

## A Low-Tech Bioreactor System for the Enrichment and Production of Ureolytic Microbes

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Submitted 16 May 2017, revised 15 September 2017, accepted 27 September 2017

### Abstract

Ureolysis-driven microbially induced carbonate precipitation (MICP) has recently received attention for its potential biotechnological applications. However, information on the enrichment and production of ureolytic microbes by using bioreactor systems is limited. Here, we report a low-tech down-flow hanging sponge (DHS) bioreactor system for the enrichment and production of ureolytic microbes. Using this bioreactor system and a yeast extract-based medium containing 0.17 M urea, ureolytic microbes with high potential urease activity ( $> 10 \mu\text{mol}$  urea hydrolyzed per min per ml of enrichment culture) were repeatedly enriched under non-sterile conditions. In addition, the ureolytic enrichment obtained in this study showed *in vitro* calcium carbonate precipitation. Fluorescence *in situ* hybridization analysis showed the existence of bacteria of the phylum *Firmicutes* in the bioreactor system. Our data demonstrate that this DHS bioreactor system is a useful system for the enrichment and production of ureolytic microbes for MICP applications.

**Key words:** DHS bioreactor system, enrichment culture, microbially induced carbonate precipitation (MICP), ureolytic microbes

### Introduction

Microbially induced carbonate precipitation (MICP) occurs as a by-product of microbial activities in the environment (*e.g.*, ureolysis, photosynthesis, denitrification, ammonification, sulfate reduction, and methane oxidation; Zhu and Dittrich, 2016). In recent years, MICP processes have received attention for their potential biotechnological applications, including remediation of heavy metals and radionuclides, carbon dioxide sequestration, and biocementation (Anbu *et al.*, 2016; Zhu and Dittrich, 2016). In ureolysis-driven calcium carbonate ( $\text{CaCO}_3$ ) precipitation, urease (urea [ $\text{CO}(\text{NH}_2)_2$ ] amidohydrolase; EC 3.5.1.5), a nickel-containing metallo-enzyme, catalyzes the hydrolysis of urea to ammonium ion ( $\text{NH}_4^+$ ) and carbonate ion ( $\text{CO}_3^{2-}$ ; eq. 1). Following the production of  $\text{CO}_3^{2-}$ ,  $\text{CaCO}_3$  can be precipitated in the presence of calcium ion ( $\text{Ca}^{2+}$ ; eq. 2).



Urease activity is widely distributed in the environment, and urease is produced by a wide range of microbes (Anbu *et al.*, 2016; Mobley and Hausinger, 1989). *Sporosarcina pasteurii* (previously known as *Bacillus pasteurii*) has been extensively used for ureolysis-driven MICP applications because this bacterium is non-pathogenic, has high urease activity, and can tolerate the highly alkaline pH required for effective carbonate precipitation (Anbu *et al.*, 2016). Several research groups have successfully isolated microbes with the capacity for ureolysis-driven MICP (Achal *et al.*, 2012; Dhami *et al.*, 2013; Hammes *et al.*, 2003; Kang *et al.*, 2016; Li *et al.*, 2013; Vahabi *et al.*, 2013; Wei *et al.*, 2015). However, fundamental knowledge on the development of bioreactor systems for the enrichment and production of ureolytic microbes is quite limited.

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The major cost associated with ureolysis-driven MICP applications is the production of ureolytic microbes (Cheng and Cord-Ruwisch, 2013; Whiffin, 2004). The cost of sterilizing the cultivation medium is in the range of US\$0.46–0.66 per l of medium, which is comparatively higher than the cost of producing a yeast extract-based medium for culture of *S. pasteurii* (Whiffin, 2004). Therefore, to minimize the cost associated with MICP applications, the production of microbes with high urease activity without sterilization is a significant challenge. Whiffin (2004) demonstrated that the level of urease activity in a *S. pasteurii* culture was not adversely affected by the presence of a significant amount (*e.g.*, 50% [v/v]) of wastewater treatment sludge contaminants in the inoculum. In addition, Chen and Cord-Ruwisch (2013) reproducibly enriched and continuously produced highly ureolytic microbes from activated sludge by using a non-sterile chemostat under selective conditions (high pH and high concentration of urea). These results indicated that, under certain conditions, ureolytic microbes can be enriched and produced without sterilization.

Here, we report the enrichment and production of ureolytic microbes from a soil sample by using a down-flow hanging sponge (DHS) bioreactor system. This bioreactor system was originally developed as a low-tech biofilm-type sewage treatment technology (Agrawal *et al.*, 1997; Uemura and Harada, 2010). The distinctive feature of a DHS bioreactor system is the use of polyurethane sponges, which provide increased surface areas for retaining greater microbial biomass. In addition, the sponge cubes are not submerged in medium but are hanging freely in the air. Thus, adequate oxygen supply for robust growth of aerobic microbes is possible under non-turbulent conditions. Furthermore, in terms of operation, this simple, low-tech bioreactor system can be operated and maintained without highly skilled personnel (Tandukar *et al.*, 2007). The aim of this study was to evaluate the applicability of a DHS bioreactor system for the enrichment and production of ureolytic microbes under laboratory conditions. In addition, we also tested whether the enrichment and production of ureolytic microbes is feasible under non-sterile conditions.

## Experimental

### Materials and Methods

**Bioreactor and enrichment conditions.** A schematic diagram of the DHS bioreactor system used in this study is shown in Fig. 1. The bioreactor system was composed of an acrylic column with 10 polyurethane sponge cubes (32 mm × 32 mm × 32 mm; poros-

ity=98%). A surface soil sample (from ~0–10 cm depth; 100 g) was collected from the schoolyard of the National Institute of Technology at Wakayama College (33°50'00"N, 135°10'34"E), and was used as the inoculum for the bioreactor. The sponge cubes were soaked in the soil sample diluted in 500 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O, and 1.5 mM KH<sub>2</sub>PO<sub>4</sub> [pH 7.5]), and then, the sponge cubes were inserted into cylindrical plastic frames (32 mm diameter, 32 mm tall, 2 mm thick) connected vertically using plastic clamping bands. The total volume of the sponge cubes in the plastic frames was 197 cm<sup>3</sup>. The bioreactor operation period (130 days) was divided into four phases based on the yeast extract concentrations in the supplied medium and the presence/absence of an external air supply. The composition of the yeast extract-based medium (pH 8.0) was as follows: 0.17 M urea, 1 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM MgSO<sub>4</sub> · 7H<sub>2</sub>O, 10 μM NiCl<sub>2</sub>, and yeast extract at 1 g/l in phases 1 and 2 (day 0–57), 5 g/l in phase 3 (day 58–99), and 10 g/l in phase 4 (day 100–130; Becton-Dickinson and Company, NJ, USA). The urea and NiCl<sub>2</sub> concentrations were set based on the reports by Cheng and Cord-Ruwisch (2013) and Gat *et al.* (2016). The medium was stored in a refrigerator at 3 ± 2°C and

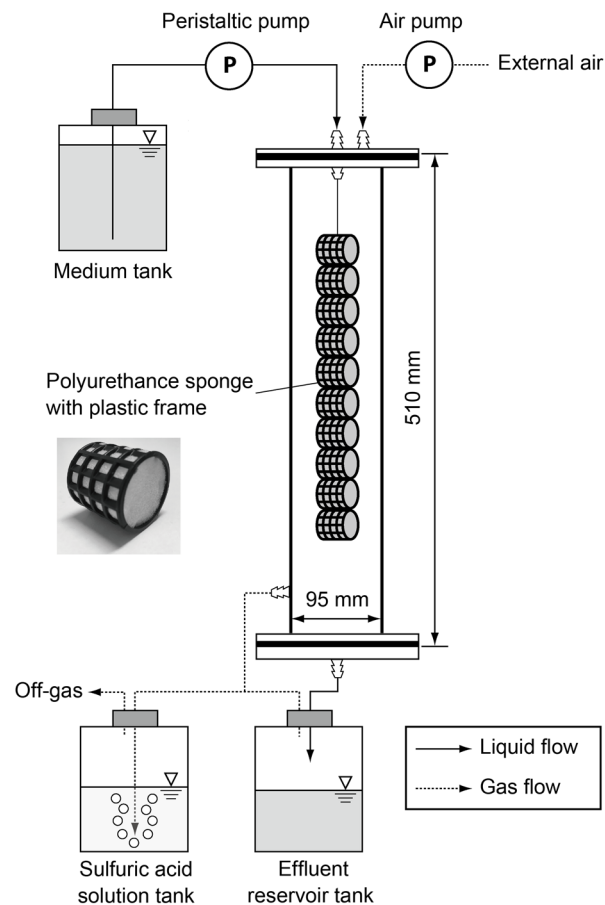


Fig. 1. Schematic diagram of the down-flow hanging sponge bioreactor system used in this study.

was continuously supplied to the bioreactor with a peristaltic pump (MP-2000; Eyela, Tokyo, Japan) and Viton tubing (Cole-Parmer, Vernon Hills, IL, USA). The theoretical hydraulic retention time, which was calculated based on the total void volume of the sponge cubes, was set at 5.5 h. Beginning on day 24, external air was continuously supplied from the upper part of the bioreactor *via* an air pump with a discharge air-flow rate of 8.0 l/min (C-5BN; Techno Takatsuki Co., Ltd., Osaka, Japan). To prevent significant atmospheric diffusion of ammonia species produced by ureolysis, the bioreactor was a closed system, and effluent air was passed through a 2N sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) solution. Effluent medium was collected in an effluent reservoir tank and was manually removed before the tank was filled. The bioreactor system was operated in a dark, temperature-controlled room at 25 ± 2°C.

**Analytical methods for bioreactor effluent and enrichment.** The pH and oxidation-reduction potential (ORP) values of freshly collected bioreactor effluent samples were measured using a pH meter (LAQUAtwin B-712; HORIBA, Ltd., Kyoto, Japan) and an ORP meter (ULTRAPEN PT3; Myron L Company, CA, USA), respectively.

Enrichment samples squeezed from even-numbered sponge cubes (counting from the top) were used to determine urease activity and biomass concentration and for fluorescence *in situ* hybridization (FISH) analysis, as described below. After every sampling, the sponge cubes were re-inserted into the cylindrical plastic frames to recover the ureolytic enrichments.

Potential urease activity was determined using a conductivity method as reported by Cheng and Cord-Ruwisch (2013), with slight modification. In brief, the relative conductivity changes in 10 ml of urea solution (1.5 M final concentration) containing 1 ml of enrichment culture at 25°C was measured using a conductivity meter (LAQUAtwin B-771; HORIBA) at 3–15 min intervals. The rate increase in conductivity was converted to potential urease activity as described previously (Cheng and Cord-Ruwisch, 2013; Whiffin, 2004). One unit (U) of potential urease activity was defined as the enzyme activity that hydrolyzes 1.0 μmol of urea per minute at 25°C. All assays were performed in triplicate.

Biomass concentrations were recorded as mg of dry weight per ml of volume. The dry weight of the biomass was determined after drying samples to a constant weight at 105°C. Subsampled enrichment cultures used for the dry weight determination were washed twice with sterilized distilled water before drying. All assays were performed in triplicate.

**16S rRNA-targeted FISH analysis.** 16S rRNA-targeted FISH was performed according to a previously described method (Snaidr *et al.*, 1997), with some modifications. Samples were fixed by adding 16% par-

aformaldehyde (PFA) solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to obtain a final concentration of 4% PFA and incubation overnight at 4°C, and then stored in a 1:1 mix of PBS and 99.5% ethanol at –20°C until analysis. The PFA-fixed samples were sonicated six times on ice for 15 s each at output setting 1 with an ultrasonic disrupter (UD-211; TOMY SEIKO Co., Ltd., Tokyo, Japan). The sonicated samples were embedded in 0.1% MetaPhor agarose (Cambrex Bio Science Rockland, Inc., ME, USA) in 8-well glass slides (Matsunami Glass Ind., Ltd., Osaka, Japan) and treated with 10 mg/ml lysozyme (from egg whites; Wako Pure Chemical Industries, Ltd.) in TE buffer (100 mM Tris-HCl and 50 mM EDTA [pH 8.0]) at 37°C for 60 min. To dehydrate the samples, the glass slides were immersed in 50%, 80%, and 99.5% ethanol for 2 min each, and then air-dried at 46°C. The 16S rRNA-targeted oligonucleotide probes used in this study were 5'-Cy3-labeled EUB338mix (an equimolar mixture of probes EUB338 [5'-GCT GCC TCC CGT AGG AGT-3'], EUB338-I [5'-GCA GCC TCC CGT AGG AGT-3'], EUB338-II [5'-GCA GCC ACC CGT AGG TGT-3'], and EUB338-III [5'-GCT GCC ACC CGT AGG TGT-3']; Amann *et al.*, 1990; Daims *et al.*, 1999) for most bacteria and 5'-6-FAM-labeled LGC354mix (an equimolar mixture of probes LGC354A [5'-TGG AAG ATT CCC TAC TGC-3'], LGC354B [5'-CGG AAG ATT CCC TAC TGC-3'], and LGC354C [5'-CCG AAG ATT CCC TAC TGC-3']; Meier *et al.*, 1999) for the phylum *Firmicutes*. The hybridization was performed in a hybridization buffer containing 20 mM Tris-HCl (pH 7.4), 0.9 M NaCl, 0.01% sodium dodecyl sulfate (SDS), 0.5 μM oligonucleotide probe, and either 20% formamide (for EUB338mix probe) or 35% formamide (for LGC354mix) for 3 h at 46°C in the dark. To remove excess probe, the glass slides were washed for 15 min in a washing buffer containing 20 mM Tris-HCl (pH 7.4), 0.01% SDS, and either 225 mM NaCl (for EUB338mix) or 80 mM NaCl (for LGC354mix) at 48°C. The glass slides were finally counterstained with ProLong Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, MA, USA) for quantification of total cells. An epi-fluorescence microscope (ECLIPSE E600; Nikon, Tokyo, Japan) equipped with a digital camera (DXM1200; Nikon) was used for microscopic observation. To determine the percentage of FISH-positive cells, 10 different microscopic fields with at least 1 × 10<sup>2</sup> DAPI-stained cells per field were examined for each sample.

**CaCO<sub>3</sub> precipitation test.** DHS bioreactor enrichment collected on day 105 was subjected to a CaCO<sub>3</sub> precipitation test. To precipitate CaCO<sub>3</sub>, 2 ml of the bioreactor enrichment, with average potential urease activity of 27.0 U/ml of culture, was aliquoted into three 50-ml polypropylene tubes containing 40 ml of

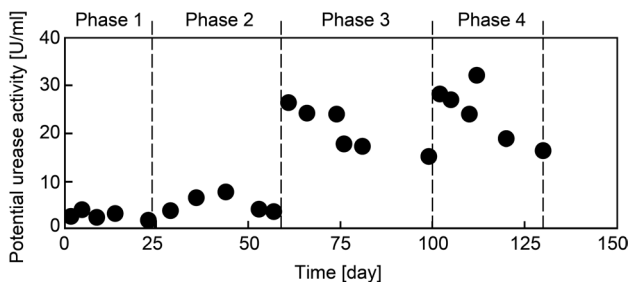


Fig. 2. Time course changes in the potential urease activity of down-flow hanging sponge bioreactor enrichments.

The standard errors of the mean of potential urease activity values ( $n=3$ ) were smaller than the size of the filed circles; therefore, they are not shown.

filter-sterilized  $\text{CaCl}_2$ -urea solution (1.0 M urea and 1.0 M  $\text{CaCl}_2$ ). Under the experimental conditions, the  $\text{CaCl}_2$  in the solution should be completely dissolved according to eq. (3):



The tubes were incubated at 25°C for 24 h with shaking (at 120 rpm). Negative controls, without the enrichment, ( $n=3$ ) were used to detect any non-biological  $\text{CaCO}_3$  precipitation. After the incubation period, precipitates were filtered with Whatman filter paper No. 1 (GE Healthcare UK Ltd., Buckinghamshire, UK) and washed with distilled-deionized water. Then, the filters were dried at 105°C for 24 h and were weighed. The weight of precipitates ( $W_a$ ) was determined from eq. (4):

$$W_a = W_b - W_c \quad (4)$$

where,  $W_b$  is the weight of the filter paper with precipitates and  $W_c$  is the weight of the filter paper without precipitates.

To reveal the mineralogy and surface morphology of the precipitates, a representative sample from the  $\text{CaCO}_3$  precipitation test was subjected to X-ray diffraction (XRD) and scanning electron microscope (SEM) analyses. The XRD analysis was performed on a SmartLab (Rigaku, Tokyo, Japan) with Cu K $\alpha$  radiation operated at 40 kV and 30 mA. Prior to the XRD analysis, the collected precipitates were powdered with a mortar and pestle. The XRD data were collected on the

scale of diffraction angle ( $2\theta$ ) = 5°–90°. The XRD analysis was performed by the Industrial Technology Center of Wakayama Prefecture (Wakayama, Japan). The SEM analysis was performed with a JSM-6510 (JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 5 kV. The collected precipitates used for the SEM analysis were sputter coated with platinum prior to the analysis.

## Results and Discussion

**Enrichment of ureolytic microbes with a DHS bioreactor system.** A previous study by Cheng and Cord-Ruwisch (2013) demonstrated that a non-sterile chemostat system has great potential for reproducibly enriching and continuously producing ureolytic microbes with approximately 60 U of urease activity per ml of culture. In the present study, a high concentration (0.17 M) of urea in the supplied yeast extract-based medium was used as a selective factor for the enrichment and production of ureolytic microbes under non-sterile laboratory conditions (Cheng and Cord-Ruwisch, 2013).

To confirm the enrichment and production of ureolytic microbes in the DHS bioreactor system, the potential urease activity of enrichment samples retrieved from the sponge cubes was determined. Although no obvious potential urease activity (*i.e.*,  $\geq 0.1$  U/ml of inoculum) was detected in the soil slurry inoculum (data not shown), relatively high potential urease activity was repeatedly detected in the enrichments (Fig. 2). The higher pH values of the bioreactor effluents compared to that of the supplied medium (*i.e.*, pH 8.0; Fig. 3) were likely due to ammonia production by ureolysis.

In the initial phase 1 (day 0–23), the yeast extract concentration in the supplied medium was set at 1 g/l. The average potential urease activity observed in this phase was 2.9 U/ml (Fig. 2). Since the bioreactor system used in this study was a closed system, we expected that oxygen could not be effectively supplied to any aerobic ureolytic microbes that colonized the sponge cubes. In

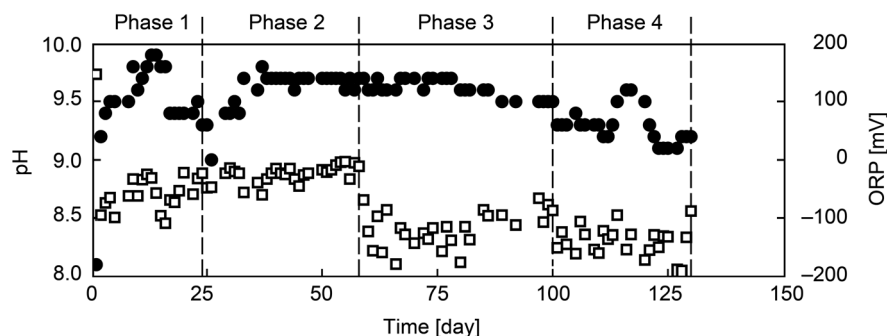


Fig. 3. Time-course changes in the pH (●) and oxidation-reduction potential (ORP; □) values of the effluent from the down-flow hanging sponge bioreactor.

fact, the negative ORP values of the bioreactor effluents (Fig. 3) suggested depletion of the dissolved oxygen in the supplied medium as the medium flowed down into the sponge cubes. Therefore, from day 24 to 130 (*i.e.*, phases 2–4), external air was continuously supplied to the bioreactor to enhance the growth of aerobic ureolytic microbes. It should be noticed that the negative ORP values of the bioreactor effluents were also confirmed, even in the presence of an external air supply (Fig. 3). This phenomenon may be explained by enhanced oxygen consumption by the increasing aerobic biomass in the sponge cubes. During phase 2 (day 24–57), the operational conditions of the bioreactor system were same as those during phase 1, except for the external air supply. The average potential urease activity observed during phase 2 was 5.2 U/ml (Fig. 2). However, the potential urease activity values in phase 2 did not exceed 10 U/ml, which could be required for biocementation applications (Whiffin, 2004). Since yeast extract was thought to be the growth-limiting substrate for the ureolytic microbes in the bioreactor system, the yeast extract concentrations in the supplied medium were increased to 5 and 10 g/l in phases 3 (day 58–99) and 4 (day 100–130), respectively. In phases 3 and 4, the average potential ureolytic activity values were increased to 20.8 and 24.4 U/ml (Fig. 2), respectively. These average values were similar to those of the highly ureolytic *S. pasteurii* (17–34 U/ml of culture; Harkes *et al.*, 2010), and this level of ureolytic activity could be sufficient for biocementation applications.

A positive correlation (coefficient of determination  $R^2=0.604$ ) between the potential urease activity values and the biomass concentrations of the DHS bioreactor enrichment (Fig. 4) suggested that the biomass concentration of the enrichment significantly affected the potential urease activity values. In addition, based on the correlation shown in Fig. 4, an average potential urease activity of 1.5 U/mg of biomass was calculated for the bioreactor enrichment. The types of ureolytic microbes present in the inoculum could have significantly affected the potential urease activity per dry weight of biomass, and the average potential urease activity of the DHS bioreactor enrichment was roughly 8 and 10 times lower than that of *S. pasteurii* under normal growth conditions (Whiffin, 2004), and the ureolytic chemostat enrichment reported by Cheng and Cord-Ruwisch (2013), respectively. Here, it should be noted that the biomass concentrations observed in phases 3 (day 58–99) and 4 (day 100–130; Fig. 4) were 1.5–4.0 times higher than the maximum average biomass concentration observed in the ureolytic chemostat system (Cheng and Cord-Ruwisch, 2013). The high biomass-retention capacity of DHS bioreactor systems has been discussed elsewhere (Onodera *et al.*, 2013; Tandukar *et al.*, 2007). Taken together, it seems that the

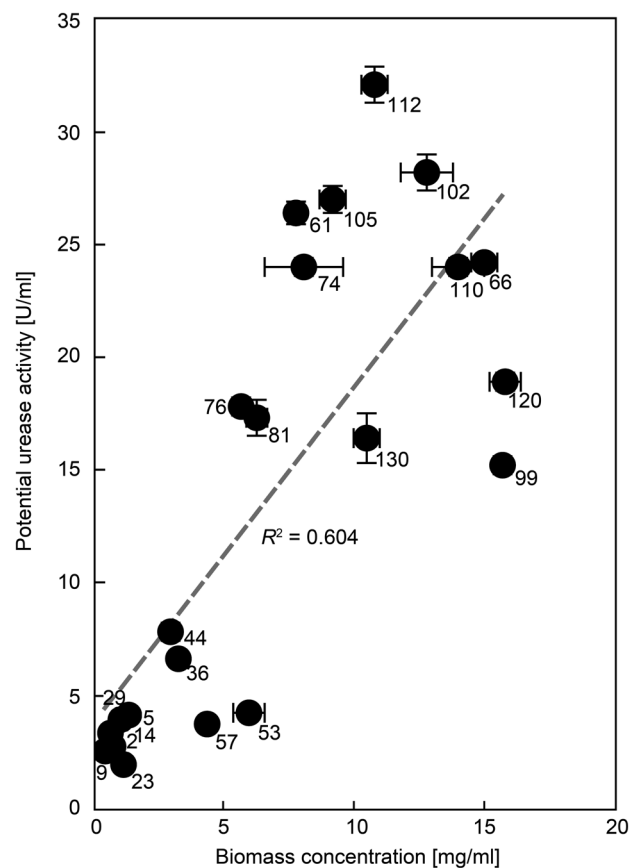


Fig. 4. Scatter plot of potential urease activities and biomass concentrations observed in the down-flow hanging sponge bioreactor enrichments.

The numbers associated with the filled circles are the sampling times for the bioreactor enrichments. The error bars are the standard error of the mean ( $n=3$ ), and filled circle symbols without error bars have standard errors that are smaller than the size of the circle.

high biomass retention capacity allowed the successful establishment of ureolytic enrichments that could be applicable for biocementation applications.

**Detection of the active microbial components by FISH.** The genera *Bacillus* and *Sporosarcina* within the phylum *Firmicutes* are well-known bacterial genera with the capacity for ureolysis-driven MICP (Anbu *et al.*, 2016; Zhu and Dittrich, 2016). In addition, bacteria in these genera have been repeatedly isolated from various environments under ureolytic conditions (Dhami *et al.*, 2013; Hammes *et al.*, 2003; Kang *et al.*, 2016; Li *et al.*, 2013; Vahabi *et al.*, 2013; Wei *et al.*, 2015). Therefore, we performed a FISH analysis using the universal bacterial probe EUB338mix and the *Firmicutes* specific LGC354mix probe to obtain preliminary information on the active microbial components in the DHS bioreactor system. Our FISH analysis revealed that the existence of bacteria in the phylum *Firmicutes* in the bioreactor system (Fig. 5). Since microbial cultivation in a bioreactor selects a simplified microbial community that is optimally adapted to the experimental conditions, these LGC354mix-detected *Firmicutes*

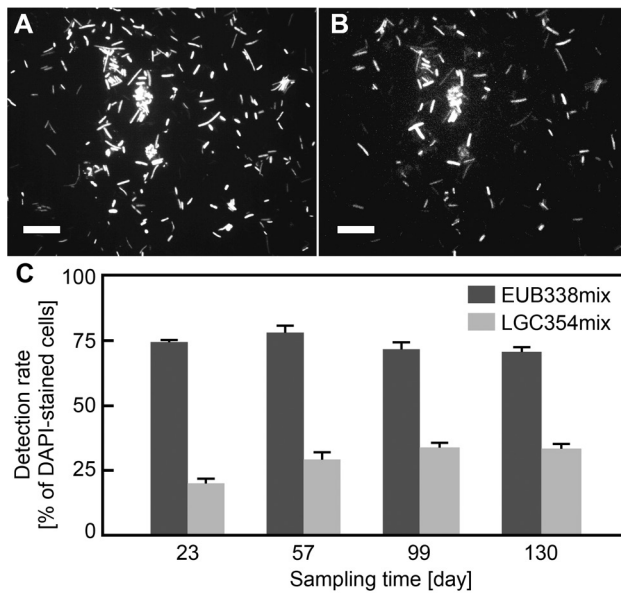


Fig. 5. Fluorescence *in situ* hybridization analysis.

(A) and (B) Representative photomicrographs of a down-flow hanging sponge bioreactor enrichment collected on day 130 after *in situ* hybridization with the phylum *Firmicutes*-targeted LGC354mix probe. Photomicrographs of DAPI-stained cells (A) and the LGC354mix-stained cells (B) in an identical field. Scale bars = 10  $\mu\text{m}$  [in (A) and (B)]. (C) Detection rates (% DAPI-stained cells) of EUB338mix and LGC354mix probe-targeted microbial populations in down-flow hanging sponge bioreactor enrichments. The error bars in (C) are the standard error of the mean from 10 different microscopic fields.

populations might be associated with ureolysis. Considering previous enrichment results (Cheng and Cord-Ruwisch, 2013; Gat *et al.*, 2016), it seems that the high concentration (0.17 M) of urea in the supplied medium functioned as a selective factor for the enrichment of bacteria in the phylum *Firmicutes*. It is a well known fact that the composition of cultivation medium affects the microbial community structure of enrichment cultures (*e.g.*, Gat *et al.*, 2016).

**CaCO<sub>3</sub> precipitation by DHS bioreactor enrichment.** In the CaCO<sub>3</sub> precipitation test using the DHS bioreactor enrichment collected on day 105, the average amount of precipitates ( $\pm$  standard error of the mean,  $n = 3$ ) obtained by the CaCl<sub>2</sub>-urea solutions plus the enrichment was  $92 \pm 7$  mg/ml of CaCl<sub>2</sub>-urea solution. This amount was close to the theoretical value (100 mg/ml of CaCl<sub>2</sub>-urea solution), which was calculated according to the sum of eqs. (1), (2), and (3). In contrast, the negative controls (CaCl<sub>2</sub>-urea solution without the enrichment;  $n = 3$ ) did not show much precipitate (*i.e.*,  $\geq 1$  mg/ml of CaCl<sub>2</sub>-urea solution). These results indicated that the precipitates were produced by the enrichment. XRD analysis of a representative sample from the CaCO<sub>3</sub> precipitation test showed that the dominant precipitate was calcite (Fig. 6A), which is one of the most common CaCO<sub>3</sub> polymorphs induced by

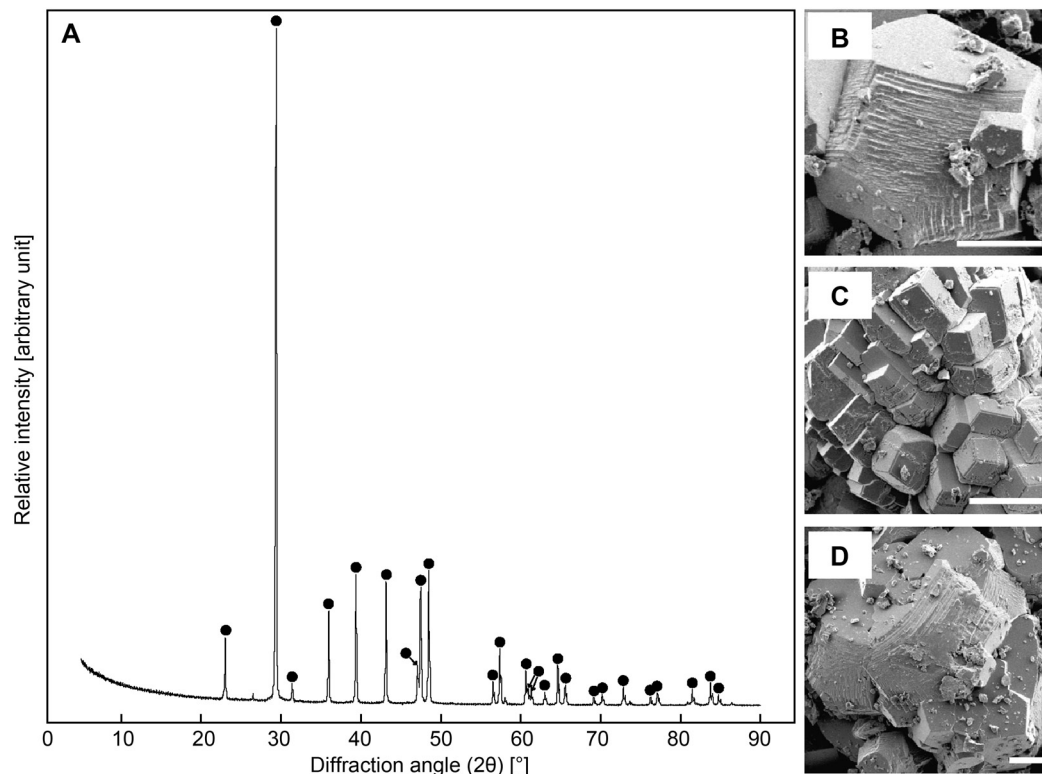


Fig. 6. Mineralogy and surface morphology of precipitates obtained by a down-flow hanging sponge bioreactor enrichment collected on day 105.

(A) X-ray diffraction pattern of the induced precipitates. The peaks marked with filled circles arose from calcite. (B)-(D) Representative scanning electron microscopy images of the induced precipitates. (B) Dodecahedron-like crystals. (C) A cluster of hexahedral-like crystals. (D) A cluster of irregular-shaped crystals. Scale bar = 10  $\mu\text{m}$  [in (B)-(D)].

ureolytic bacteria (Wei *et al.*, 2015). The SEM analysis demonstrated the existence of dodecahedron-like crystals (Fig. 6B) and clusters of hexahedral-like (Fig. 6C) and irregular-shaped crystals (Fig. 6D) in the sample. It has been reported that not only microbial cells, but also extracellular polymeric substances (the major component of biofilm matrix) can function as nucleation sites for CaCO<sub>3</sub> precipitation (Dupraz *et al.*, 2009; Zhu and Dittrich, 2016). In addition, it seems that microbes with biofilm-forming capacity are capable of occupying ecological niches in the DHS bioreactor system because of the bioreactor's characteristics. Therefore, the effective CaCO<sub>3</sub> precipitation observed in this study might be attributed to the possible biofilm-forming capacity of the ureolytic microbes in the bioreactor enrichment.

### Conclusion

Our data demonstrate that the low-tech DHS bioreactor system described in this study has potential for the enrichment and production of ureolytic microbes under non-sterile conditions. In addition, the ureolytic enrichment obtained from the bioreactor system showed effective CaCO<sub>3</sub> precipitation capacity *in vitro*. The results presented in this study provide profound insights into the development of large-scale systems for the enrichment and production of ureolytic microbes for industrial MICP applications.

### Acknowledgments

We would like to thank Dr. Keizo Kashihara for the operation of the scanning electron microscope. This work was partly supported by JSPS KAKENHI Grant Number 15K06218.

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