Environmental Technology & Innovation 27 (2022) 102403

Contents lists available at ScienceDirect

Environmental Technology & Innovation

journal homepage: www.elsevier.com/locate/eti

# Effect of different concentrations of sodium selenite on anaerobic digestion of waste sewage sludge

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ARTICLE INFO

*Article history:*
Received 3 June 2021
Received in revised form 11 January 2022
Accepted 6 February 2022
Available online 14 February 2022

*Keywords:*
Waste sewage sludge
Sodium selenite
Methanogenesis
Anaerobic digestion
Microbial community

ABSTRACT

The effect of sodium selenite on anaerobic digestion of waste sewage sludge (WSS) was studied. Interestingly, methane production was enhanced at 2 mg/L and inhibited at 100 mg/L of sodium selenite during the anaerobic digestion of WSS. Hydrolytic activity increased in correlation with increased concentrations of sodium selenite (0 to 100 mg/L). At the acidogenesis stage, acetic acid accumulated at the concentrations more than 25 mg/L of sodium selenite whereas it was consumed for 10 days at the concentration of 2 mg/L of sodium selenite. Also, the concentration of propionic acid decreased and that of butyric acid increased at 100 mg/L of sodium selenite. Although methanogenic activities were totally inhibited at 100 mg/L of sodium selenite, a high hydrogenotrophic methanogenic activity was observed at 2 mg/L of sodium selenite. The abundance of hydrogenotrophic methanogens (*Methanospirillum* and *Methanocorpusculum*) increased in the WSS at 2 mg/L of sodium selenite. Moreover, the results of pure-culture experiments using *Methanosarcina acetivorans* C2A show that the growth promotion or inhibition was observed at 2 mg/L or at 100 mg/L of sodium selenite. In conclusion, sodium selenite has the double-sided effect during the anaerobic digestion of WSS as shown by the results that methane production was promoted at a low concentration and inhibited at a high concentration.



## 1. Introduction

Waste sewage sludge (WSS) is one of the industrial wastes, which is produced from the biological wastewater treatment process (Appels et al., 2008). Nowadays, a large amount of WSS is produced daily; therefore, there is still a strong need to improve anaerobic digestion using WSS as a raw material with carbon neutral properties (Gherghel et al., 2019) although there are several issues to be figured out; for example, it is time-consuming and unstable (Gaby et al., 2017; Ye et al., 2018). Thus, the use of WSS resource contributes to the reduction of greenhouse gas along with another approach to directly convert carbon dioxide into beneficial compounds (Morimoto et al., 2018; Takatsuji et al., 2019).

One of the approaches to improve anaerobic digestion is to change the activity of microbial communities in WSS because various microbial groups are involved in the reactions of hydrolysis, acidogenesis, and methanogenesis during the anaerobic digestion (Venkiteshwaran et al., 2015). To date, there are several reports that methane production was

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https://doi.org/10.1016/j.eti.2022.102403

altered in the presence of an antibiotic (Mustapha et al., 2016), a quorum quenching compound (Nguyen et al., 2019), or an iron material (Mustapha et al., 2020). These results indicate that by changing the microbiota activity, WSS should be efficiently utilized for not only producing bioenergy but also reducing the cost to treat it. To date, trace elements have a certain potential to change the composition and function of microbiota and microbiota diversity (Ringelberg et al., 2009; Kasaikina et al., 2011; Zhu et al., 2018). For example, the trace elements improve C1 gas bioconversion by an enriched anaerobic sludge (Chakraborty et al., 2020). In the experiment using food industrial waste by Feng et al. methane production was altered in the presence of trace elements (Feng et al., 2010). However, how the trace elements act in the microbial community or which of the elements has an effect has not been fully investigated to date although there is a report that trace elements are the most important nutrients for the enzymes related to the anaerobic digestion (Molaey et al., 2018). Among the elements, selenite has been reported to have not only an antibacterial ability (Alam et al., 2016) but also an antioxidant property (Grman et al., 2019); therefore, selenite can affect the activity of complex microorganisms. In addition, selenium has actually been detected as one of the contaminants in WSS (Fijalkowski et al., 2017). Therefore, the target of our study was to clarify the effect of selenite on the anaerobic digestion of WSS. To date, there are several reports investigating the effect of sodium selenite on anaerobic digestion of pig manure (Liang et al., 2020), ruminal fermentation (Czauderna et al., 2012), and anaerobic digestion of rice straw (Cai et al., 2018); however, to date, no studies have investigated the effect of sodium selenite on the anaerobic digestion of WSS in detail. In addition, although selenium has been reported to inhibit hydrogenotrophic and acetoclastic methanogenesis (Lenz et al., 2008), the detail mechanism of selenite toward the methanogens which are key microorganisms to explain enhanced or reduced methane production is still unclear. In this study, the effect of sodium selenite during the anaerobic digestion was investigated by using WSS. Then, microbial community composition was compared in the WSS sample with or without sodium selenite by high-throughput 16S rRNA gene sequencing.

## 2. Material and methods

### 2.1. Preparation of waste sewage sludge

Waste sewage sludge (WSS) was collected from the secondary treatment stage of Hiagari Wastewater Treatment Plant in Kitakyushu City, Japan. Prior to the experiments, the washing steps were performed for the WSS by the centrifuged at 8000 × g for 10 min at 4 °C to remove the supernatant and the residuals pellet was resuspended with distilled water by vigorous shaking. Three times washing were done before adjusting the final sewage sludge concentration of 10% (wet sludge [w/w]).

### 2.2. Sodium selenite

Sodium selenite used in this study was purchased from Wako Pure Chemical Industries, Ltd. (Japan). The reagent was dissolved into distilled water to make 10 mg/mL stock solution and directly used for methane fermentation and other analyses. The final concentrations of sodium selenite used was adjusted to 0.1 mg/L, 1 mg/L, 2 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L.

### 2.3. Measurement of methane, $CO_2$, and hydrogen

Anaerobic digestion was prepared in a 66-mL vial to fundamentally perceive the role of sodium selenite for methane production and microbial activity in WSS. The total volume of 10% (w/v) WSS (30 mL) was prepared to be different concentrations of sodium selenite: 0 (as control), 0.1 mg/L, 1 mg/L, 2 mg/L, 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L. Each vial was tightly sealed using a butyl rubber stopper and aluminum cap and then sparged with nitrogen gas for 2 min to create an anaerobic condition. All the vials were then incubated at 37 °C and 120 rpm for 15 days. Each experiment was conducted at least in triplicate. The amount of methane, $CO_2$, and hydrogen was measured by a GC-3200 gas chromatograph with a thermal conductive detector (GL Sciences, Japan) by injecting 50 μL of headspace gas from the vials during the anaerobic fermentation for 15 days. A molecular sieve 13 × 60/80 mesh column and a Steel Use Stainless (SUS) 2 m × 3 mm inner diameter (GL Science, Japan) was used as a column to detect methane and hydrogen. For the detection of carbon dioxide, a WG-100SUS 1.8 m × ⌀ 1/4" O.D column (GL Science, Japan) was set. Helium gas was used as a carrier gas and set at 40 mL/min for methane and $CO_2$ or 20 mL/min for hydrogen. The gas chromatography conditions were set as follows: current, 100 mA; oven, injector, and detector temperatures, 40 °C, 50 °C, and 65 °C, respectively (Mustapha et al., 2020).

### 2.4. Analytical methods

Each sample during the fermentation was used for the following analyses: pH, protein concentration, protease activity, and organic acids. Initially, the WSS samples incubated were centrifuged at 12,300 × g for 7 min to collect the supernatant and was further filtered with a 0.2-μm pore membrane syringe filter. The filtrate was utilized to measure organic acids by high performance liquid chromatography (HPLC) (Shimadzu LC-10 AD) as described previously (Mohd Yusoff et al., 2012)

and pH by a compact pH meter (AS ONE, AS-211, Japan). In addition, the soluble protein concentration was measured by the Lowry method using BSA (bovine serum albumin) as a standard protein (Lowry et al., 1951). Protease activity was analyzed as described previously (Maeda et al., 2011). One unit of protease activity was defined as the quantity of tyrosine (μmol) produced from casein by 1 mg of enzyme per minute. Sludge reduction ratio was measured as described previously by (Maeda et al., 2011). WSS samples (before and after the fermentation) were collected (10 g wet weight) and centrifuged at 18,000×*g* for 10 min. The pellets were transferred to porcelain dishes and dried in an oven (D-300, Iuchi, Japan) at 105 °C for 2 days to determine the dry weight. Each experiment was conducted at least in triplicate.

### 2.5. Analyses of methanogenic activity

Initial WSS was first centrifuged at 8000 × g for 10 min at 4 °C and the residuals pellet was resuspended with distilled water by shaking thoroughly. The sludge pellets were washed three times and resuspended with distilled water to be the final concentration to 5% (w/w). The 5% (w/v) WSS (30 mL) was mixed with four antibiotics (benzyl penicillin, 0.5 mg/mL; streptomycin sulfate, 0.5 mg/mL; vancomycin-HCl, 0.2 mg/mL; and ampicillin, 0.2 mg/mL) to inactivate bacterial activity (Battumur et al., 2017). For the acetoclastic methanogenesis, 20 mM sodium acetate was further mixed with the above vials with WSS and 4 antibiotics. For the hydrogenotrophic methanogenesis, $H_2/CO_2$ (80:20) gas was replaced in place of nitrogen gas in the headspace of the vials. All the vials were tightly sealed with butyl rubber stoppers and aluminum caps and sparged with nitrogen gas for 2 min to make an anaerobic atmosphere (except vials for the assay of hydrogenotrophic methanogenesis). Finally, all the vials were incubated at 37 °C and shaken at 120 rpm for 10 days and methane production was evaluated as mentioned above.

### 2.6. Total RNA extraction, cDNA synthesis and quantitative RT-PCR

RNA extraction and cDNA synthesis from the pellets of the control WSS and the WSS with sodium selenite were performed as previously described (Mustapha et al., 2017). The total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA), and the total RNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (SCRUM Inc., Japan). The cDNA was synthesized using the PrimeScript RT Reagent Kits (TAKARA Bio Inc., Shiga, Japan). The cDNA was used as a template to determine bacterial and archaeal communities using Illumina MiSeq. The qRT-PCR was performed using a TaqMan system with specific primers and probes for quantify total bacteria and archaea by StepOne Real Time PCR System (Applied Biosystems). In our previous paper, we defined the detailed procedures and the standard curve for universal bacteria and archaea (Mohd Yasin et al., 2015). The calculation of copy numbers based on the amount of DNA was performed as described previously (Lenz et al., 2008).

### 2.7. High-throughput 16S rRNA sequencing

Two sets of primers were used for the identification of bacterial and archaeal communities using the high-throughput sequencing by MiSeq. The primers including overhang adapter for Nextera XT Index kit (Illumina Inc., CA, USA) 341F (5'- TCGTCGGCAGCGTCAGATGTGTAT AAGAGACAGCCTACGGGNGGCWGCAG-3') and 785R (5'– GTCTCGTGGGCTCG-GAGAT GTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013) targeting the V3 and V4 regions of each 16S rRNA gene were used for bacteria whereas the primers including overhang adapter A2Fa (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGAC AGTTCCGGTTGATCCYGCCGGA-3') (Baker et al., 2003) and 519R (5'- GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGWATTACCGCGGCKGCT-3') (Lane et al., 1985) targeting the V1–V3 regions were used for archaeal community. The amplification and library preparation were conducted according to the 16S metagenomics sequencing library preparation for Illumina MiSeq system as described in details previously (Mustapha et al., 2018). For archaea, the first round PCR amplification was slightly modified. The PCR mixture (25 μL) consisted of PCR grade water (10.4 μL), 10×Taq buffer (2.5 μL) and Taq polymerase (0.35 μL) (BioLabs), 10 μM of each primer (1.25 μL), 2 mM each dNTPs mix (6.25 μL), 25 mM of $MgSO_4$ (1 μL) (Toyobo, Osaka, Japan), and the cDNA template (2 μL). PCR was carried out under the following conditions: initial denaturation at 94 °C for 3 min followed by 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min 50 s of 30 cycles, and a final extension at 72 °C for 15 min. Then, the rest of library preparation steps were according to the 16S metagenomics sequencing library preparation for Illumina MiSeq. The high-throughput data analysis was done using LotuS pipeline (Hildebrand et al., 2014) and classified as previously described in details (Mustapha et al., 2018) into different taxonomic levels. All the raw sequence data were deposited into NCBI Sequence Reads Archive (SRA) database under the accession number of SRP072534.

### 2.8. Growth of Methanosarcina acetivorans C2A

*Methanosarcina acetivorans* C2A (ATCC 35395) was used to determine the effect of sodium selenite on its growth and methane production. The strain was cultured in HSYE-methanol medium as previously reported (McAnulty et al., 2017). The cell culture (0.6 mL) was inoculated to the same medium (29.4 mL) with or without 2 mg/L or 100 mg/L sodium selenite and incubated at 37 °C for 6 days. Cell turbidity was measured with time at 600 nm by using a UV–Vis spectrophotometer (JASCO, V-530) and methane production was analyzed as mentioned above.

### 2.9. Statistical analysis

WSS samples with different concentrations of sodium selenite were compared with control WSS using means from at least triplicate data ($n = 3$). Comparison was performed using means and standard deviations by the Student's t test (GraphPad software) and ANOVA test at a significance level of $p < 0.05$.

## 3. Results and discussion

### 3.1. Methane and hydrogen production in the presence of sodium selenite

Eight concentrations of sodium selenite (0.1, 1, 2, 5, 10, 25, 50, and 100 mg/L) during the anaerobic digestion of WSS for 15 days were compared to see the effect of sodium selenite on methane production from WSS. As a result, methane production using WSS was significantly inhibited according to the increased concentrations of sodium selenite (Fig. 1-A). At 100 mg/L of sodium selenite, a complete inhibition of methane production was observed. Other concentrations of sodium selenite showed almost the same amount of methane production as the control (data not shown). . Instead, the amount of hydrogen gas detected in the presence of 100 mg/L sodium selenite reached to 164 ± 4 μmol/g-VS at 7 days whereas WSS with 2 mg/L sodium selenite and control WSS could not produce hydrogen after 5 days (Fig. 1-B). On the other hand, methane production at 2 mg/L of sodium selenite was 1.7- times higher than that at the control WSS at day 10. At the same time, the amounts of $CO_2$ produced were recorded as 67 ±1, 72 ± 2, 60± 0.5, 64 ± 1, and 50 ± 3 μmol/gVS in the WSS with 0 mg/L, 2 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L at day 10, respectively. The results corroborate that there is no significant effect on $CO_2$ production. Thus, it was found that even in the anaerobic digestion of WSS, methane production is improved or repressed at a low or high concentration of sodium selenite. There results indicate that the desired bioenergy such as methane and hydrogen can be produced by adjusting the concentration of sodium selenite. Therefore, the study was motivated to understand the mechanism how sodium selenite affects the anaerobic digestion of WSS. However, the effect of this reagent in a long-time operation test still remains as another important checking point to clarify if the improvement of methane production at 2 mg/L of sodium selenite is due to the increased reaction speed or the expanded available substrates (able to increase the yield of methane). In addition, it is necessary to confirm how long methane production is repressed in the WSS with 100 mg/L of sodium selenite.

### 3.2. Impact of sodium selenite at hydrolysis and acidogenesis stages

Anaerobic digestion of WSS is a process consisting of reactions of hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Sikora et al., 2017). Therefore, the effect of sodium selenite on the hydrolytic reaction was first investigated by monitoring the protease activity that mainly acts in the hydrolysis (Maeda et al., 2009). In addition, the concentration of soluble proteins was also determined as a product of hydrolytic reactions in the presence of protease enzymes. Fig. 2-A shows the results of protease activity at 2, 6, and 10 days in the different concentrations of sodium selenite. Protease activity at the 2nd day increased in correlation with increased concentrations of sodium selenite. Then, the protease activity at the concentration of 100 mg/L sodium selenite still maintained high at 6 and 10 days. The results of protease activities were in well agreement with those of soluble protein concentrations which increased according to the increased concentrations of sodium selenite (Fig. 2-B). These results demonstrate that sodium selenite promotes the reaction of hydrolysis process. Furthermore, sludge reduction was evaluated with or without sodium selenite to obtain the corroborating evidence for the hydrolysis process. As a result, the highest sludge reduction ratio was observed in the WSS sample in the presence of 100 mg/L sodium selenite: the sludge degradation ratio was approximately 38 ± 3% at 100 mg/L of sodium selenite whereas that of the control WSS was 27 ± 3%. On the other hand, sludge reduction at 2 mg/L sodium selenite was comparable to the control. Thus, sodium selenite has a certain ability to promote the hydrolysis stage, which is known to be the rate-limiting step during the anaerobic digestion (Ma et al., 2013). An interesting point of the result is to show a relatively-high high protease activity despite the inhibition of methane production in the WSS with 100 mg/L of sodium selenite. The discrepancy between protease activity and methane-producing activity is similar to our previous result of anaerobic digestion of WSS in the presence of chloramphenicol (Mustapha et al., 2016). It is suggested that the inhibition of methane-producing activity at 100 mg/L of sodium selenite can be due to the other reactions than hydrolysis one.

Next, organic acids which are precursor compounds for methane production (Jiang et al., 2007) were analyzed to evaluate the acidogenesis and acetogenesis steps during the anaerobic digestion of WSS with or without sodium selenite. Organic acids produced were determined after the 2, 6, and 10-day incubation (Fig. 3). As a result, acetic acid was detected as a main product. In addition, the concentration of acetic acid ($p < 0.01$) was lowered at 2 mg/L sodium selenite whereas that of acetic acid ($p < 0.01$) increased at more than 25 mg/L of sodium selenite (Fig. 3-A). On the other hand, the concentrations of propionic acid and isobutyric acid decreased slightly as the concentration of sodium selenite increased (Fig. 3-B and Fig. 3-C). Also, the concentration of butyric acid increased in a dose-dependent manner of sodium selenite (Fig. 3-D). Taken together, the addition of sodium selenite to WSS did not negatively influence the bacterial activity at the acidogenesis/acetogenesis stage although different patterns of organic acids were observed for each concentrations of sodium selenite. The profile of acetic acid seems to be consistent with the results of methane production in the presence

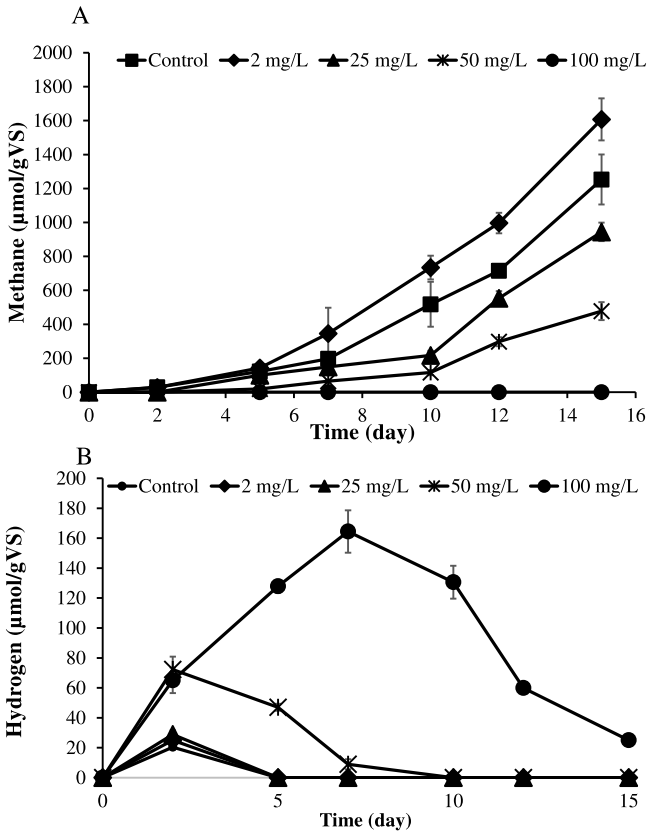


**Fig. 1.** Effect of sodium selenite on the production of methane (A) and hydrogen (B) during the anaerobic digestion of waste sewage sludge (WSS). Two gases (methane and hydrogen) produced from the process of anaerobic digestion of WSS at different concentrations of sodium selenite: 0 mg/L as the control (dark squares), 2 mg/L (dark diamonds), 25 mg/L (dark triangles), 50 mg/L (dark star), or 100 mg/L (dark circles) were measured for 15 days. Error bars indicate standard errors ($n = 3$).

of sodium selenite. In detail, acetic acid consumed at 10 days under the condition of enhanced methane production at 2 mg/L of sodium selenite. On the other hand, the accumulation of acetic acid was observed at the concentrations of sodium selenite in which methane production was repressed. Regarding the profile of propionic acid, it is necessary to consider the balance between the reactions that produce and consume propionic acid. As a reaction that consumes propionic acid, a propionate-oxidizing pathway is known to produce acetic acid and hydrogen from propionate (Worwag and Kwarciak-Kozłowska, 2019) although the reaction is difficult to proceed from a view point of Gibbs free energy (Patón et al., 2020). The oxidation of propionic acid is a reasonable reaction due to the accumulation of acetic acid and hydrogen at 100 mg/L of sodium selenite. However, since a propionate-producing reaction has been reported to be also present among the anaerobic digestion reactions (Han et al., 2020), sodium selenite may inhibit the reaction as another possible reason. Although butyric acid has been reported to be basically converted into acetic acid and hydrogen (Ciani et al., 2013; Youcai and Ran, 2021), the accumulation of butyric acid was observed at a high concentration of sodium selenite (Fig. 3-D). Therefore, although the detail mechanism remains unclear, butyrate-producing pathway or butyric-consuming pathway may be inhibited by sodium selenite. Although different patterns of organic acids were detected in the presence of sodium selenite, pH values during the anaerobic digestion ultimately ranged from pH 6.5 to pH 7.1 in all the samples including the control WSS.

### 3.3. Effect of sodium selenite on methanogenesis stage

Methane production from $H_2/CO_2$ gas or acetate as a substrate was examined by initial WSS samples under different concentrations of sodium selenite (0 mg/L, 2 mg/L, and 100 mg/L) (Fig. 4). For the assay, 4 antibiotics (benzyl penicillin, 0.5 mg/mL; streptomycin sulfate, 0.5 mg/mL; vancomycin-HCl, 0.2 mg/mL; and ampicillin, 0.2 mg/mL) were used to inactivate bacterial activity as mentioned in a previous report that isolated several strains of methanogens through killing all of the bacteria present in the sample (Battumur et al., 2017). In fact, no methane production was detected in the WSS with 4 antibiotics (Fig. 4). Therefore, it allows us to investigate the methanogenic activities (acetoclastic methanogenesis in case of acetate and hydrogenotrophic methanogenesis in case of hydrogen/carbon dioxide as a substrate) by adding

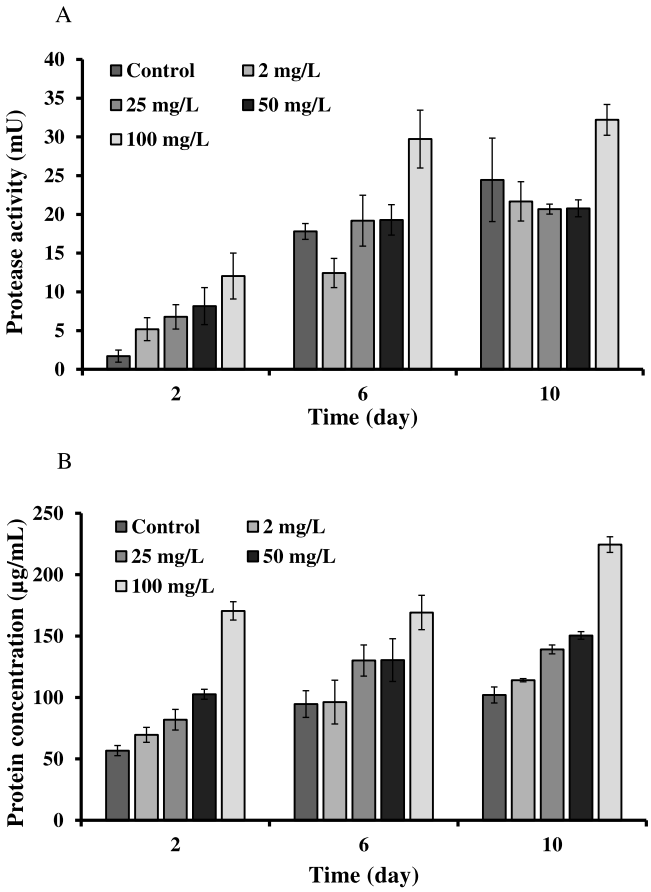


**Fig. 2.** Effect of sodium selenite on the hydrolysis process of anaerobic digestion using waste sewage sludge (WSS). Protease activity (A) and soluble protein concentration (B) were monitored in the WSS samples with sodium selenite (0 mg/L, 2 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L) during the 10-day anaerobic incubation. Error bars indicate standard errors ($n = 3$).

acetate or $H_2/CO_2$ as a substrate at different concentrations of sodium selenite. As a result, methane production from $H_2/CO_2$ was enhanced in the presence of 2 mg/L sodium selenite (Fig. 4-A). On the other hand, a slight low methane production was observed in the methane production from acetate in the presence of 2 mg/L sodium selenite (Fig. 4-B). Besides, no methane production was recorded when $H_2/CO_2$ or acetate was incubated in the presence of 100 mg/L sodium selenite (Fig. 4-A and Fig. 4-B). These results indicate that enhanced activity of hydrogenotrophic methanogenesis which is a pathway to produce methane from $H_2/CO_2$ is one of the reasons for the improved methane production at 2 mg/L of sodium selenite.

### *3.4. Bacterial and archaeal activity at the different concentrations of sodium selenite*

Next, bacterial and archaeal activities were quantified by qRT-PCR using RNA as a template to evaluate the number of active bacteria and archaea in the WSS with or without sodium selenite. Three WSS samples with 0 mg/L (control), 2 mg/L, and 100 mg/L sodium selenite were used in this measurement. (Fig. 5). As a result, there were no differences in the active bacterial activities between the three WSS samples. On the other hand, the archaeal activity in the presence of WSS with 100 mg/L sodium selenite was slightly lower than that of control WSS or 2 mg/L sodium selenite. This results indicate that sodium selenite may slightly decrease the number of methanogens during the anaerobic digestion. Therefore, it was found that the inhibition of methane-producing activity at 100 mg/L of sodium selenite is due to the inactivation of methanogenesis pathway.

### *3.5. Richness of bacteria and archaea*

In the anaerobic digestion of WSS, methane production was repressed at a high concentration of sodium selenite (100 mg/L). The result is considered to be due to the suppression of the activity of methanogens under a high concentration of sodium selenite as described in the previous reports (Lenz et al., 2008). This explanation is consistent with the result

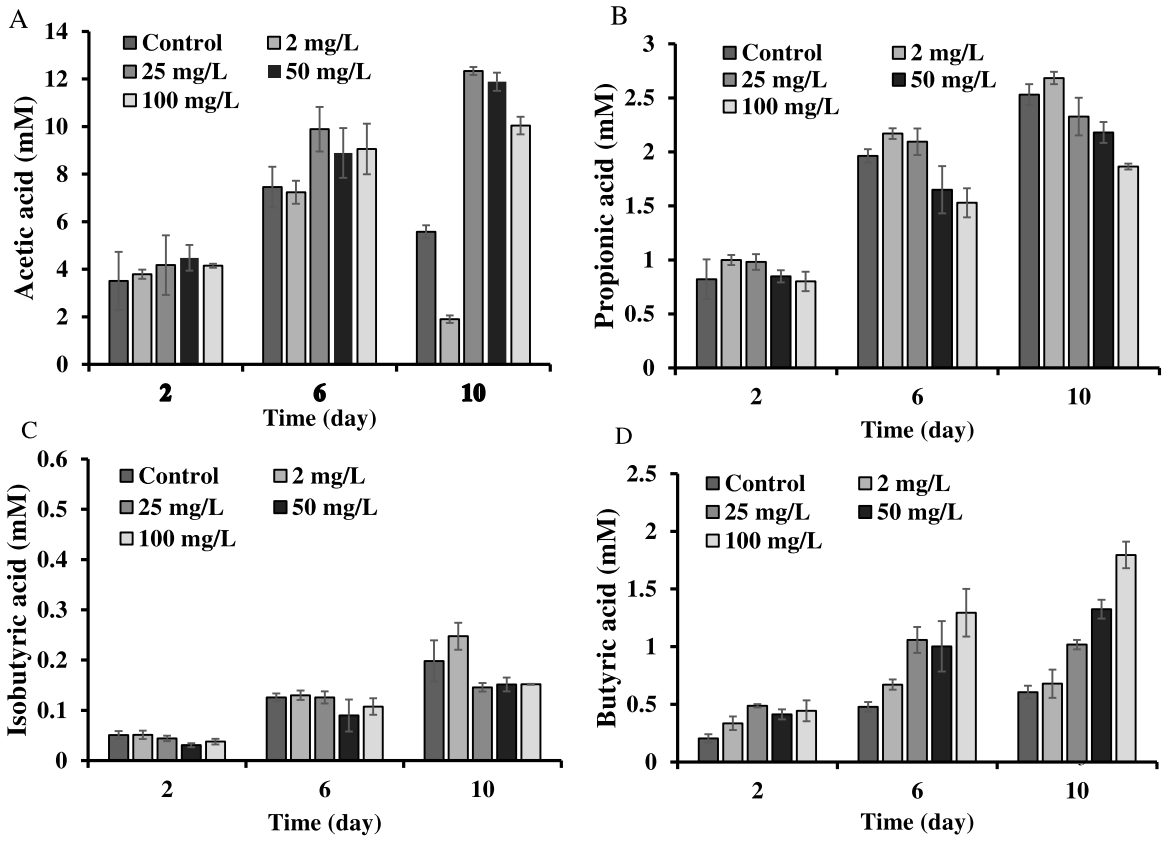


**Fig. 3.** Profile of organic acids detected in the acidogenesis process of anaerobic digestion using waste sewage sludge (WSS). The concentrations of acetic acid (A), propionic acid (B), isobutyric acid (C), and butyric acid (D) were monitored during the 10-day anaerobic incubation. Error bars indicate standard errors ($n = 3$).

that archaeal activity reduces in the presence of 100 mg/L sodium selenite (Fig. 5). On the other hand, a high concentration of sodium selenite at 100 mg/L has been shown to have high hydrolytic activities (Fig. 2) along with different profiles of organic acids (Fig. 3) during the anaerobic digestion. In contrast, 2 mg/L of sodium selenite has been shown to promote methanogenesis (Fig. 1); however, the bacterial hydrolysis and acidogenesis processes are not significantly different from those of the control (Fig. 2 and Fig. 3). Therefore, in the anaerobic digestion of WSS, microbial community structure analysis was performed by targeting bacteria at 100 mg/L and archaea at 2 mg/L of sodium selenite. Each community with or without sodium selenite was analyzed by the next-generation sequencing for 16S ribosomal RNA gene using RNA samples to evaluate only the active microbial community in WSS. Table 1 summarizes the number of operational taxonomic unit (OTU), Chao1 richness index, and Shannon diversity index of bacterial communities at 0 mg/L and 100 mg/L of sodium selenite. In the bacterial community at 10 days, the numbers of OTUs and Chao1 index in the control WSS were slightly higher than those of WSS with sodium selenite. In addition, the Shannon index, which estimates the diversity of the bacterial population, showed slightly increased at 10 day in WSS with sodium selenite compared to control WSS. This indicates that sodium selenite can slightly influence the bacterial diversity compared to the control WSS. On the other hand, the numbers of OTUs, Chao1 index, and Shannon index of archaeal community at 2 mg/L of sodium selenite were higher at 10 days than those of control WSS (Table 1). The results indicate that the richness of archaeal community and diversity increase at 2 mg/L of sodium selenite.

### 3.6. Comparison of bacterial community with or without sodium selenite

The addition of sodium selenite to WSS at 100 mg/L altered the dynamics of bacterial community during the anaerobic digestion of WSS. The changes in the abundance ratio of the remarkable bacterial genera at 100 mg/L of sodium selenite are summarized in Table 2. The relative abundance of *Turneriella, Rhodabacter, Planctomyces, Allochromatium, Nannocystis, Rhodoferax,* or *Candidatus microthrix* was higher at day 10 in the WSS at 100 mg/L of sodium selenite than that of the control WSS (Table 2). Some previous reports indicate that *Rhodabacter, Allochromatium,* and *Candidatus microthrix* are key

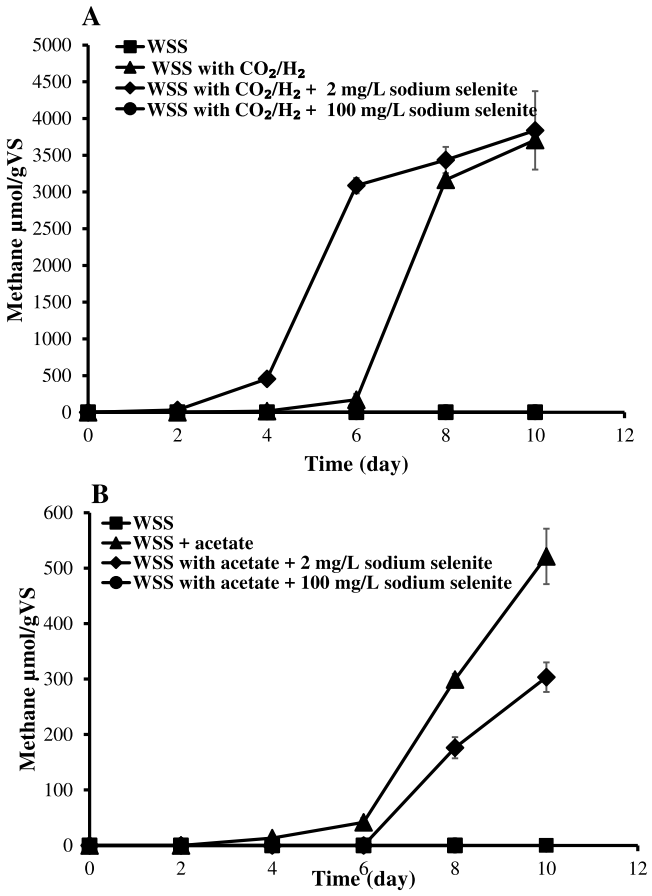


**Fig. 4.** Effect of sodium selenite on the methanogenesis process in waste sewage sludge (WSS). Hydrogentrophic methanogenesis (A) or acetoclastic methanogenesis (B) was evaluated by using $H_2/CO_2$ mix gas or acetate as a substrate for methane in initial WSS samples at 0 mg/L (dark triangles), 2 mg/L (dark diamonds), and 100 mg/L (dark circles) of sodium selenite along with 4 antibiotics (benzyl penicillin, streptomycin sulfate, vancomycin-HCl, and ampicillin) which inactivate the bacterial activity that is detrimental to this assay. Only WSS without any substrate and sodium selenite (displayed as WSS) was set as a negative control to verify that no methane was detected during the anaerobic incubation for 10 days in the presence of 4 antibiotics. Error bars indicate standard errors ($n = 3$).

**Table 1**
Diversity of the bacterial and archaeal community in WSS with sodium selenite and control WSS after the anaerobic digestion for 10 days.

| Sample | OTUs[a] | Chao1[a] | Shannon Index |
|---|---|---|---|
| (Bacterial community) | | | |
| Control WSS | 785.98 | 1024.91 | 3.297 |
| WSS with 100 mg/L sodium selenite | 744.01 | 958.52 | 3.381 |
| (Archaeal community) | | | |
| Control WSS | 45.95 | 57.56 | 0.7665 |
| WSS with 2 mg/L sodium selenite | 71.35 | 79.78 | 2.446 |

[a]Values were defined using a dissimilarity level of 0.03.

bacteria for the efficient degradation of wastewater under an anaerobic condition (Maza-Márquez et al., 2019; Unuofin et al., 2019; Wen et al., 2016); therefore, these bacteria may be active to utilize the hydrolytic compounds during the anaerobic digestion. In addition, genus *Nannocystis* has been well-known as a microbe able to utilize glucose, propionate, butyrate, and acetate (Ariesyady et al., 2007). Besides, *Planctomycetes* should have a key role in carbohydrate metabolism to produce volatile fatty acids (Elshahed et al., 2007). Moreover, *Rhodoferax* can utilize pyruvate, acetate, and hydrogen as electron donors for the dissimilatory iron reduction (Finneran, 2003). Changes in the activity of these bacteria should be factors to influence the reactions at the hydrolysis and acidogenesis stages at 100 mg/L of sodium selenite.

**Table 2**
Comparison of relative abundance of bacterial communities detected specifically in the anaerobic digestion using WSS with 100 mg/L sodium selenite and control WSS for 10 days.

| Bacterial genus | Relative abundance % | |
|---|---|---|
| | Control WSS | WSS with sodium selenite |
| *Turneriella* | 3.10 | 5.05 |
| *Rhodabacter* | 2.89 | 2.99 |
| *Planctomyces* | 0.88 | 5.35 |
| *Allochromatium* | 2.39 | 4.05 |
| *Nannocystis* | 0.80 | 1.32 |
| *Rhodoferax* | 1.18 | 2.22 |
| *Candidatus microthrix* | 2.06 | 4.83 |

**Table 3**
Comparison of relative abundance percentage of archaeal communities detected specifically in the anaerobic digestion using WSS with 2 mg/L sodium selenite and control WSS for 10 days.

| Archaeal group | Relative abundance % | |
|---|---|---|
| | Control WSS | WSS with 2 mg/L sodium selenite |
| *Methanosarcina* | 0.11 | 16.88 |
| *Candidatus Nitrososphaera* | 2.13 | 0.52 |
| *Methanospirillum* | 2.52 | 13.51 |
| *Methanosaeta* | 0.09 | 5.44 |
| *Methanocella* | 0.02 | 1.54 |
| *Methanocorpusculum* | 0.15 | 6.83 |

### 3.7. Comparison of archaeal community with or without sodium selenite

Community structure analysis targeting archaea was performed to seek a key microorganism for enhanced methane production in the WSS at 2 mg/L of sodium selenite. There are two major methanogens which are functional to the production of methane through anaerobic digestion (Venkiteshwaran et al., 2015). One of them is acetoclastic methanogens which can utilize acetate to produce methane and $CO_2$. The other one is hydrogenotrophic methanogens which can use $H_2$ or formate to convert $CO_2$ into methane. Table 3 shows the difference percentage of archaeal communities in the WSS sample with or without 2 mg/L sodium selenite. As a result, the abundance ratio of *Methanosarcina*, *Methanospirillum*, *Methanosaeta*, and *Methanocorpusculum* increased significantly during the 10-day anaerobic digestion, changing from 0.11% to 16.88%, from 2.52% to 13.51%, from 0.09% to 5.44%, and from 0.15% to 6.83%. In particular, since *Methanospirillum* and *Methanocorpusculum* are hydrogenotrophic methanogens (Shehab et al., 2013; Venkiteshwaran et al., 2015), the increase of these methanogens supports the result that the activity of hydrogenotrophic methanogenesis was high at 2 mg/L of sodium selenite (Fig. 4-A). Also, *Methanosarcina* can produce methane by both the acetoclastic and hydrogenotrophic methanogenesis pathways (Liu et al., 2011; Thauer et al., 2008) whereas *Methanosaeta* has only the acetoclastic methanogenesis (Venkiteshwaran et al., 2015). Thus, the result of archaeal community structure suggests that 2 mg/L sodium selenite increases methane production via increased the relative abundance of some key methanogens.

### 3.8. Effect of sodium selenite on the growth of Methanosarcina acetivorans C2A

The effect of sodium selenite was further investigated by checking the growth of pure culture of *Methanosarcina acetivorans* C2A (ATCC 35395) which is the only utilizable strain in our laboratory. As a result, the growth of *M. acetivorans* C2A was improved at 2 mg/L and repressed at 100 mg/L of sodium selenite (Fig. 6). On the other hand, no methane production was observed at 100 mg/L of sodium selenite (data not shown). These results using the pure culture of *M. acetivorans* C2A corroborate that sodium selenite have a positive impact at a low concentration and a negative one at a high concentration for the activity of methanogens.

## 4. Conclusion

In this study, the effect of sodium selenite on anaerobic digestion of WSS was investigated at difference concentrations. Our results showed that methane production was enhanced at a low concentration (2 mg/L) or repressed at a high concentration of sodium selenite (100 mg/L) during the anaerobic digestion of WSS. In detail, the hydrolysis process was activated at 100 mg/L of sodium selenite. The notable differences in organic acids at the acidogenesis stage were that the concentration of propionic acid decreased and that of butyric acid increased at 100 mg/L of sodium selenite. Both the acetoclastic and hydrogenotrophic methanogenic activities were totally inhibited at 100 mg/L of sodium selenite. In contrast, sodium selenite at 2 mg/L enhanced the activity of hydrogenotrophic methanogenesis. Whereas archaeal activity decreased at 100 mg/L of sodium selenite, the abundance of hydrogenotrophic methanogens (*Methanospirillum*

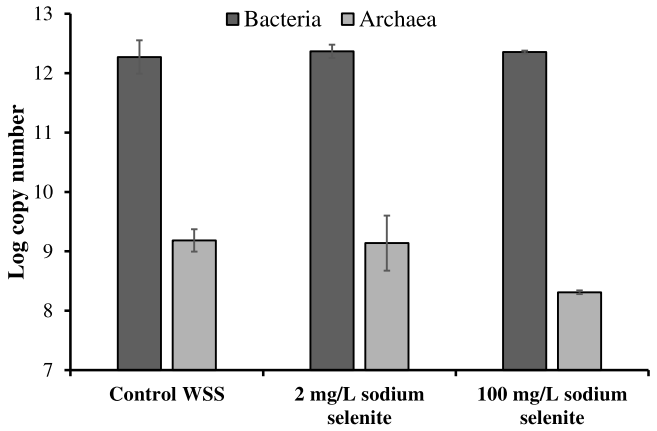


**Fig. 5.** Comparison of active bacterial and archaeal activities between WSS samples with sodium selenite (0 mg/L, 2 mg/L, and 100 mg/L) after the 10-day anaerobic digestion. Error bars indicate standard errors ($n = 3$).

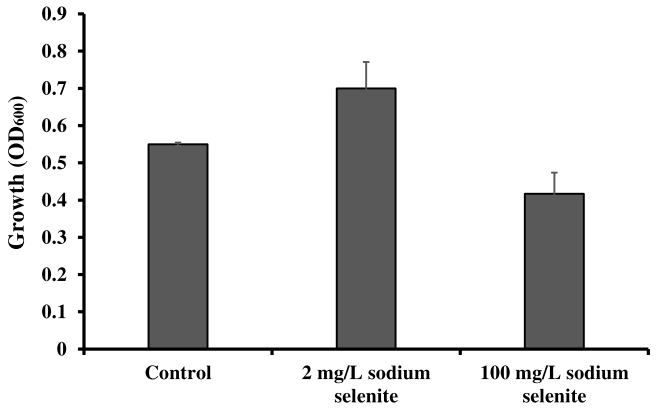


**Fig. 6.** Effect of sodium selenite on the growth of *Methanosarcina acetivorans* C2 A. *M. acetivorans* culture was inoculated in HSYE-methanol medium with or without sodium selenite (indicated as control, sodium selenite 2 mg/L and 100 mg/L) and the growth was compared at day 7. Error bars indicate standard errors ($n = 3$).

and *Methanocorpusculum*) increased in the WSS at 2 mg/L of sodium selenite. Furthermore, in pure-culture experiments using *M. acetivorans* C2A, the growth was promoted at 2 mg/L and inhibited at 100 mg/L of sodium selenite. Taken together, sodium selenite is one of the good reagents to regulate the anaerobic digestion of WSS for an appropriate recycling management of WSS.

## CRediT authorship contribution statement

**Chapol Kumar Roy:** Investigation, Writing – original draft. **Yuki Hoshiko:** Methodology, Formal analysis, Validation, Data curation. **Shotaro Toya:** Methodology, Formal analysis, Resources. **Toshinari Maeda:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquistion.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

The study was supported by a grant (No. JPMJAS2004) of aXis program (Accelerating Social Implementation for SDGs Achievement) of the Japan Science and Technology Agency.

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