Development of an effective method for isolating and culturing non-cultureable microbes using a specialized cellulose sheet

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Research aims:

In recent years, it has been determined that the conventional method for culturing microbes (agar plate culture method) can cultivate less than 1% of microorganisms existing in nature[1)], and its limitations have been pointed out. The remaining 99% of uncultured microorganisms are called “microbial dark matter”[2)]. These uncultured microorganisms are expected to be used in various strata of research and industry. So, a comprehensive, world-wide environmental genome analysis is being carried out. If the remaining 99% of microorganisms can be cultured and their specific attributes can be efficiently applied, then they may solve current challenges. Base sequence information of microbial genomes is helpful in the prediction of microbial functions, although it is always necessary to confirm simulated predictions via wet lab experiments of the isolation and cultivation of uncultured microorganisms (dark matter microbes). Against this background, various culture methods have been developed but have not been generally and widely used.

In this study, a culture substratum was developed using a specialized cellulose sheet (CS-SCS) for the isolation and cultivation of uncultured microorganisms in attempt to eliminate the problems of the conventional agar plate culture method. Application of the CS-SCS led to the isolation and cultivation of uncultured microorganisms from various environmental samples; the successful growth of uncultured microbes demonstrated the practicability and flexibility of the CS-SCS.

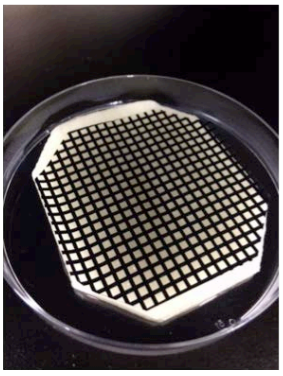

Fig. 1. Photograph of CS-SCS installed in petri dish (DIA : 9 cm).

Methods:

Various environmental samples isolated from soil, freshwater, seawater and plants were treated appropriately, and cultured at 25 °C for about 2 weeks using the CS-SCS (**Fig. 1**) and a conventional agar plate medium. The extracted DNA of the cultured microorganisms was analyzed using the denaturing gradient gel electrophoresis (PCR-DGGE) method and compared comprehensively using next-generation sequencing.

The grown colonies were randomly isolated, and the entire length of the 16S rDNA was amplified by direct colony PCR. Sequence analysis was performed to identify the isolated microorganism species.

An attempt was also made to use the CS-SCS under acidic cultivation conditions where it was difficult to use an agar plate.

Results:

Cultivation of various microbes using the CS-SCS were performed to optimize the specification of the CS-SCS. Soil samples collected at the University of Tsukuba were cultivated on the CS-SCS and agar plate medium. Any cultured microbial communities were compared using the PCR-DGGE method. The results indicated that the cultured microbial community was distinct in the CS-SCS compared to the agar plate medium (**Fig. 2**). These results suggested the applicability of the CS-SCS in the isolation and cultivation of novel microorganisms. A similar trend was observed in studies using environmental samples such as those isolated from freshwater, seawater, etc. The soil samples were cultured on the CS-SCS and agar plate media using R2A medium; later, the cultured microbial communities and the existing microbial communities in the soil samples were analyzed using a next-generation sequencer. These results showed that compared to the agar plate medium, the CS-SCS supported a cultured microbial community with a structure close to that of a soil sample.

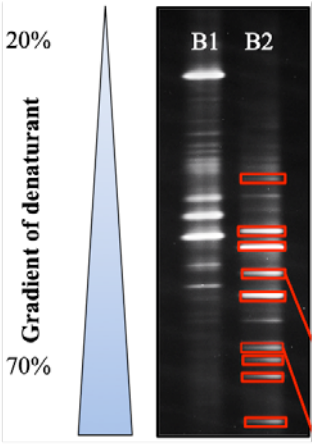

Fig. 2. SYBR Gold-stained DGGE gel. B1: R2A agar plate medium. B2: R2A medium with CS-SCS. The frame indicates microbes cultured only with CS-SCS.

While culturing various samples from plants (*Wasabia japonica* and *Chamaecyparis obtuse*, etc.) and using the CS-SCS and an agar plate medium, it was found that the cultured microorganism communities obtained by the CS-SCS and the agar plate medium were greatly different (as per the result of statistical analysis, the similarity was low among the obtained microorganisms). Some novel microorganisms (less than 97% of 16S rDNA) could be isolated and cultivated using the CS-SCS.

The CS-SCS was further explored for its applicability under acidic conditions where agar plates cannot be used. The elastic value of the agar plate medium, in which *Escherichia coli* can maintain the shape of the colony, was measured (the limiting elastic value was 27.8 kPa). Incubation of agar plates with various pH values and the change in elasticity over time showed that the agar plates with pH 1 could be used within 3 d of cultivation. The comparison of cultivation on the agar plate medium and the CS-SCS with pH <3.0 revealed a great variation among the cultured microbial communities. The CS-SCS

could withstand the strong acidic conditions and supported the cultivation of acidophilic microorganisms. Thus, novel acid-resistant microorganisms could be isolated and cultured from environmental samples using the CS-SCS under pH 1.

Conclusion

The culture substratum developed in this study (CS-SCS) could solve the problems of conventional agar plate medium. The CS-SCS was found to be effective for the isolation and cultivation of novel microorganisms. The CS-SCS could be generally and widely used and is expected to acquire novel microbial resources.

References

1) Amann, R. I., Ludwig, W., Schleifer, K. H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev*. **59**:143-169.
2) Ling, L. L. *et al*. (2015) A new antibiotic kills pathogens without detectable resistance. *Nature* 517: 455-459.