Applied and Environmental Microbiology, July 2009, p. 4616–4619 Vol. 75, No. 13
0099-2240/09/$08.00+0 doi:10.1128/AEM.00519-09

Versatile Solidified Nanofibrous Cellulose-Containing Media for Growth of Extremophiles▽

Mikiko Tsudome,[1] Shigeru Deguchi,[1]* Kaoru Tsujii,[2] Susumu Ito,[3] and Koki Horikoshi[1]

Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan[1]; Nanotechnology Research Center, Research Institute for Electronic Science, Hokkaido University, N21, W10, Kita-ku, Sapporo 001-0021, Japan[2]; and Department of Bioscience and Biotechnology, Faculty of Agriculture, University of the Ryukyus, 1 Senbaru, Nishihra-cho, Okinawa 903-0213, Japan[3]

Received 2 March 2009/Accepted 27 April 2009

Solidified media that employ a porous matrix of nanofibrous cellulose are described. The physicochemical stability of the porous structure allows the development of solidified media that can support the growth of extremophiles, such as acidophilic *Acidiphilium*, alkaliphilic *Bacillus*, thermophilic *Geobacillus* and *Thermus*, alkalithermophilic *Bacillus*, and acidothermophilic *Sulfolobus* microbes. The cellulose-supported media have several advantages over agar- and gellan gum-derived media, including versatility and stability.

Solidified media using agar as a solidifying agent are indispensable in microbiology. For solid cultures of mesophilic microorganisms, agar is an ideal solidifying agent and has been used essentially unchanged since it was first introduced in the late 19th century (2, 15). However, the situation is very different when it comes to culturing extremophiles on solidified media. For example, agar media are not suitable for culturing thermophiles and hyperthermophiles because the solidification of agar is thermoreversible at around 50 to 60°C (21), and the media are unstable at temperatures much above 70°C for extended periods (1). Culturing extremophiles on solidified media under acidic or alkaline conditions presents a similar problem of instability.

We reported previously the use of porous plates made of nanofibrous cellulose for microbial culture (4). Detailed accounts of the preparation procedure, fine structure of the cellulose plate, and its application to culturing representative mesophilic microorganisms (*Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*) appeared in that paper.

Interestingly, the porous structure of the cellulose plate has a structural robustness comparable to that of highly crystalline cellulose (6–8) despite its seemingly fragile structure (4). In situ optical microscopic observation in hot and compressed water (5, 18) revealed that the cellulose plate remained unchanged in water up to 260°C at 25 MPa (4, 7). This finding suggested that the cellulose plate could be used, in principle, as a versatile platform for developing solidified media that support the growth of extremophilic microbes under a wide range of extreme culture conditions. A proof-of-concept experiment was done by successfully culturing *Thermus thermophilus* on the cellulose plate at 80°C (4), but its true potential, especially its versatility, still remained to be corroborated.

In this paper, we show that a wide variety of extremophiles, including an acidophile, an alkaliphile, thermophiles, an acidothermophile, and an alkalithermophile, can be cultured on the cellulose plates. Another advantage of the cellulose-supported media, that no solidifying aid is needed for solidification regardless of culture conditions, is also discussed.

Preparation of cellulose plate. Plates of nanofibrous cellulose were prepared as previously described (4). Cellulose (Funacel SF from Funakoshi, Tokyo, Japan, or microcrystalline cellulose, for thin-layer chromatography, from Merck, Darmstadt, Germany) was dispersed in a saturated aqueous solution of $Ca(SCN)_2 \cdot 4H_2O$ (Wako Pure Chemical, Osaka, Japan) at a concentration of 3% (by weight). Twenty-milliliter aliquots of the dispersion were placed in glass culture dishes and heated to 121°C for 1 min in an autoclave to dissolve the cellulose. The solutions were allowed to solidify at room temperature overnight. $Ca(SCN)_2$ was removed from the solidified plates by washing the plates with methanol and then a copious amount of water. The cellulose plates thus obtained were highly porous materials that consisted of nanofibers (20 to 50 nm in width) of crystalline cellulose and had a typical pore size on the order of several hundred nanometers (4). The cellulose plates were autoclaved at 121°C for 20 min, and then 20 ml of a double-strength (2×) medium was laid on top of the plates. The nutrient components were allowed to diffuse into the pores of the cellulose plates with gentle agitation on an orbital shaker for 4 h, after which the excess supernatant was discarded.

Stability of cellulose plate under extreme culture conditions. Cellulose, agar, and gellan gum (often referred to by the trade name Gelrite) plates containing Luria-Bertani (LB) broth, nutrient broth (NB), or soybean casein digest (SCD) broth at three different pH values (2, 7, and 12) were prepared. The pH was adjusted with H_2SO_4 or NaOH-Na_2CO_3. The plates were kept in an incubator set to 60, 70, or 80°C and examined by visual inspection for syneresis, softening, or melting. The stability of agar and gellan gum plates depended strongly on the type of medium, temperature, and pH. Only LB-agar and SCD-agar plates at pH 7 remained solid at 70°C. Gellan gum plates showed better stability: LB-gellan gum at pH 2, 7, and 12 and SCD-gellan gum at pH 7 and 12 remained solid at 70°C. However, none of them remained solid at 80°C. The NB-gellan

* Corresponding author. Mailing address: Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan. Phone: 81-46-867-9679. Fax: 81-46-867-9715. E-mail: shigeru.deguchi@jamstec.go.jp.

▽ Published ahead of print on 1 May 2009.

TABLE 1. Growth of extremophiles on media solidified with cellulose, agar, and gellan gum[a]

Organism	Basal medium	pH	Incubation		No. of CFU (mean ± SD) on medium with indicated solidifying agent[b]		
			Temp (°C)	Time	Cellulose	Agar	Gellan gum
A. acidophilum	9-K Glucose medium[c]	3.5	25	10 days	285 ± 21 (3)	0 (1.5)	240 ± 27 (0.7)
B. clarkii	Horikoshi-II medium[d]	10.5	30	6 days	65 ± 23 (3)	63 ± 18 (1.5)	ND
G. stearothermophilus	NB	6.8	55	24 h	215 ± 32 (3)	259 ± 31 (2)	— (0.8)
T. thermophilus	TM medium[e]	7.2	80	2 days	379 ± 30 (3)	— (3[d])	— (1)
	TM medium + 0.1% (by wt) $MgCl_2 \cdot 6H_2O$	7.2	80	2 days	335 ± 66 (3)	ND	430 ± 65 (0.7)
S. acidocaldarius	*Sulfolobus* medium[f]	2.0	80	7 days	<100[h] (3)	ND	<100[h] (0.7)
Bacillus sp. strain TX-3	Alkaline NB[g]	10.0	55	24 h	153 ± 39 (3)	115 ± 24 (1.5)	ND

[a] All cultures were incubated aerobically. The experiments were done at least three times.
[b] The numbers in parentheses show the concentration (%) of the solidifying agent. ND, not determined; —, medium did not remain solid.
[c] ATCC medium 738.
[d] JCM medium 181 with soluble starch added instead of glucose.
[e] JCM medium 273.
[f] DSM medium 88.
[g] DSM medium 31.
[h] Colonies were small and could not be enumerated reliably.

gum medium did not solidify even at room temperature, probably because the concentrations of divalent cations in NB were too low to induce solidification. To develop agar- or gellan gum-supported media for these conditions, the medium formulations, such as agar concentration, had to be modified empirically to ensure solidification (14). In contrast, the cellulose plates remained solid under all conditions tested.

Culture of extremophiles on cellulose media. Various extremophiles, including acidophilic *Acidiphilium acidophilum* ATCC 27807^T, alkaliphilic *Bacillus clarkii* DSM8720^T, thermophilic *Geobacillus stearothermophilus* ATCC 12016 and *Thermus thermophilus* JCM10941^T, acidothermophilic *Sulfolobus acidocaldarius* DSM639^T, and alkalithermophilic *Bacillus* sp. strain TX-3 (JCM9162) (13), were cultured on the cellulose plates. Culture media and culture conditions are given in Table 1. The plates were inoculated from liquid cultures and incubated for 1 to 10 days (see Table 1 for details). Conventional agar plates and/or gellan gum plates containing the same nutrients were used for control experiments. In culturing thermophilic microorganisms, care was taken to prevent water evaporation. The lid and body of the culture dish were sealed with adhesive tape, wrapped with a wet paper towel, and sealed in a zip-lock bag during incubation at high temperature.

Culture of an acidophile and an alkaliphile. Hydrolysis of agar in acidic media and the toxicity of hydrolysates prevented growth of *A. acidophilum* on the agar-solidified medium (10, 12). In contrast, visible colonies of *A. acidophilum* were formed on the cellulose and gellan gum plates after incubation for 8 days, showing their chemical inertness in the acidic environment. In the case of alkaliphilic *B. clarkii*, no difference was observed in the number of colonies formed on the cellulose and agar plates.

Culture of thermophiles. *G. stearothermophilus* grew as well on nutrient cellulose at pH 6.8 and 55°C as it did on nutrient agar. The cellulose plate could also be used successfully for culturing thermophilic *T. thermophilus* at 80°C (Fig. 1A). At this temperature, agar and gellan gum media do not remain solid (4). *T. thermophilus* formed colonies on a TM medium (JCM medium 273) solidified by gellan gum and a magnesium

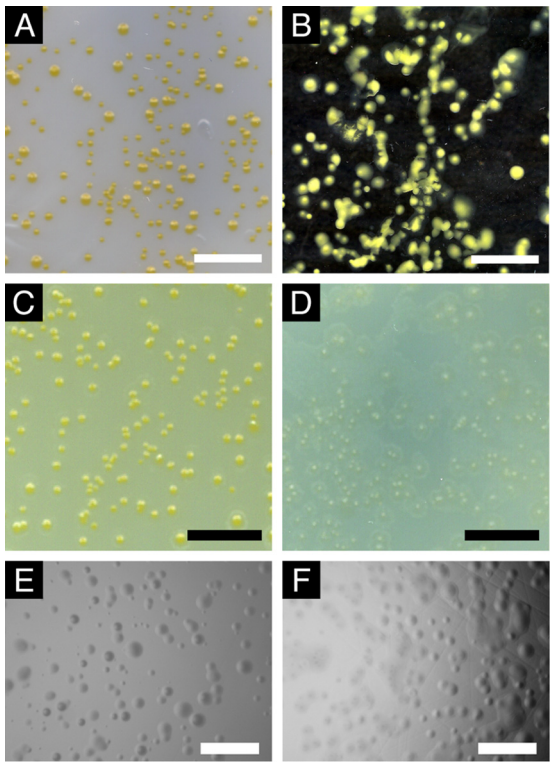

FIG. 1. (A and B) Colonies of *T. thermophilus* formed on a cellulose plate containing TM broth (A) and a gellan gum plate containing TM broth plus 0.1% $MgCl_2 \cdot 6H_2O$ (B). Images were taken after incubation at 80°C for 2 days. Syneresis prevented formation of well-isolated colonies on the gellan gum plate. Scale bar represents 1 cm. (C and D) Colonies of *T. thermophilus* formed on a cellulose plate containing TM broth (C) and TM broth plus 0.1% $CaCl_2 \cdot 2H_2O$ (D). Images were taken after incubation at 80°C for 4 days. Scale bar represents 5 mm. (E and F) Optical micrographs showing colonies of *S. acidocaldarius* formed on the cellulose plate (E) and the gellan gum plate (F) after incubation for 7 days at pH 2 and 80°C. Scale bar represents 1 mm.

gelling aid (14), but the effect of syneresis was evident, leading to irregular colony shapes and merger of neighboring colonies (Fig. 1B).

We also examined the effects of magnesium and calcium gelling aids on the growth of *T. thermophilus* because the addition of a gelling aid negatively affects microbial growth in some cases (14). *T. thermophilus* was cultured at 80°C for 4 days on cellulose plates that contained TM broth, TM broth plus 0.1% $MgCl_2 \cdot 6H_2O$, or TM broth plus 0.1% $CaCl_2 \cdot 2H_2O$. Culturing *T. thermophilus* on solidified TM medium that was free from a gelling aid was made possible only by using cellulose.

T. thermophilus formed yellow circular colonies on a TM-cellulose plate (Fig. 1C). The pigmentation was so intense that the entire plate became yellowish after incubation for 4 days. The pigmentation is due to the formation of carotenoids and is one of the important phenotypic characteristics of *Thermus* strains (22). Interestingly, the pigmentation became less intense when the cellulose plate was supplemented with 0.1% $MgCl_2 \cdot 6H_2O$, although the colony shape remained unaffected (data not shown). *T. thermophilus* formed nonpigmented umbonate colonies (Fig. 1D) on the cellulose plate that contained 0.1% $CaCl_2 \cdot 2H_2O$. The results show that the color and shape of *T. thermophilus* colonies are significantly affected by the type of gelling aid.

Culture of an acidothermophile and an alkalithermophile. Acidothermophilic *S. acidocaldarius* formed colonies on both cellulose and gellan gum plates at pH 2 and 80°C, although the colonies were smaller than 300 μm in diameter and difficult to enumerate accurately (Fig. 1E and F). Close examination revealed significant effects of syneresis only on the colonies on the gellan gum plate. Alkalithermophilic *Bacillus* sp. strain TX-3 was cultured successfully on the cellulose plate at pH 10 and 55°C. Colony formation was also confirmed at 65°C with cellulose, whereas culture on the agar plate met with sporadic success due to syneresis. Gellan gum was unusable because the addition of a calcium or magnesium gelling aid to alkaline NB resulted in precipitation.

Conclusions. Solidified media using nanofibrous cellulose plates can be used for culturing various extremophiles. The cellulose-supported media have several advantages over conventional solidified media using agar or gellan gum (9, 14) for culture of extremophiles, including versatility, stability, and ease of preparation. First, essentially any medium can be solidified with the cellulose plate, regardless of the culture conditions, while the solidification of conventional solidified media depends strongly on the composition of the medium, such as the concentrations of solidifying agent and gelling aids (11, 16, 17). The cellulose plate, for example, allows the preparation of solidified media for extremophiles that contain very dilute and low-nutrient fluids. Various previously uncultured microorganisms have been propagated successfully by using such dilute, low-nutrient media (3, 19, 23). However, solidifying such media with agar or gellan gum for use under extreme culture conditions is difficult. Second, the stability of the cellulose plate is unparalleled. It remains unchanged even at temperatures much higher than the known upper temperature limit for life (122°C) (20). Because oxygen does not affect the stability of the cellulose plate, it may be possible to use the plates to culture anaerobic hyperthermophiles at temperatures at or above 100°C, although this has not been tested. Third, syneresis of the cellulose plate is negligible. Finally, preparation of the cellulose medium obviates such hassles as handling very hot solutions, which is often necessary for preparing gellan gum medium (1).

One drawback may be that preparation of the cellulose plate is somewhat time-consuming compared with reported solidified media. However, unlike conventional solidified media, the cellulose plate can be reused by treating the used plate with 2% (by weight) aqueous NaOH at 120°C for 20 min (4).

We thank Yuichi Nogi and Tohru Kobayashi, JAMSTEC, for stimulating discussions and critical reading of the manuscript.

M.T. acknowledges financial support from the Science and Technology Foundation of Japan.

REFERENCES

1. **Baross, J. A.** 1995. Isolation, growth, and maintenance of hyperthermophiles, p. 15–23. *In* F. T. Robb, A. R. Place, K. R. Sowers, H. J. Schreier, S. DasSarma, and E. M. Fleischman (ed.), Archaea: a laboratory manual. Thermophiles. Cold Spring Harbor Laboratory Press, Plainview, NY.
2. **Codner, R. C.** 1969. Solid and solidified growth media in microbiology, p. 427–454. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 1. Academic Press, London, England.
3. **Connon, S. A., and S. J. Giovannoni.** 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl. Environ. Microbiol. **68:**3878–3885.
4. **Deguchi, S., M. Tsudome, Y. Shen, S. Konishi, K. Tsujii, S. Ito, and K. Horikoshi.** 2007. Preparation and characterisation of nanofibrous cellulose plate as a new solid support for microbial culture. Soft Matter **3:**1170–1175.
5. **Deguchi, S., and K. Tsujii.** 2002. Flow cell for *in situ* optical microscopy in water at high temperatures and pressures up to supercritical state. Rev. Sci. Instrum. **73:**3938–3941.
6. **Deguchi, S., K. Tsujii, and K. Horikoshi.** 2006. Cooking cellulose in hot and compressed water. Chem. Commun. **2006:**3293–3295.
7. **Deguchi, S., K. Tsujii, and K. Horikoshi.** 2008. Crystalline-to-amorphous transformation of cellulose in hot and compressed water and its implications for hydrothermal conversion. Green Chem. **10:**191–196.
8. **Deguchi, S., K. Tsujii, and K. Horikoshi.** 2008. Effect of acid catalyst on structural transformation and hydrolysis of cellulose in hydrothermal conditions. Green Chem. **10:**623–626.
9. **Deming, J. W., and J. A. Baross.** 1986. Solid medium for culturing black smoker bacteria at temperatures to 120°C. Appl. Environ. Microbiol. **51:** 238–243.
10. **Johnson, D. B.** 1995. Selective solid media for isolating and enumerating acidophilic bacteria. J. Microbiol. Methods **23:**205–218.
11. **Kang, K. S., G. T. Veeder, P. J. Mirrasoul, T. Kaneko, and I. W. Cottrell.** 1982. Agar-like polysaccharide produced by a *Pseudomonas* species: production and basic properties. Appl. Environ. Microbiol. **43:**1086–1091.
12. **Kelly, D. P., and A. P. Harrison.** 1989. Genus *Thiobacillus beijerinck*, p. 1842–1858. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. Springer-Verlag, New York, NY.
13. **Kitada, M., Y. Dobashi, and K. Horikoshi.** 1989. Enzymatic properties of purified D-xylose isomerase from a thermophilic alkalophile, Bacillus TX-3. Agric. Biol. Chem. **53:**1461–1468.
14. **Lin, C. C., and L. E. Casida, Jr.** 1984. Gelrite as a gelling agent in media for the growth of thermophilic microorganisms. Appl. Environ. Microbiol. **47:** 427–429.
15. **Madigan, M. T., J. M. Martinko, and J. Parker.** 1997. Brock biology of microorganisms, 8th ed. Prentice Hall, Upper Saddle River, NJ.
16. **Miyoshi, E., and K. Nishinari.** 1999. Effects of sugar on the sol-gel transition in gellan gum aqueous solutions. Prog. Colloid Polym. Sci. **114:**83–91.
17. **Moorhouse, R., G. T. Colegrove, P. A. Sanford, J. Baird, and K. S. Kang.** 1981. PS-60: a new gel-forming polysaccharide, p. 111–124. *In* D. A. Brand (ed.), Solution properties of polysaccharides. American Chemical Society, Washington, DC.
18. **Mukai, S., S. Deguchi, and K. Tsujii.** 2006. A high-temperature and -pressure microscope cell to observe colloidal behaviors in subcritical and supercritical water: Brownian motion of colloids near a wall. Colloids Surf. A **282–283:**483–488.
19. **Rappé, M. S., S. A. Connon, K. L. Vergin, and S. J. Giovannoni.** 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature **418:**630–633.
20. **Takai, K., K. Nakamura, T. Toki, U. Tsunogai, M. Miyazaki, J. Miyazaki, H. Hirayama, S. Nakagawa, T. Nunoura, and K. Horikoshi.** 2008. Cell proliferation at 122°C and isotopically heavy CH_4 production by a hyperthermo-

philic methanogen under high-pressure cultivation. Proc. Natl. Acad. Sci. USA **105:**10949–10954.
21. **Watase, M., and K. Nishinari.** 1987. Dynamic viscoelasticity and anomalous thermal behaviour of concentrated agarose gels. Makromol. Chem. **188:** 1177–1186.
22. **Williams, R. A., K. E. Smith, S. G. Welch, J. Micallef, and R. J. Sharp.** 1995. DNA relatedness of *Thermus* strains, description of *Thermus brockianus* sp. nov., and proposal to reestablish *Thermus thermophilus* (Oshima and Imahori). Int. J. Syst. Evol. Microbiol. **45:**495–499.
23. **Zengler, K., G. Toledo, M. Rappé, J. Elkins, E. J. Mathur, J. M. Short, and M. Keller.** 2002. Cultivating the uncultured. Proc. Natl. Acad. Sci. USA **99:**15681–15686.