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Short communication

# Digested sludge-degrading and hydrogen-producing bacterial floras and their potential for biohydrogen production

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ABSTRACT

Digested sludge (DS) is a recalcitrant waste produced by anaerobic digestion of excess sludge. Although biological wastes have received attention as a substrate for hydrogen fermentation, DS has not been well examined as a substrate for the fermentation. In this study, we found and characterized DS-degrading/hydrogen-producing bacterial floras. Soil and cattle faecal samples were enriched by DS supplementation, and repeated subculture of the samples produced three bacterial floras showing stable hydrogen production (0.95–1.62 ml $H_2$/g-dried DS). They comprised unique members with some belonging to novel genera (*Fonticella*, *Gracilibacteri* and *Romboutsia*). The culture supernatant exhibited xylanase, chitinase and keratinase activities, suggesting that bacterial members in the floras degrade xylan, chitin, and protein in DS to produce hydrogen. While there are many articles of hydrogen fermentation from excess sludge, our study is the first report on hydrogen fermentation from untreated DS as a fermentation substrate.



## 1. Introduction

Although the activated sludge method is employed in sewage treatment worldwide, it produces a huge amount of excess sludge (Liu and Tay, 2001). Anaerobic digestion is generally used to reduce the volume of excess sludge and produce biogas. However, the overall efficiency of sludge degradation during anaerobic digestion is only ~30%–60% (Appels et al., 2008; Radaideh et al., 2010; Rapport et al., 2012), and a large quantity of digested sludge (DS) is produced as recalcitrant waste. Recently several thermophilic bacteria that assimilate excess sludge under aerobic conditions have been reported (Kim et al., 2002; Li et al., 2009; Liu et al., 2010, 2011). However, these thermophiles have been shown to digest up to 50% of sewage sludge under optimal conditions, leaving DS-like residue. Because the shortage of final disposal sites has become a serious problem recently, it is imperative to reduce DS volume by utilizing DS as a bioresource.

Since biohydrogen is considered as a renewable energy, anaerobic bacteria that can produce hydrogen from DS are attractive for establishing technologies for DS utilization. Hydrogen-producing bacteria are known to exist ubiquitously in anaerobic environments, and many bacteria have been studied thus far for biohydrogen production from agricultural wastes, food wastes and excess sludge, which contain copious amounts of fermentable substrates (Guo et al., 2010). For instance, Cai et al. (2004) reported that 8 ml biohydrogen was produced from 1 g of untreated excess sludge, and 17 ml of biohydrogen from 1 g alkaline-treated excess sludge. Zhao et al. (2010) reported that 20 ml of biohydrogen was produced from 1 g of heat-treated excess sludge. Sato et al. (2016) recently reported that the digestion liquor supplemented with fungal hydrolase enzymes yields hydrogen from DS, but the sludge used in their study was pretreated by $H_2SO_4$ prior to fermentation. There are many articles of hydrogen fermentation from excess sludge or isolation of excess sludge-fermenting bacteria from DS. To the best of our knowledge, therefore, there are no reports on biohydrogen production from untreated DS. In this study, we tried to enrich and characterize hydrogen-producing bacterial floras that can assimilate untreated DS as a fermentation substrate.

## 2. Materials and methods

### 2.1. Chemicals and materials

Avicel, carboxymethyl cellulose, birchwood xylan, azure chitin and azure keratin were purchased from Sigma-Aldrich (St. Louis,

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MO, USA). Cellulase (*Trichoderma reesei* ATCC26921), xylanase (*Thermomyces lanuginosus*), and laccase (*Trametes versicolor*) were also purchased from Sigma-Aldrich. Zymolyase 100T was obtained from Seikagaku Corporation (Tokyo, Japan). Standard hydrogen and methane gases were purchased from GL Sciences (Tokyo, Japan). Reagents for molecular biology were purchased from Toyobo (Osaka, Japan), Life Technologies (Carlsbad, CA, USA), Thermo Fisher Scientific (Waltham, MA, USA) and MP Biomedicals (Santa Ana, CA, USA). All other chemicals were purchased from Wako Pure Chemicals (Kyoto, Japan). Glass and plastic labwares used for hydrogen fermentation were purchased from Maruemu Corporation (Osaka, Japan) and AS ONE Corporation (Osaka, Japan).

### 2.2. Sample collection

Dewatered DS (17% total solids) was obtained from a municipal sewage treatment plant in Yamaguchi City, Japan, dried completely in FSP450 dry oven (Advantec, Tokyo, Japan) at 105 °C for 24 h and then used as a substrate for bacterial culture. The Tyurin and Kjeldahl methods (Marumoto et al., 1978; Bremner and Mulvaney, 1982; Sato et al., 2001; Horwitz, 1995) were employed to determine the organic carbon and nitrogen contents of the DS, which were found to be 410 and 68 mg per g-dried DS, respectively. Cellulose, xylan, chitin and lignin contents in DS were determined by enzymatic hydrolysis of DS. Digested sludge (30 mg) was suspended in 1 ml of 50 mM phosphate buffer (pH 5.0) containing 2 mg/ml cellulase or xylanase, and incubated at 50 °C for 4 h to hydrolyze cellulose or xylan, respectively, in DS. For hydrolysis of chitin, DS (30 mg) was suspended in 1 ml of 50 mM phosphate buffer (pH 6.0) containing 2 mg/ml zymolyase and incubated at 50 °C for 4 h. Reducing sugars in the hydrolysates were measured by a dinitrosalicylic acid method (Miller, 1959; Wood and Bhat, 1988). For determination of lignin content, DS (30 mg) was suspended in 1 ml of 50 mM phosphate buffer (pH 6.0) containing 2 mg/ml laccase and incubated at 25 °C for 4 h, after which absorbance (280 nm) for supernatant of the reaction mixture was measured. Lignin content was calculated based on extinction coefficient of 15.6 ($l \cdot g^{-1} \cdot cm^{-1}$), according to the method of Gindl et al. (2000). For determination of protein content, DS (30 mg) were ground for 30 s using the Mini Bead Beater-8 (Biospec, Oklahoma, USA) in 500 μl MilliQ water containing 50 mg of 0.5-mm glass beads and 50 mg of 0.1-mm zirconia silica beads to extract protein. The resultant cell homogenate was microfuged at 14,500 rpm for 5 min at 4 °C, and concentration of the extracted protein was analyzed by the BCA method (Smith et al., 1985). Carbohydrates, lignin, and protein content of DS is shown in Table 1.

To determine acetate, lactate, and ethanol contents in DS, dried DS powders (10 g) were homogenized in 100 ml deionized water for 1 min using an Osterizer blender, and the homogenate was subsequently centrifuged at 6000×g, 10 min at 4 °C to recover supernatant. Acetate, lactate, and ethanol concentration in the supernatant were analyzed to determine their contents in DS using F-kit acetate, lactate, and ethanol enzymatic assay kits (Roche, Basel, Switzerland) respectively, according to the manufacturer's manual. However, those metabolites were not detected in DS used for the present study, implying that they were lost through liquid-solid separation, dewater, and drying processes.

To determine the metal content of DS, 400 mg of DS was incubated in 2 ml of $H_2SO_4$ for 3 h, after which 34 ml deionized water was added to the mixture, which was then autoclaved at 121 °C for 1 h to extract metals. The resulting hydrolysate was filtered through an Advantec GB-140 glass fiber filter, and the metals in the filtrate were analyzed using an Optima 8300 ICP-OES Spectrometer (PerkinElmer, MA, USA). Metal content of DS is shown in Table 1.

To isolate the bacteria that assimilate DS and produce hydrogen, seven environmental samples were collected from several locations in Yamaguchi University, as shown in Table 2. A total of 1 g of the sample was suspended in 9 ml of sterile water. Half of the suspension was boiled for 15 min to inactivate hydrogen-consuming bacteria and enrich spore-forming hydrogen producers (Lay et al., 1999; Lay, 2000), and used as the bacterial inoculum. The other half was used as the inoculum without the heat pretreatment.

**Table 1**
Chemical characteristics of digested sludge (DS) used in this study.

| Constituents | | Content (mg/g-dried DS) |
|---|---|---|
| Organic compounds | Cellulose | 4.2 |
| | Xylan | 4.1 |
| | Chitin | 9.0 |
| | Lignin | 24.2 |
| | Protein | 42.9 |
| Metals | Al | 8.9 |
| | Fe | 19.7 |
| | Cu | 0.3 |
| | Mn | 0.2 |
| | Zn | 0.5 |

### 2.3. Cultivation of the bacterial flora

Enrichment and subculture of bacterial floras were performed in a 17-ml glass vial. For enrichment of the environmental samples, 1 ml of the inoculum was mixed with 9 ml of sterile water and 100 mg of dried DS. The pH of the mixture was adjusted to 5.0 with 1 M HCl. The gas phase in the vial was then flushed with nitrogen for 1 min and the vial was sealed with a butyl-rubber stopper and an aluminum cap. Fermentation was allowed to proceed for 1 week at 50 °C. Control experiments using glucose (10 mg/vial) as a substrate were also carried out for three times.

Subsequently, gas phase in a headspace of the vial for the 1-week culture was sampled for hydrogen analysis, and the supernatant was recovered for enzyme assay. The culture remains were inoculated into a new vial containing 9 ml of sterile water and 100 mg of DS and cultivated for subculture.

For the isolation of bacteria, a 10 μl aliquot of each subculture was inoculated onto Bacto Anaerobic agar (Becton Dickinson, Franklin Lakes, NJ, USA), and cultivated anaerobically in an Anaeropack jar (Mitsubishi Gas Chemical, Tokyo, Japan) at 50 °C.

### 2.4. Gas and metabolites analysis

Hydrogen produced during the 1-week fermentation in a headspace (7 ml) of the vial was analyzed using a GC-2014 gas chromatograph with a thermal conductivity detector (Shimadzu, Kyoto, Japan) under the following conditions: column, Micropacked ST column (2.0 m × 1.1 mm internal diameter [i.d.], Shinwa Chemical Industries, Kyoto, Japan); injection volume, 0.5 ml; carrier gas, argon (43.5 ml $min^{-1}$) and column temperature, 100 °C.

Metabolites (acetate, lactate, and ethanol) concentration in 1-week-old culture was analyzed using the F-kit enzymatic assay kits. One ml of the bacterial culture was microfuged at 15,000 rpm for 10 min at 4 °C, and the supernatant was directly assayed to determine metabolites concentration.

### 2.5. Enzyme assay

An aliquot (2 ml) of the 1-week culture was microfuged at 15,000 rpm for 10 min at 4 °C, and the supernatant was recovered as the enzyme solution. Chitinase and keratinase activities in the enzyme solution were quantified using azure chitin and azure keratin as substrates, according to the methods of Ramírez et al.