barely decreased from 10^5 to 10^4 after storage for 1 week at 4℃ or for 2 weeks at 37℃. Likewise, the viability of cells of *Methanothermobacter thermautotrophicus* NBRC 100330^T remained at 10^5 after the L-drying procedure and storage for 1 week at 4℃, and barely decreased from 10^5 to 10^4 after storage for 2 weeks at 37℃. Thus, the numbers of viable cells of all strains used in this study were found not to decrease fatally during preservation by using the improved L-drying method, and were preserved stably after an accelerated storage test at 37℃ for 2 weeks, which approximately corresponds to the normal storage at 4℃ for 20 years (Sakane *et al*., 1997). These strains are expected to be maintained stably for a long term by the improved L-drying method.

In the case of the three strains of methanogens grown under H_2/CO_2, namely *Methanobrevibacter arboriphilus* NBRC 101200, *Methanoculleus chikugoensis* NBRC 101202^T, and *Methanothermobacter thermautotrophicus* NBRC 100330^T, their L-dried samples were found to be preserved stably, even

Table 2 Revival of methanogens preserved by L-drying method

Strain	Preparation	Viability (cells/m*l*)	Methanogenesis (%)
Methanobrevibacter arboriphilus NBRC 101200	Before preservation	10^6	–
	Subculturing	10^6	24.6
	Immediately after drying	10^4	27.7
	After storage at 4℃ for 1 week	10^5	29.6
	After storage at 37℃ for 2 weeks	10^4	26.6
Methanoculleus chikugoensis NBRC 101202^T	Before preservation	10^6	–
	Subculturing	10^6	46.3
	Immediately after drying	10^6	31.4
	After storage at 4℃ for 1 week	10^6	36.0
	After storage at 37℃ for 2 weeks	10^6	33.7
Methanosarcina mazei NBRC 101201	Before preservation	10^5	–
	Subculturing	10^5	45.8
	Immediately after drying	10^5	47.1
	After storage at 4℃ for 1 week	10^4	47.2
	After storage at 37℃ for 2 weeks	10^4	45.5
Methanothermobacter thermautotrophicus NBRC 100331^T	Before preservation	10^5	–
	Subculturing	10^5	46.7
	Immediately after drying	10^5	44.3
	After storage at 4℃ for 1 week	10^5	47.6
	After storage at 37℃ for 2 weeks	10^4	46.8

when cells were harvested during the stationary phase, and they could be recovered well from the L-dried samples stored at 37℃ for 2 weeks. In contrast, *Methanosarcina mazei* NBRC 101201, which is a methanogen grown on acetate, grew well from an L-dried sample prepared by using the cells harvested during the logarithmic phase, whereas only a few cells (1–10 cells/m*l*) were recovered when cells harvested during the stationary phase were used (data not shown). Although the reason is not clear in the present study, this observation suggests that we have to pay attention to the culture conditions, especially during the growth phase, for long-term preservation of methanogens by the L-drying method, in particular when acetate is used for growth.

As described above, the L-drying method improved in this study was designed to keep anaerobic conditions throughout all procedures without using an anaerobic chamber. The improved L-drying method saves time and labor compared with the L-drying method with skim-milk plug by using an anaerobic chamber reported by Morinaga and Inoue (1990). It was found that all of the methanogens tested could be preserved stably by the improved L-drying method, and retained their high viability. Generally, L-drying is an advantageous and useful method for maintaining microorganisms. The improved L-drying method described here will be applicable to the preservation of anaerobes that are sensitive to freezing or freeze-drying. However, attention should be paid to the culture conditions of the cells used for L-drying, especially during the growth phase. Cross-contamination, which is one of the serious problems during the handling of anaerobes in an anaerobic chamber, must be prevented by using plugged cotton wool and a deoxygenized gas injector. The microbial cells dried were predicted to remain viable after long-term preservation by an accelerated storage test. In addition to the low cost of maintenance, L-drying is safer than freezing even in the case of a power outage. Furthermore, the L-drying ampoules can easily be transferred to other laboratories or institutions as they can be stably maintained at room temperature for a short time (Sakane & Kuroshima, 1997; Sakane *et al*., 1992). Consequently, the method improved in this study expands the applicability of L-drying for the preservation and distribution of various methanogens and other anaerobes especially for culture collection works.

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カルチャー・コレクションにおけるメタン生成古細菌の長期保存のための嫌気的 L-乾燥保存法の確立

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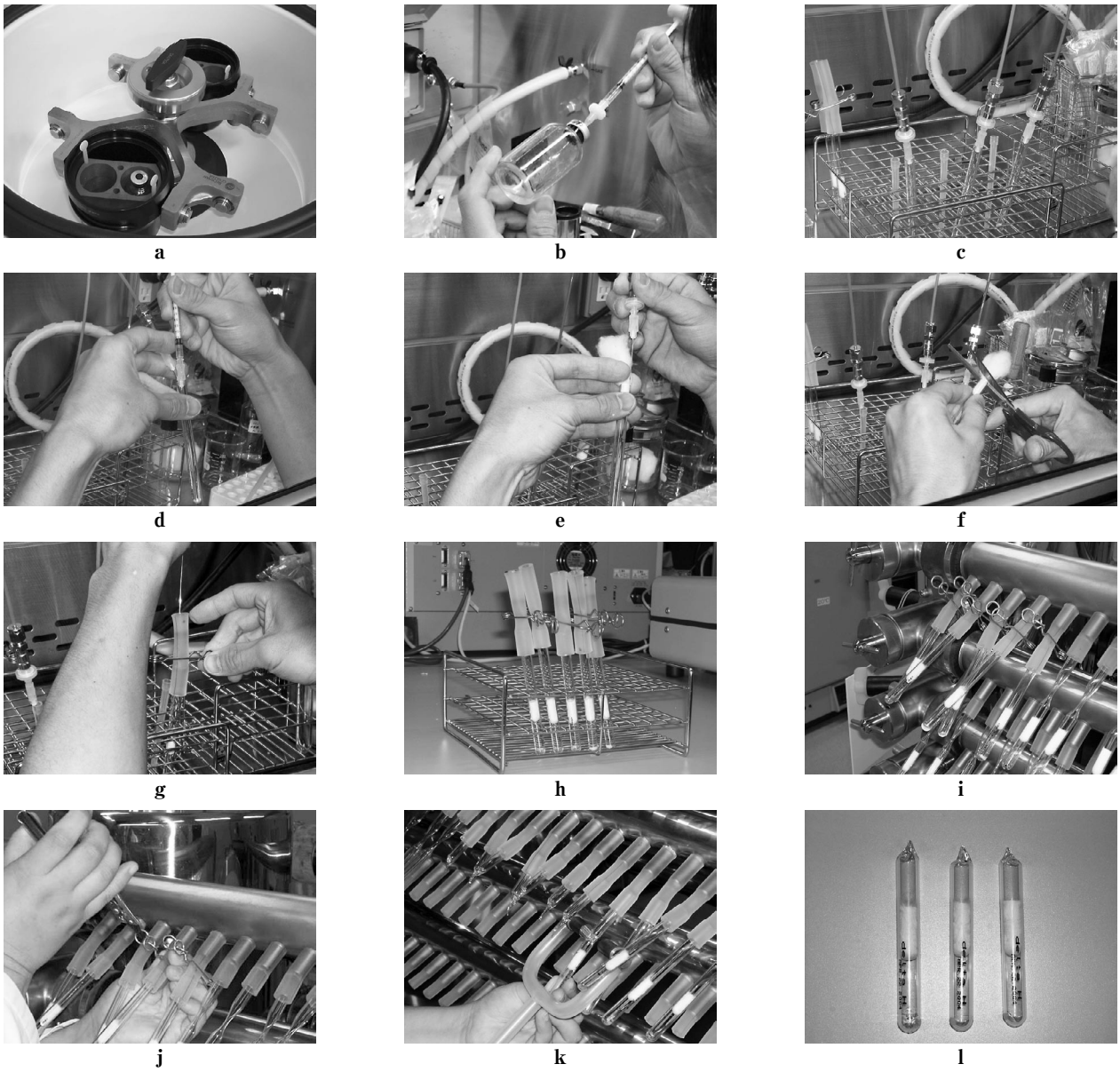
L-乾燥保存法（L-乾燥法）は微生物の長期保存のために一般に用いられる方法の１つである．我々はこの L-乾燥法を高度嫌気性古細菌であるメタン菌に適用するため，作業工程を嫌気環境に維持した L-乾燥法を開発した．供試した *Methanobrevibacter arboriphilus* NBRC 101200，*Methanoculleus chikugoensis* NBRC 101202^{T}，*Methanothermobacter thermautotrophicus* NBRC 100330^{T} の３株は本 L-乾燥法により良好に保存され，乾燥標品を２週間 37℃に保管した加速保存試験後も良好に復元した．これらの生残菌数は，継代培養時は 10^{5} ～ 10^{6} cells/m*l* であったのに対し，L-乾燥保存後は 10^{4} ～ 10^{6} cells/m*l* であった．一方，酢酸を資化する *Methanosarcina mazei* NBRC 101201 は，対数増殖期の細胞を L-乾燥保存に供した時は良好に保存され，10^{4} cells/m*l* が復元したが，定常期に達した細胞を供した時，著しく生残菌数が減少し，10 cells/m*l* 程度しか復元しなかった．このことより，メタン菌の保存には培養条件に注意を払わなければならないことが示唆された．本改変 L-乾燥法はメタン菌のほか，高度嫌気性菌の長期保存や分譲の迅速化を可能にし，カルチャーコレクションの事業に貢献することが期待される．

APPENDIX L-drying procedure for methanogens



a) Cells of methanogens were collected by centrifugation at 3,000 or 5,000 rpm for 10 min at room temperature. **b)** Cells were washed with the protective medium. Washed cells were re-suspended in 2 m*l* of the protective medium. **c)** The air in the sterilized ampoules was replaced by an anaerobic gas by using a hollow needle (0.70×90 mm). **d)** 200 μl aliquots of archaeal cell suspension were dispensed into the sterilized ampoules by using the hollow needle. **e)** The needle for replacing the air was removed from the ampoules, and the cotton wool was immediately plugged in. **f)** The cotton wool plugs were trimmed at the top of the glass ampoules to make it about 1.5 cm in length, and pushed down to the glass ampoules till the middle. **g)** Rubber tubes (9×70 mm) were then connected to the top of the glass ampoules, and the anaerobic gas was flushed again to replace the remaining air. The needle for replacing the air was removed from the ampoules, and the rubber tubes were immediately plugged with a stopper (Tozai Tsusho Co. Ltd., Tokyo, Japan). **h)** The glass ampoules flushed with anaerobic gas. **i)** The glass ampoules were attached to the horizontal manifold of a drying apparatus (FREEZVAC 4C, Tozai Tsusho Co. Ltd.). **j)** The valve attached to the manifold was opened, and then the stoppers were removed. The temperature of cell suspension was observed to fall below 10℃ in the glass ampoules, but not resulting in fallen below the freezing point because the cotton wool plugs impeded it as reported by Sakane *et al*. (1992, 1997). **k)** After drying *in vacuo* for 3 hours, the ampoules were sealed at about 1 cm above the cotton plug by applying a gas flame. **l)** Dried ampoles containing cells of methanogens.