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Research Paper

Effect of oyster shell on mesophilic anaerobic digestion of fish waste

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ABSTRACT

Marine product processing industry discharges large amounts of fish waste, which could be used for anaerobic digestion because of its high organic matter content. Fish waste, however, is a protein-rich substrate and its degradation products (ammonium nitrate and ammonia) inhibit anaerobic digestion. Such inhibition could be reduced by oyster shell as it contains high levels of calcium ion. In this study, the effects of oyster shell on anaerobic digestion were investigated. Results showed that the addition of oyster shell accelerated methane production in both batch tests and semi-continuous experiments. Oyster shell also acted as a substrate for the attachment of hydrogenotrophic methanogenic *Methanoculleus* spp. In conclusion, oyster shell is effective for the anaerobic digestion of fish waste because it can play two roles: as a source of calcium ions that counteract the inhibitory effect of ammonia and as a carrier that ensures microbial retention.

Key words: Oyster shell, calcium ion, ammonium nitrate, anaerobic digestion, fish waste.

INTRODUCTION

The combined production of fish, crustaceans, molluscs and other aquatic animals has continued to increase globally, reaching 158 million tons in 2012, according to the Food and Agriculture Organization of the United Nations (FAO, 2014). In Japan, the total production of fishery products amounted to 4.86 million tons in 2012 (Ministry of Agriculture, Forestry, and Fisheries of Japan, 2013) and the Japanese fishery industry produces significant amounts of fishery wastes with fish waste amounting to 2.11 million tons in 2002 (National Research Institute of Fishery Science, 2004).

Fish waste can be a potentially valuable resource for anaerobic digestion due to its high organic matter content. However, fish waste contains high concentrations of protein (Folador et al., 2006), making anaerobic digestion difficult due to the inhibitory effects of the protein degradation products, ammonium nitrate and ammonia (Anegelidaki and Ahring, 1993; Chen et al., 2008).

It has been reported that calcium ions antagonize the inhibitory effects of ammonia (McCarty and McKinney, 1961; Tada et al., 2005). Calcium is also essential for the growth of methanogens (Murray and Zinder, 1985) and important in the formation of microbial aggregates (Thiele et al., 1990). Oyster shell is a kind of fishery waste containing large amounts of calcium ions in the form of $CaCO_3$ and has an estimated production volume of 0.2 million tons in Japan (Norinchukin Research Institute Co. Ltd., 2004). Assuming calcium ions contained in oyster shell are derived from $CaCO_3$, then 93.5% of oyster shell consists of $CaCO_3$ according to a study published by Kwon et al. (2004). Yoon et al. (2003) also reported that approximately 96% of oyster shell is composed of $CaCO_3$.

In a reactor, the retention of microbes is an integral factor in accelerating anaerobic digestion. Retention is largely influenced by the physical characteristics of the supporting material including surface area, porosity, surface roughness, pore size and orientation of the packing material (Elmitwalli et al., 2000). Researchers have investigated various carriers such as bentonite (Angelidaki et al., 1990), polyvinyl alcohol (Hanaki et al., 1994), fibrous sponge (Sasaki et al., 2010) and cedar charcoal (Watanabe et al., 2013). Using biomass as a carrier could facilitate resource circulation and control the cost of anaerobic digestion.

Table 1: Characteristics of raw materials.

Raw materials	Total COD (mg/l)	TS (%)	VS (%)	C/N ratio
Seed sludge	5122	2.24	33.20	-
Cod waste	244054	29.54	20.65	3.36
Activated sludge	9355	0.38	16.04	6.20

In this study, crushed oyster shell was added to fish waste in an anaerobic digestion reactor. The effects on methane production were assessed in both batch and semi-continuous experiments. Furthermore, the effect of oyster shell as a carrier was analyzed using denaturing gradient gel electrophoresis (DGGE).

MATERIALS AND METHODS

Seed sludge was obtained from a mesophilic anaerobic digester treating pig slurry and cultured with fish waste before using it as the seed. Cod waste was obtained from a fishery factory (Watarai Co., Ltd., Miyagi, Japan), crushed with a mixer and used as an experimental material for anaerobic digestion. Activated sludge was collected from the wastewater treatment plant in the same factory. The characteristics are shown in Table 1. Oyster shell was obtained from Chiba Iron Works Co., Ltd. (Miyagi, Japan) and crushed to ~5 mm^2.

$CaCO_3$ reagent (99.5%) was purchased from Wako Chemical Industries Ltd. (Tokyo, Japan). Type e-4 carbon felt (3 cm × 3 cm × 0.7 cm × 10 pieces) (Tsukuba Materials Information Laboratory, Ltd., Ibaraki, Japan) was also used as a carrier.

Batch test of anaerobic digestion

Batch tests were performed to examine the effect of oyster shell on anaerobic digestion of fish waste as the calcium additive. The batch operation of anaerobic digestion was conducted at 35°C in a 500 ml reactor. The reactor was filled with 300 ml of inoculated seed sludge, 100 ml of activated sludge, and 20 g of crushed cod waste. Also, 25 g of $CaCO_3$ reagent or 25 g of crushed oyster shell was then added as the calcium additive and carbon felt (3 cm × 3 cm × 0.7 cm × 10 pieces) was added as the carrier. The test with no calcium additive was conducted as a control.

Semi-continuous anaerobic digestion experiment

The semi-continuous operation of anaerobic digestion was conducted at 35°C in a 500 ml reactor. A volume of 300 ml of inoculated seed sludge was added to the reactor. 10 or 20% fish slurry, mixed with activated sludge was added to the reactor as the supply material once a day. Crushed oyster shell (25 g) was added as the calcium additive. Carbon felt (3 cm × 3 cm × 0.7 cm × 10 pieces) was added to each reactor as a carrier and calcium additive-free negative controls were performed. The hydraulic retention time (HRT) was set up as: 70 days until day 7; 40 days from day 7 to 35; and 20 days from day 35 to 54.

Analytical methods

Total solids (TS) and volatile solids (VS) were analyzed according to the Wastewater Examination Method (Japan Sewage Works Association, 1997). C/N ratio was determined using a CNS elemental analyzer (Variomax CNS, Elementar, Hanau, Germany). Gas samples were collected in Polyvinylidene difluoride gas bags and CH_4 and CO_2 contents were determined using a gas chromatograph (GC-8A; Shimadzu Corporations, Kyoto, Japan) with a thermal conductivity detector equipped with a Porapak-Q column (Shinwa Chemical Industries Ltd., Kyoto, Japan) with an inject/detect temperature of 100°C. Nitrogen was used as the carrier gas. Total chemical oxygen demand (COD) was measured using a colorimetric method (Jirka and Carter, 1975). Volatile fatty acids (VFAs) were determined using high performance liquid chromatography (HPLC) (Jasco Corporation, Tokyo, Japan), equipped with an ion-exchange column (Shodex RSpak KC-811; Showa Denko K. K., Tokyo, Japan), and UV detector (870-UV; Jasco Corporation). The experimental conditions were as follows: 60°C; 3 mM $HClO_4$ eluent; flow rate of 0.8 ml/min. Ammonium nitrate and calcium ions were analyzed by ICS-1000 ion chromatography (Dionex, Sunnyvale, CA, USA), equipped with Dionex IonPac CS16 column (Dionex) with a column temperature of 40°C. The experimental conditions were as follows: 30 mM methanesulfonic acid eluent at a flow rate of 1 ml/min. The samples for the analyses of VFAs, ammonium nitrate and calcium ions were filtered through cellulose filters with a pore size of 0.45 μm.

Polymerase chain reaction–denaturing gradient gel electrophoresis and sequencing

Samples of the attached support materials were collected at the end of the continuous anaerobic digestion operation. DNA was extracted using a Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Archaeal 16S rRNA gene

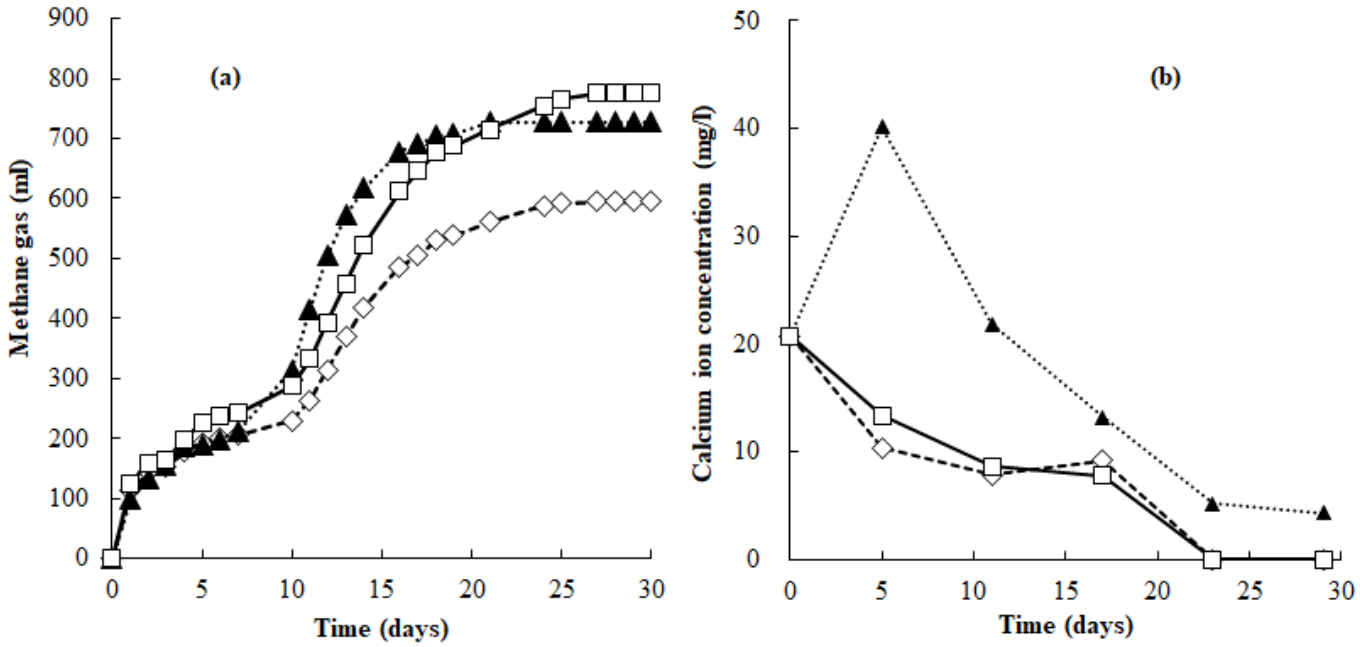

Figure 1: Effects of oyster shell on anaerobic digestion in batch test. (a) methane volume; (b) calcium ion. ◇; control, ▲; oyster shell, □; $CaCO_3$.

sequences were amplified by PCR with the primer pair 1106F (5'-TTWAGTCAGGCAACGAGC-3'), containing a 40-bp GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3'), and 1378R (5'TGTGCAAGGAGCAGGGAC-3') (Watanabe et al., 2006). The archaeal PCR reaction consisted of 30 cycles of 30 s at 94°C, 30 s at 52°C, and 30 min at 72°C. The PCR products were used for denaturing gradient gel electrophoresis (DGGE) analysis. DGGE of the PCR amplified 16S rRNA genes was performed using a D-code multiple system (Bio-Rad, Hercules, CA, USA). Polyacrylamide gels were prepared as follows: 8% polyacrylamide–bisacrylamide mixture (37.5:1.0) in 0.5× TAE buffer with a gradient of 30 to 70%. Denaturants were prepared according to the manufacturer's guidelines (Muyzer et al., 1993). The 30% denaturant contained 2.1 M urea and 12% formamide. Gels were run for 12 h at 100 V in 1× TAE buffer at a constant temperature of 60°C. After electrophoresis, the gels were stained for 15 min in 1× TAE buffer containing 100 ng/ml GelStar™ Nucleic Acid Gel Stain (Lonza Rockland, Rockland, ME, USA), and the DNA bands excised and transferred into 1.5 ml tubes containing 70 µl TE buffer. Of each aliquot, 1 µl was used as template for sequencing the DNA bands using the primer sets without the GC clamp. The PCR product was purified by polyethylene glycol (13% PEG-6000, 1.6 M NaCl) precipitation. The purified DNA was sequenced using a Big Dye Terminator v. 1.1 Cycle Sequencing Kit, according to manufacturer's instructions (Applied Biosystems, Foster City, CA, USA) and an ABI Prism310 automated sequence analyzer (Applied Biosystems, Foster City, CA, USA). The acquired sequences were compared with 16S rRNA sequences in the BLAST database using nucleotide-nucleotide alignment (http://blast.ddbj.nig.ac.jp/top-j.html).

RESULTS

Batch test

Figure 1a shows the methane gas production by anaerobic digestion with and without calcium additives. Methane concentrations were similar in all samples, including the negative control at approximately 65%. Methane production until day 7 is considered derived from seed sludge. As shown in Figure 1a, methane production was accelerated by the addition of a calcium source. Methane volumes peaked at 595, 728 and 777 ml in the negative control, oyster shell, $CaCO_3$ samples, respectively. Time courses of calcium ion concentrations with and without calcium additives are shown in Figure 1b. Calcium ion concentrations were decreased with time with control and $CaCO_3$. On the other hand, the increase of calcium ion concentration was observed with oyster shell on day 5, when calcium ion concentration was 40 mg/L.

Figure 2 shows time courses of acetic acid and propionic acid in the batch test. Acetic acid and propionic acid were increased rapidly until day 5. Maximum Acetic acid and propionic acid were about 3000 to 3500 mg/L and 550 to 600 mg/L in all the experiments. While acetic acid and propionic acid concentrations were decreased by adding oyster shell from day 5 and 11, respectively, acetic acid and propionic acid concentrations in the other operations were

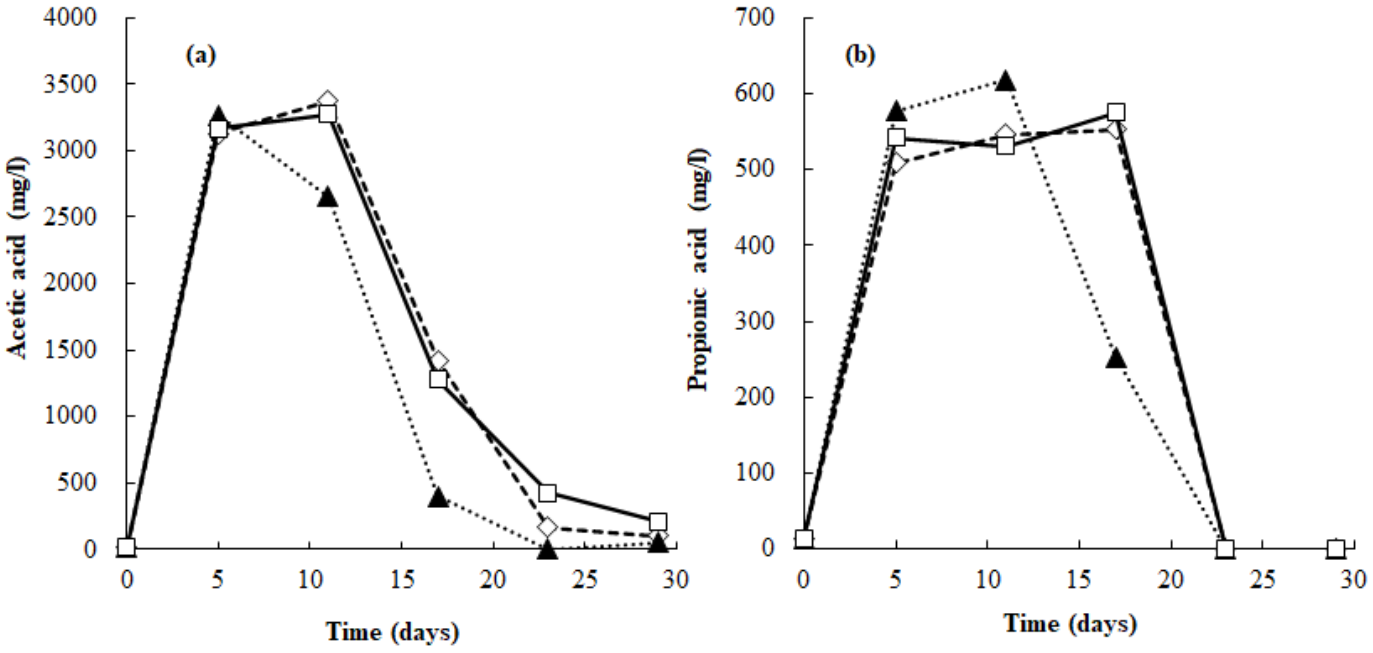

Figure 2: Time courses of VFAs in batch test. (a) acetic acid; (b) propionic acid. ◇; control, ▲; oyster shell, □; $CaCO_3$.

Table 2: VFAs under various conditions in batch test.

	Time (days)					
VFAs	**0**	**5**	**11**	**17**	**23**	**29**
Control						
Acetic acid	19	3129	3372	1424	164	101
Propionic acid	12	509	547	553	N.A.	N.A.
Isobutyric acid	N.A.	215	221	N.A.	N.A.	N.A.
Butyric acid	N.A.	43	24	N.A.	N.A.	N.A.
Isovaleric acid	N.A.	527	567	359	N.A.	N.A.
Oyster shell						
Acetic acid	19	3269	2655	400	N.A.	20
Propionic acid	12	578	618	253	N.A.	N.A.
Isobutyric acid	N.A.	245	222	N.A.	N.A.	N.A.
Butyric acid	N.A.	195	N.A.	N.A.	N.A.	N.A.
Isovaleric acid	N.A.	415	535	386	N.A.	N.A.
$CaCO_3$						
Acetic acid	19	3164	3273	1277	424	208
Propionic acid	12	542	531	575	N.A.	N.A.
Isobutyric acid	N.A.	243	225	N.A.	N.A.	N.A.
Butyric acid	N.A.	122	N.A.	N.A.	N.A.	N.A.
Isovaleric acid	N.A.	585	522	371	N.A.	N.A.

delayed to day 11 and 17, respectively. As shown in Table 2, isobutyric acid, butyric acid and isovaleric acid were also detected during the process of the anaerobic digestion.

Semi-continuous experiment

Figure 3a represents the methane yields of samples under

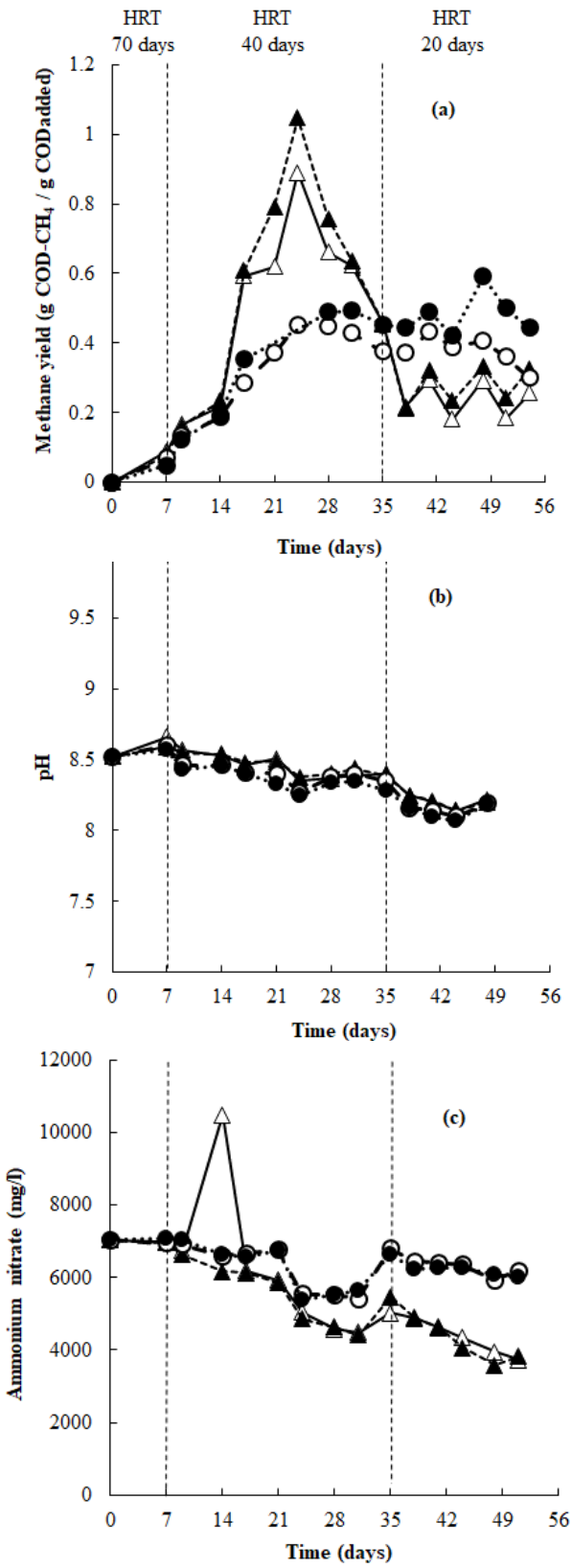

Figure 3: Effects of oyster shell on anaerobic digestion in semi-continuous experiment. (a) methane yield; (b) pH; (c) ammonium nitrate. △; 10% fish, ▲; 10% fish + oyster shell, ○; 20% fish, ●; 20% fish + oyster shell.

various conditions during semi-continuous anaerobic digestion. During HRT 40 days, the methane yield of the reactor containing 10% fish with oyster shell reached 1 g COD-CH_4/g COD added, which was higher than the oyster shell-free control. During HRT 20 days, the methane yield of the reactor containing 20% fish supplemented with oyster shell was higher than the oyster shell-free control from day 47 to 52. Therefore, methane production was enhanced by the addition of oyster shell during the semi-continuous anaerobic digestion of fish waste. In Figure 3b, pH changes were shown and pH was over 8 in each condition. Figure 3c indicates ammonium nitrate behavior in each condition. Ammonium nitrate concentrations were about 3800 and 6000 mg/L with fish loading 10 and 20% regardless of the existence of oyster shell on day 51, respectively, and not significantly affected by the addition of oyster shell in any of the samples. Figure 4 shows VFA levels during semi-continuous anaerobic digestion. Contrary to the batch test, isobutyric acid and butyric acid were undetected in the semi-continuous experiments. During HRT 20 days, the acetic acid concentration in the reactor containing 20% fish with oyster shell decreased from 2500 mg/L on day 47 to 2000 mg/L on day 49. During the same period, acetic acid concentrations in the oyster shell-free control experiment increased from 2700 to 2800 mg/L.

Analysis of microbial communities on the carriers

Figure 5 shows the banding patterns on the acrylamide gel from the DGGE analysis of archaea present on the oyster shell and carbon felt carrier obtained from semi-continuous experiments. DNA extracted from the carbon felt was separated into three bands (A1, A2 and A3) regardless of the existence of oyster shell with 10% fish as shown in (a) and (b). However, only one band (A1) was obtained with 20% fish. DNA extracted from oyster shell was separated into two bands (A1 and A2) when sampled from the reactor containing 10% fish (e). On the other hand, only one band (A1) was detected.

Table 3 shows the results of the sequence analyses of the three bands excised from the polyacrylamide gel (Figure 5a). All of the bands were related to *Methanoculleus* spp., which are hydrogenotrophic methanogens. These microorganisms have previously been detected in reactors used for anaerobic digestion of various materials (Zellner et al., 1998; Feng et al., 2010). In this study, *Methanoculleus* spp. were detected not only on the carbon felt carrier, but more importantly, on the oyster shell.

DISCUSSION

The effect of an added calcium source depends on its solubility in water. Previous researches have shown the optimum calcium ion concentration using $CaCl_2$ (Ahn et al., 2006; Dang et al., 2014). However, $CaCl_2$ is relatively rare in

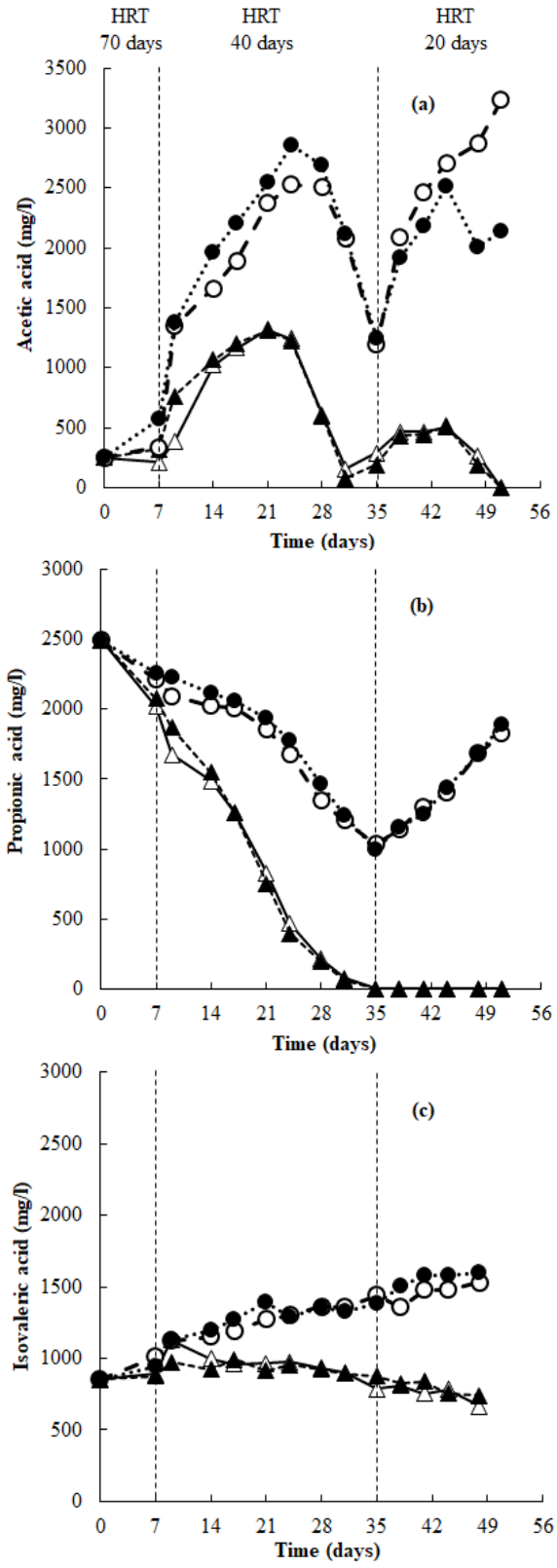

Figure 4: Time courses of VFAs in semi-continuous experiment. (a) acetic acid; (b) propionic acid; (c) isovaleric acid. △; 10% fish, ▲; 10% fish + oyster shell, ○; 20% fish, ●; 20% fish + oyster shell.

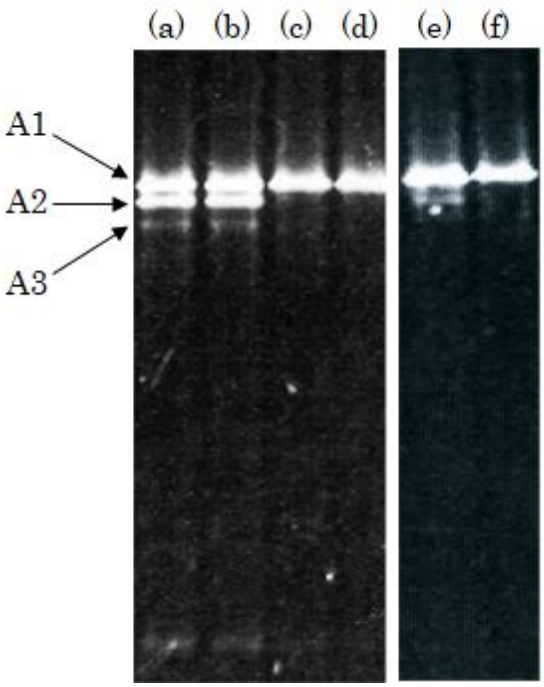

Figure 5: DGGE profiles in semi-continuous experiment. (a) carbon carrier (10% fish); (b) carbon carrier (10% fish + oyster shell); (c) carbon carrier (20% fish); (d) carbon carrier (20% fish + oyster shell); (e) oyster shell (10% fish + oyster shell); (f) oyster shell (20% fish + oyster shell).

nature and needs to be manufactured industrially. Alternatively, $CaCO_3$ exists naturally in the form of oyster shell and seashell, and can easily be utilized. Furthermore, $CaCO_3$ has low water solubility compared to $CaCl_2$, which can minimize the inhibitory effect that an excessive supply of calcium ions gives on anaerobic digestion. Thus, the use of oyster shell as a calcium ion source has a potential for the improved anaerobic digestion.

The methane production rate increased at a faster pace by adding oyster shell than $CaCO_3$ (Figure 1a). This result is supported by the steep reduction in VFAs in the sample with oyster shell (Figure 2). Crushed oyster shell has larger exposed surface area and increases its solubility compared to $CaCO_3$ reagent. Elevated solubility, in turn, would cause an accelerated rise in calcium concentrations (Figure 1b), which could improve microbial activities. The drop in calcium ion concentrations can be attributed to various factors: growth of archaea, recruitment of calcium ions for microbial aggregates and co-precipitation with carbonate and phosphate.

Methane production was accelerated by adding oyster shell especially, in 10% fish as shown in Figure 3a. On the other hand, Figure 3c indicates that the ammonium nitrate concentrations were not significantly affected by the addition of oyster shell. Thus, it was found that there was no correlation between methane production and ammonia

Table 3: Species attached to carbon carrier and oyster shell.

Band No.	Related species	Similarity (%)	Kinds of archaea
A1	*Methanoculleus* sp. Dm2 (AJ550158.1)	99	Hydrogenotrophic methanogen
A2	*Methanoculleus bourgensis* strain NF-1 (DQ150254.1)	100	Hydrogenotrophic methanogen
A3	*Methanoculleus olentangyi* (AF095270.1)	100	Hydrogenotrophic methanogen

nitrite concentration, suggesting that oyster shell enhanced microbial activities by an alternative mechanism except the ammonia nitrite reduction.

In Figure 4a, acetic acid concentration in the reactor containing 20% fish with oyster shell decreased from 2500 mg/L on day 47 to 2000 mg/L on day 49 during HRT 20 days. During the same period, acetic acid concentrations in the oyster shell-free control experiment increased from 2700 to 2800 mg/L. These results indicate that acetic acid was broken down into methane in the reactor containing 20% fish supplemented with oyster shell at an HRT of 20 days.

In the semi-continuous experiment, hydrogenotrophic methanogens were detected on the oyster shell. Oyster shell could act as a carrier for the retention of microbes, especially hydrogenotrophic methanogens. Hydrogenotrophic methanogens are important in reducing hydrogen partial pressure to between 0.1 to 10 Pa which is the optimal range for the decomposition of propionic acid into acetic acid (Harper and Pohland, 1986). In the batch test, propionic acid levels rapidly decreased by the addition of oyster shell. This could be because oyster shell acts as a microbial carrier, thereby, initiating and/or increasing the activity of hydrogenotrophic methanogens. If oyster shell, which is one of fishery wastes, was used as a carrier, the cost needed for the anaerobic digestion operation could be significantly reduced.

Conclusion

This study was performed to investigate the effects of crushed oyster shell on methane production from fish waste during anaerobic digestion. The results indicated that oyster shell enhanced anaerobic digestion. It is suggested to be because the calcium ions from the oyster shell decrease the anaerobic digestion inhibition. In addition, oyster shell acted as a microbial carrier for hydrogenotrophic methanogenic *Methanoculleus* spp., which is integral to the anaerobic digestion process. In view of these findings, oyster shell could be compatible for application in the anaerobic digestion of fish waste.

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