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Identification of microorganisms in the granules generated during methane fermentation of the syrup wastewater produced while canning fruit

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A B S T R A C T

The wastewater produced in the process of canning fruit contains a syrup that consists mainly of sucrose. This syrup wastewater was treated by methane fermentation in an upflow anaerobic sludge blanket reactor. The organic loading rate of syrup wastewater was increased gradually as fermentation progressed. The higher the organic loading rate, the more methane gas evolved until the organic loading rate reached 30.3 kg COD m^{-3} d^{-1}, at which point methane generation abruptly diminished because the loading rate was too high to stably operate the reactor. The changes in the microbial community, that of both bacteria and archaea in the granules, were analyzed simultaneously using PCR-DGGE during the fermentation process. *Methanosaeta* spp., which are methanogenic archaea that produce extracellular polymers indispensable for the formation of granules, were dominant when the methane gas vigorously evolved, and the iron-reducing bacterium belonging to genus *Geobacter*, which outcompetes methanogens, grew proportionally with the deterioration of methane fermentation.

1. Introduction

Recently, circumstances surrounding the fruit-canning industry in Japan have changed. Fruit canning companies import large containers of canned fruits mainly from Asia [1]. They remove the fruit from the large containers and adjust the syrup taste to repackage the fruit and syrup into smaller cans more suitable for each household in Japan. This process produces a large amount of waste syrup at the canning facilities, and adequate treatment of syrup wastewater is urgently required.

For many years, methane fermentation of biomass waste has been of interest in the production of alternative energy in order to reduce the use of fossil fuels, as well as for the treatment of organic waste [2]. Methane fermentation of biomass materials normally proceeds in 4 steps—hydrolysis, acidogenesis, acetogenesis, and methanogenesis—in which hydrolytic bacteria, fermentative bacteria, acetogens, and methanogens, respectively, play distinct roles. Therefore, methane fermentation involves many groups of microorganisms, and several intermediate reactions.

One of the most appropriate methods for methane fermentation is to use an upflow anaerobic sludge blanket (UASB) reactor because of its simplicity; low investment and operation costs; and the long, favorable experience in the treatment of a wide range of industrial wastewaters [3–6]. The formation of anaerobic granular sludge is essential for successful UASB operation of wastewater. In the UASB reactor, granules, mostly with diameter of about 2–5 mm, are produced, and various types of microorganisms are immobilized in the granules. Molecular biology methods have been used to analyze the microbial community in the anaerobic fermentation reactor, and the composition of the microbial community was directly determined by these methods without using one that was culture dependent. The fluorescence *in situ* hybridization method, a molecular biology technique, was performed to understand the spatial distribution of microorganisms responsible for methane fermentation in the granules, and was useful in analyzing the microbial community of both bacteria and archaea in the granules derived from full-scale UASB reactors [7,8]. Another molecular biology technique, polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE), has an advantage in that it can precisely determine the type of microorganisms in the granules [9–12]. Moreover, there have been some studies which addressed the changes in the microbial community associated with the change in the organic loading rate [13–15]. However, to the best of our knowledge, there have been no studies on the changes in the microbial community in granules produced during methane fermentation of the syrup wastewater from fruit-canning. The waste syrup is rather special in its composition in that it is composed of sucrose, a homogenous low-molecular-weight substance, which seems to degrade easier than do organic wastes made of heterogeneous high-molecular-weight substances, such as animal manure,

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Table 1
Characteristics of waste syrup.

Parameters	Unit	Values
TOC	kg m^{-3}	70.8 ± 4.8
COD	kg m^{-3}	189.0 ± 9.4
pH	–	4.2 ± 0.1
Brix	%	15.2 ± 0.1
Protein	kg m^{-3}	1.0 ± 1.0
Phosphorous	g m^{-3}	21.5 ± 9.1
Potassium	g m^{-3}	339.8 ± 205.7
Manganese	g m^{-3}	2.3 ± 1.4
Magnesium	g m^{-3}	147.0 ± 99.1
Nickel	g m^{-3}	ND[a]
Cobalt	g m^{-3}	ND[a]

Values are average ± 95% confidence intervals for the averaged values ($n = 3$).
[a] ND, not detected.

sewage sludge, and kitchen garbage. Moreover, the waste syrup usually does not contain substantial amounts of proteins, lipids, and trace metals.

In the present study, syrup wastewater was treated using UASB methane fermentation, and the relationship between the performance of the UASB reactor and the changes in the microbial community was determined when the organic loading rate of the syrup wastewater into the fermentor increased with the progress of fermentation.

2. Materials and methods

2.1. Methane fermentation

Waste syrup discharged from Yamanashi Kanzume Co. Ltd., Shizuoka, a fruit-canning factory, was treated using a methane fermentation process. The syrup consisted mainly of sucrose at a concentration of 15.2° Brix; its other characteristics are shown in Table 1. The waste syrup contained the trace metals Mn^{2+} and Mg^{2+} at a concentration of approximately 2.3 and 147 ppm, respectively; however, the syrup was diluted with water, which did not contain Ni^{2+}, Co^{2+}, Mn^{2+}, or Mg^{2+}, to prepare the syrup wastewater. Therefore, the concentration of these metals in the syrup wastewater was quite low. In particular, Ni^{2+} and Co^{2+} were under detectable levels.

The upflow anaerobic sludge blanket (UASB) reactor was 5000 mm long and 2500 mm in diameter, with a capacity of 20 m^3. Approximately 15 m^3 of granular sludge obtained from a brewery was packed into the fermentor. At the beginning of the fermentation process, the temperature of the fermentor was adjusted to 39 °C and left for 2 d before the syrup wastewater was added. On the third day, the waste syrup was diluted with water to produce 3.03 kg COD m^{-3} d^{-1}, adjusted to a temperature of 39 °C, and introduced into the fermentor. The OLR was then adjusted by both diluting the waste syrup with water and changing the hydraulic retention time (HRT). The HRT was set at 7.5 d during the initial stages of from days 3 to 19, and decreased stepwise by 5.0 d from days 20 to 33 to 3.8 d from days 34 to 61, and down to 3.1 d until day 145 of fermentation, after which time it was increased back up to 3.8 d at day 146. The OLR was increased stepwise as fermentation progressed, and reached a maximum value of 30.3 kg COD m^{-3} d^{-1} at approximately 127 d, but it decreased after 146 d of fermentation (Fig. 1). The temperature inside the UASB reactor was maintained at 39 °C throughout the fermentation process. The pH value of the fermenting liquid was adjusted to 7.5 by adding 25% NaOH solution to the syrup wastewater, which was introduced into the reactor. The amount of NaOH added to the reactor was associated with the daily inflow of the syrup wastewater and was equivalent to approximately 3 kg.

Because it has been shown that trace metals enhance microbial activity [16,17], after 49 d of fermentation, metal salts such as $CoCl_2$, $MgCl_2$, $MnCl_2$, and $NiCl_2$ (20 g each) were added to the UASB reactor once a week to maintain microorganism activity in the syrup wastewater, which contributes to methane fermentation. The syrup wastewater used in the present study had a rather peculiar characteristic in that it contained few amount of trace metals; therefore, it was presumed effective to add these trace metals to the wastewater. In addition, after 64 d of fermentation, the carbon-to-nitrogen (C/N) ratio was adjusted to 15.0 by adding urea. The exhaust-gas volume was continuously measured using a flow meter (CMS0500, Yamatake Corporation, Tokyo, Japan) installed at the top of the fermentor, and the pH value was monitored using a pH meter (pH100, Yokogawa Electric Corporation, Tokyo, Japan) installed inside the fermentor. A sample of the exhaust gas was captured in a Tedlar bag once a day, and its components were measured using gas chromatography (G1011, Yanagimoto Corporation, Kyoto, Japan) installed with the column packed with Molecular Sieve 5A 80/100 mesh 1 m × 1/8″ × 2 mm ID SilcoSmooth™ tubing (80440-800, Restek, PA, USA). Approximately 200 mL of the granular sludge together with the fermentation liquid inside the fermentor were withdrawn at adequate time intervals from the sampling port installed 1700 mm from the bottom and 750 mm from the wall inside the fermentor. Volatile fatty acid (VFA) concentrations in the fermentation liquid sample were measured by high-performance liquid chromatography (HPLC) (LC-2000 plus HPLC system, Jasco, Tokyo, Japan) equipped with a YMC-UltraHT Pro C18 column (AS12S02-1003WT, YMC Corporation, Kyoto, Japan). Ferrous ion concentration in the fermentation liquid sample was determined using PACKTEST WAK-Fe^{2+} (Kyoritsu Chemical Check Laboratory, Corp., Tokyo, Japan), which is based on the chelating ability of o-phenanthroline with ferrous ions, and the color change was quantitatively measured using digital PACKTEST (DPM-MT, Kyoritsu Chemical Check Laboratory, Corp., Tokyo, Japan). In addition, the microbial community in the granules was analyzed, as described in the following section.

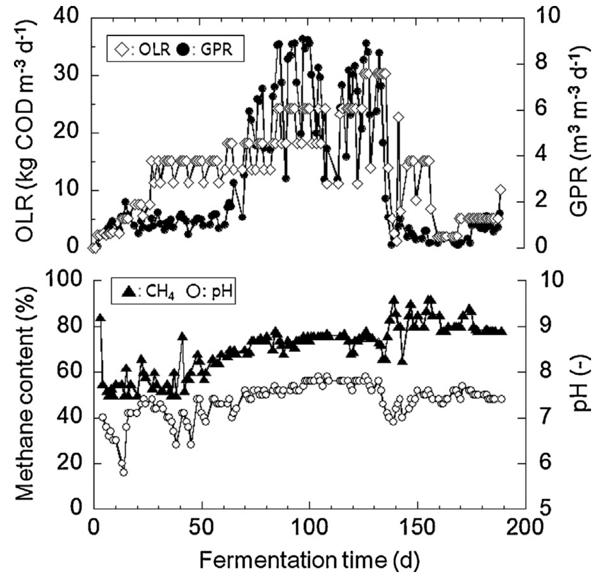

Fig. 1. The courses of gas production rate (GPR), organic loading rate (OLR), gas compositions, and pH value during methane fermentation.

2.2. DNA extraction

A 3-mL fermentation liquid sample containing granules was washed in 27 mL of sterilized distilled water. Then, DNA extraction was performed on a 0.5 g wet weight granule sample. DNA extraction from the granule sample was conducted using the ISOIL for Beads Beating Kit (No. 319-06201, Nippon Gene Co., Ltd., Toyama, Japan) according to the manufacturer's instructions. The extracted DNA was used as a template for the PCR.

2.3. PCR amplification

The 16S rRNA gene region of bacteria and archaea was used for PCR-DGGE analysis. The primers for the bacteria were 357FGC, 5′-*CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG* GCC TAC GGG AGG CAG CAG-3′ (*Escherichia coli* 341–357) and 518R, 5′-ATT ACC GCG GCT GCT GG-3′ (*E. coli* 518–534) [18], and the primers for archaea were PRA46F, 5′-(C/T) TAA GCC ATG C(G/A)A GT-3′ (*E. coli* 46–60); PREA1100R, 5′-(T/C)GG GTC TCG CTC GTT (G/A)CC-3′ (*E. coli* 1100–1117); PARCH 340F, 5′-*CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG* GCC CTA CGG GG(C/T) GCA (G/C)CA G-3′ (*E. coli* 340–357); and PARCH519R, 5′-TTA CCG CGG CKG CTG-3′ (*E. coli* 519–533) [19]. PRA46F and PREA1100R were used for nested PCR. The sequence in italics is a GC clamp. PCR amplification was performed using TaKaRa *Ex Taq*™ HS (RR006A, Takara Bio Inc., Otsu, Japan). Amplifications were carried out in a final volume of 50 μL, containing 2 μL of template DNA, 5 μL of 10× *Ex Taq* Buffer, 4 μL of dNTP Mix (2.5 mM each), 0.5 μL of each primer, 0.25 μL of TaKaRa *Ex Taq*™ HS (5 U μL^{-1}), and 37.75 μL of sterilized distilled water.

PCRs were performed using the following steps: initial denaturation at 94 °C for 7 min; 7 cycles at 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min; 7 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min; 21 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min for bacteria, and initial denaturation at 95 °C for 5 min; 35 cycles at 95 °C for 1 min, 53.5 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 6 min for archaea.

The PCR products of 2 μL were analyzed by electrophoresis in a 1.0× Tris–borate–EDTA (TBE) agarose gel.

2.4. Denaturing gradient gel electrophoresis (DGGE)

The Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) was used for DGGE analysis. Polyacrylamide gel (8%, wt/vol, acrylamide–bisacrylamide at 37.5:1) was used with a denaturant gradient