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Possible practical utility of an enzyme cocktail produced by sludge-degrading microbes for methane and hydrogen production from digested sludge

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Digested sludge (DS) is a major waste product of anaerobic digestion of sewage sludge and is resistant to biodegradation. In this study, we examined suitability of the hydrolases produced by DS-degrading fungal strains (DS-hydrolases) for methane and hydrogen fermentation from DS. Although the strains are mesophilic, DS-hydrolases showed strong chitinase and keratinase activity at ∼50°C. SDS-PAGE analysis suggested that the strains possess a multienzyme system, which allows the hydrolases of some strains to be stable in a wide range of temperatures. Addition of the DS-hydrolases to a vial-scale anaerobic digester enhanced methane and hydrogen production from DS at pH 9.0 and 5.0, respectively. The hydrogen production was also enhanced by the use of methacrylate ester-precipitated DS as a substrate. Further improvement of culture and reaction conditions may make these hydrolases suitable for production of renewable fuels.

Introduction

The activated-sludge method is employed for the biological treatment of sewage worldwide; however, there remain several technical issues, such as production of a huge amount of sewage sludge [1]. Anaerobic digestion is generally used to reduce the amount of sludge and to produce biogas composed mainly of methane and carbon dioxide. However, the overall efficiency of sludge degradation during anaerobic digestion is still ∼30–60% [2–4], and a large quantity of biodegradation-resistant residue (digested sludge, DS) is produced as a major waste. For instance, approximately 620 million and 170 million tons (dry weight) of the sludge are produced annually in the United States and Japan, respectively, most of which is incinerated as useless material [5,6]. Because the shortage of final disposal sites became a serious problem recently, it is imperative to reduce DS disposal by making DS to good use.

While there are many articles for physico-chemical, enzymatic, and microbial solubilization of excess sludge ([7–14], for instance), only a few works focus on pretreatment of DS using chemical or enzymatic methods [15–17] because of its hard biodegradability. Therefore, employment of hydrolases from DS-degrading microbes is of considerable interest for establishing the technology to transform DS into a biofuel. Recently, we isolated DS-degrading fungal strains (*Penicillium* sp. CedarWA2, *Fusarium* sp. OreYA, *Chaetomium* sp. GalleryYA, *Cunninghamella* sp. CedarWA4, *Neosartorya* sp. OreWA, and *Umbelopsis* sp. FernWA) from a forest soil [18]. All of the strains were found to produce hydrolases (DS-hydrolases) that degrade chitin, xylan, and keratin, which are major constituents of DS [19–21]. In this study, therefore, we tested the suitability of these hydrolases for use in methane and hydrogen production from DS.

Materials and methods

Chemicals

Birchwood xylan, azure chitin, and azure keratin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Standard hydrogen and methane gases were purchased from GL Sciences (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemicals (Kyoto, Japan) and Sigma–Aldrich.

Treatment of DS

Dewatered DS (17% total solids), hydrous DS (3% total solids), and excess sludge (1% total solids) were obtained from a municipal

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sewage treatment plant in Yamaguchi City, Japan. Digestion liquor was obtained from a thermophilic anaerobic digestion facility in the same plant.

Because the growth of DS-degrading strains is inhibited by polyaluminum chloride (PAC; a widely used inorganic flocculant for dewatering of sewage sludge), aluminum was extracted and removed from DS prior to the enzyme and fermentation experiments. Thus, we prepared DS that was free of aluminum (acid-treated DS) for the experiments. To prepare the acid-treated DS, 300 g of dewatered DS and 600 mL of 1 M sulfuric acid were mixed in a 1000-mL beaker and incubated at room temperature for 1 hour. The mixture was then filtered through threefold gauze to remove the supernatant, and the filter residue was thoroughly rinsed with tap water until their pH reached 6.0. The resultant residue was then dried completely in a FSP450 dry oven (Advantec, Tokyo, Japan) at 105°C for 24 hours.

To prepare flocculant-precipitated DS, 2.0 g of a tested flocculant was dissolved in 20 L of hydrous DS to attain the final concentration of 100 mg/L, and the mixture was remained at room temperature for 30 min and then filtered through threefold gauze to remove the supernatant. Next, the filter residue was thoroughly rinsed with tap water. The resultant residue was dried completely in a FSP450 dry oven at 105°C for 24 hours.

The excess sludge was dewatered by centrifugation (7000 × *g*, 4°C, 10 min) and dried completely at 105°C for 24 hours.

Preparation of the DS-hydrolase cocktail and an enzyme assay

Mycelium (5 mm in diameter) of the DS-degrading fungal strains was grown in the YM medium (10 g/L glucose, 5 g/L pepton, 3 g/L yeast extract, 3 g/L malt extract) and then harvested by a sterile glass tube and inoculated into 50 mL of water containing 1.0 g of acid-treated DS, excess sludge, or flocculant-precipitated DS. After that, the mixture was cultured aerobically at 30°C for one week. The supernatant of the one-week culture was recovered by centrifugation at 3000 × *g* for 10 min using an H-19F benchtop centrifuge (Kokusan, Tokyo, Japan) and was used as the DS-hydrolase cocktail. Chitinase and keratinase activity in the cocktail were quantified using azure chitin and azure keratin as substrates, according to the methods of Ramírez et al. [22] and Riffel et al. [23], respectively. Xylanase activity was quantified using birch wood xylan as a substrate by means of the DNS method [24].

To analyze the expression pattern of DS-hydrolases, 7.29 g of ammonium sulfate was dissolved in 30 mL of the culture supernatant from each strain to attain the final concentration of 40% saturation. The protein precipitate (0–40% ammonium sulfate fraction) was obtained by centrifugation (20,000 × *g*, 30 min, 4°C), solubilized in 1 mL of SDS sample buffer, and boiled for 10 min. After that, 9.54 g of ammonium sulfate was dissolved in the above-mentioned 0–40% ammonium sulfate supernatant to attain the final concentration of 80% saturation, and the mixture was centrifuged as described above. The protein precipitate (40–80% ammonium sulfate fraction) was harvested, solubilized in 1 mL of SDS sample buffer, and boiled for 10 min. Twenty microliters of each protein sample was subjected to SDS-PAGE in a 12% gel on a NA-1012 electrophoresis module (Nippon Eido, Tokyo, Japan); the gel was stained using the Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA).

Fermentation experiments

The fermentation for methane production was carried out in a 15-mL glass vial (Maruemu Corporation, Tokyo, Japan). Four milliliters of the digestion liquor as a source of the methanogenic microflora, 4 mL of the DS-hydrolase cocktail, 4 mL of sterile water, and 100 mg of dried sludge powder were mixed in the vial. The pH in the mixture was adjusted to 9.0 with 1 M sodium hydroxide. The gas phase in the vial was then flushed with nitrogen and sealed with a butyl-rubber stopper and an aluminum cap. The fermentation was allowed to proceed for one week at 50°C.

The fermentation for hydrogen production was conducted using the above method for methane production, but the digestion liquor was boiled for 15 min prior to the inoculation to inactivate hydrogen-consuming bacteria and enrich hydrogen-producing ones [25,26]. The pH of the mixture was adjusted to 5.0 with 1 M hydrogen chloride.

The methane and hydrogen that were produced during the one-week fermentation were collected by a water displacement method under 50°C and atmospheric pressure, and analyzed by a GC-2014 gas chromatograph with a thermal conductivity detector (Shimadzu, Kyoto, Japan) under the following conditions: Micropacked ST column (2.0 m × 1.1 mm internal diameter [i.d.]) from Shinwa Chemical Industries (Kyoto, Japan); injection volume, 0.5 mL; the carrier gas, argon (43.5 mL/min); column temperature, 100°C.

Flocculation

The dewatering activity of following flocculants was examined: organic flocculants including methacrylate ester (CAS 26161-33-1), acrylamide PVFY (CAS 169874-00-4), polyacrylamide (CAS 69418-26-4) and polyvinylformamide (CAS 1011461-78-1), and inorganic flocculant (polyaluminum chloride, CAS 1327-41-9). Hydrous DS was mixed with each type of flocculant and was allowed to settle in a 1000-mL cylinder for 30 min so that we could assess the percentage of sludge interface, which was calculated using the following formula:

$$\text{Sludge interface}\ (\%) = \frac{\text{volume of thickening sludge (mL)}}{\text{total volume of flocculant-sludge mixture (mL)}} \times 100$$

Results and discussion

Enzymatic characteristics of the DS-hydrolases

We previously reported that the DS-degrading strains can grow at 20–30°C but cannot grow at 37°C [18]; however, the optimal temperature for the DS-hydrolase activity has not been determined yet. Thus, enzyme activities (xylanase, chitinase, and keratinase) of the DS-hydrolase cocktail produced by the strains were evaluated at 37, 42, 50, and 57°C (Fig. 1). In terms of the xylanase activity, the hydrolase of the strains CedarWA4, OreYA, and GalleryYA showed thermostability, but the xylanase activity from other strains was not thermostable and had the optimal temperature 37°C. In contrast, all strains were found to produce a thermostable chitinase, and most strains except for GalleryYA produced a thermostable keratinase. Stability of chitinase and keratinase activity from some mesophilic bacteria and fungi at ~50°C has

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