Enhancement of Biogas Production from Sewage Sludge with the Addition of *Geobacillus* sp. Strain AT1 Culture

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Abstract

We investigated the enhancement of biogas production from anaerobic digestion of sewage sludge with the addition of an aerobic thermophilic bacterial culture of *Geobacillus* sp. strain AT1. Biogas production was significantly enhanced by the 2 ml addition of stationary-growth-phase culture (24h) of strain AT1 to 38 ml methanogenic sludge. Strain AT1 grew aerobically in F-JX synthetic medium, and optimum growth occurred at pH 7.0 and 65°C with shaking at 140 rpm. The optimum temperature for protease activity in the strain AT1 culture was around 65°C in the presence of 2 mM CaCl₂, where relative protease activity reached 210% compared with that at 35°C without CaCl₂. Protease activity was stable from 35 to 65°C, but rapidly decreased within 30 min incubation at 85°C. A protease inhibitor mixture (AEBSE, bestatin, E-64, EDTA, and pepstatin) inhibited the biogas enhancement by the strain AT1 culture. Methane production was enhanced by 2.1 times with the addition of the strain AT1 culture compared with the addition of F-JX medium, and was enhanced by 2.8 times with the additions of authentic protease and serine protease S. These results suggest that the enhancement of biogas production from anaerobic digestion of sewage sludge was caused by the protease activity of aerobic thermophilic *Geobacillus* sp. strain AT1.

Key words: anaerobic digestion, aerobic thermophilic bacteria, protease, biogas, methane, *Geobacillus*

INTRODUCTION

Vast amounts of sewage sludge are released in the aerobic treatment of wastewater. To prevent water pollution, land filling and ocean dumping of sewage sludge are now regulated. Hygienic, efficient, and energy-saving treatment technology for sewage sludge is required. Sewage sludge contains high amounts of organic matter which could be reusable as a substrate for anaerobic digestion.¹⁾ Thermochemical and biological pretreatment methods have been studied to improve the low degradation ratio and rate of conventional anaerobic digestion

of sewage sludge.²⁻⁴⁾

Aerobic thermophilic (AT) and anaerobic mesophilic treatments of sludge have been reported in terms of pathogen reduction, vector attraction reduction, volatile solids reduction, gas production, and product sludge dewaterability.⁵⁾ Solubilization of organic sludge by AT bacteria as a pretreatment for anaerobic digestion was studied.⁴⁾ We have reported that AT bacteria can be grown with sewage sludge or anaerobically digested sewage sludge, and that the addition of a small amount of AT bacterial sludge enhanced biogas production and volatile solids reduction.⁶⁾

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AT bacteria can hydrolyze organic waste.⁷⁾ The microbial enzymatic activity has a significant role in the decomposition of organic compounds under anaerobic conditions.⁸⁾ Protease activity varies from species to species and depends on the environmental adaptation of organisms.⁹⁾ Microbial enzymatic hydrolysis of organic wastes is an attractive topic in the reuse of organic wastes. The biodegradation of sewage sludge using bacterial enzymes could be useful for the improvement of biogasification.

Microorganisms are the most important sources of enzyme production.¹⁰⁾ Proteases are produced by a wide range of microorganisms, including bacteria, moulds, and yeasts. Thermophilic bacteria are widely spread in thermal environments.¹¹⁾ Currently a large proportion of commercially available proteases are derived from *Bacillus* sp.¹²⁾ Many bacteria belonging to the genus *Bacillus* and *Geobacillus* excrete large amounts of enzymes into the culture medium.¹³⁾ Thermophilic bacterial protease could be useful for the enhancement of biodegradation of sewage sludge.

In this study, we investigated the growth characteristics of *Geobacillus* sp. strain AT1 and its protease activity to reveal the mechanism of the enhancement of biogas production from the anaerobic digestion of sewage sludge with the addition of the strain AT1 culture.

MATERIALS AND METHODS

Cultivation of strain AT1 *Geobacillus* sp. strain AT1 has been recently isolated from aerobically and thermophilically acclimatized sludge. Strain AT1 was grown in 100 ml F-JX synthetic medium in a 250 ml flask. The bioreactor was maintained at 65 °C with shaking at 140 rpm. The F-JX medium contained yeast extract 0.1% (w/v) and trypticase peptone 0.1% (w/v).¹⁴⁾ The pH of the F-JX medium was adjusted to 7.0.

Growth conditions of strain AT1 We investigated the growth of strain AT1 in autoclaved F-JX medium over a pH range of 5 to 9.5. The pH was adjusted with HCl and NaOH. The bacterial culture was maintained at 65°C with aerobic shaking at 140 rpm. The

growth of strain AT1 was measured at 660 nm by a spectrophotometer at 24 h of culture.

The growth of strain AT1 was measured in F-JX medium at temperatures of 56, 58, 60, 64, 66, 68, and 72 °C under anaerobic and aerobic conditions. The anaerobic reactor was purged with nitrogen gas to eliminate air. Both reactors were maintained at 65°C with shaking at 140 rpm. All growth tests were done in triplicate.

Protease activity Protease activity was determined by the modified method of Ghorbel *et al.* (2003).¹⁵⁾ The culture medium was centrifuged, and 0.5 ml of the supernatant was mixed with 0.5 ml of 100 mM Tris-HCl (pH 8.0) containing 1% casein. The mixture was incubated for 15 min at 60 °C. The reaction was terminated by the addition of 0.5 ml trichloroacetic acid (20%, w/v). The mixture was kept at room temperature for 15 min and then centrifuged at 13,000 rpm for 15 min. The absorbance of the supernatant was measured at 280 nm. A standard curve was prepared by using solutions of 0-50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme corresponding to the absorbance of 1 µg/ml tyrosine.

Protease activity of 160 units/ml measured at 35°C and pH 7.0 without CaCl₂ using the 24 h culture of strain AT1 was defined as 100% activity. Protease activity was measured with or without 2 mM CaCl₂. The effect of incubating temperature of protease activity was examined at temperatures of 15, 35, 45, 55, 65, and 75°C. The pH was adjusted with HCl and NaOH. The stability of protease at high temperature (35, 65, and 85 °C) was determined with incubation periods of 0 to 120 min at 15-min intervals.

Effect on anaerobic digestion To a 100-ml glass reactor we added 2 ml of AT1 culture and 38 ml of methanogenic sludge. Methanogenic sludge was sampled from an anaerobic digestion tank treating sewage sludge (primary and waste activated sludges) in a communal wastewater treatment plant (Ibaraki Prefecture, Japan). The AT1 was grown at pH 7.0 and 65°C with shaking 140 rpm. The strain AT1 cultures at different

growth phases of 6, 12, 24, and 48 h were added to the methanogenic sludge. F-JX medium (2 ml) without AT1 was added to methanogenic sludge as control. Another reactor was filled with only methanogenic sludge (38 ml) and distilled water (2 ml). Each glass reactor was capped with a natural rubber sleeve stopper and then sealed with a thin aluminum cover-cap to impose anaerobic conditions. Air in the reactors was replaced with nitrogen gas. The reactors were then placed in a thermostat at $35 \pm 1^\circ\text{C}$. All the activity tests were carried out in triplicate. Biogas production and volatile solids (VS) reduction in the bioreactors were measured. The experiment was terminated after 20 days, when biogas production had stopped.

Effect of protease inhibitor We studied the effect of a protease inhibitor mixture containing AEBSF hydrochloride (final concentration $200 \mu\text{mol/l}$), bestatin ($17 \mu\text{mol/l}$), E-64 protease inhibitor ($2 \mu\text{mol/l}$), EDTA disodium ($850 \mu\text{mol/l}$), and pepstatin A ($20 \mu\text{mol/l}$). The inhibitor mixture was dissolved in 1 ml dimethyl sulfoxide (DMSO) and 4 ml distilled water. The active volume of the bioreactors was 40 ml, made up of methanogenic sludge 37 ml, AT1 culture 2 ml, and inhibitor mixture solution 1 ml. F-JX medium without bacteria, strain AT1 + DMSO, autoclaved strain AT1 culture, and strain AT1 culture alone were also added to the methanogenic sludge as controls. During anaerobic digestion, biogas production and methane concentration were measured as above.

Effect of authentic proteases Authentic protease (Wako, Tokyo, Japan) was diluted with distilled water, and serine protease S (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in 25 mM Tris-HCl (pH 7.6) with 40% ethanol. The authentic protease (final concentration 20 units/ml) and serine protease S (20 units/ml) were added (2 ml total) to the methanogenic sludge (38 ml). The AT1 culture (2 ml, final concentration of protease activity 8 units/ml) was added to the methanogenic sludge (38 ml). As a control, (final concentration 20 units/ml) 2 ml of 25 mM Tris-HCl (pH 7.6) with 40% ethanol was

added to the methanogenic sludge (38 ml).

Analysis The volatile solids (VS) content of the sludge in the reactor was measured on operational day 0 and at the end of the experiment. The VS content was determined by heating at 105°C for 24 h and after heating at 600°C for 1 h. The biogas composition was determined by gas chromatograph (model GC-12A, Shimadzu, Kyoto, Japan) with a Porapak Q column (Shinwakakou, Kyoto, Japan) at 90°C .

RESULTS AND DISCUSSION

Enhancement of biogas production

Figure 1 shows the biogas production with the addition of different growth phases of strain AT1 culture to the methanogenic sludge. The biogas production from the anaerobic digestion of sewage sludge was significantly improved with the addition of 5% (v/v) AT1 culture. AT bacteria hydrolyze sludge with extracellular enzymes.^{16, 17} The biogas production was enhanced 95% by the addition of the stationary growth phase (24

Fig. 1 Cumulative biogas production with the addition of different growth phases of *Geobacillus* sp. strain AT1 culture into the methanogenic sludge. The bioreactor was incubated at 35°C

h) culture of strain AT1 compared with the distilled water addition. We assume that strain AT1 excreted the optimum amount of enzymes during the 24-h incubation period. Another *Bacillus* sp. excreted the optimal amount of protease in the culture medium during its post-exponential and stationary growth phases.¹⁸⁾ Hydrolysis activity in the anaerobic digestion is mainly influenced by the bacterial enzymatic activity.¹⁹⁾ The enhancement of the biogas production with the addition of strain AT1 culture could be caused by the enzymatic activity excreted from strain AT1 cells. There were no marked differences in biogas production among the addition of F-JX medium without strain AT1 cells and with 6-h culture of strain AT1 to the methanogenic sludge.

Table 1 shows the VS reduction with the addition of strain AT1 culture to the methanogenic sludge. The highest VS reduction was 15% when 12-h and 24-h AT1 culture was added. The methane gas concentration ranged from 60% to 63%, which were not significantly different. The enhancement of biogas production and VS reduction were correlated.

Growth of strain AT1 *Geobacillus* sp. strain AT1 was isolated in our laboratory from AT seed sludge taken from sewage sludge. Isolated strain AT1 grew well in F-JX culture medium. Strain AT1 grew well at temperatures ranging from 56 to 70 °C (Fig. 2). The optimum growth temperature of AT1 was 65–67 °C. Figure 3 shows the growth of strain AT1 at different pH levels. The optimum growth (cell density, OD₆₆₀ = 0.55)

occurred at pH 7.0–7.5. Strain AT1 could not grow well at either high acidity or high alkalinity.

The growth curves of strain AT1 under aerobic and anaerobic conditions are shown in Fig. 4. Strain AT1 grew well in aerobic conditions, but it could not grow in anaerobic conditions. The optimum growth occurred at 24-h culture under aerobic conditions with shaking.

Protease activity Protease activity at 35 °C and pH 7.0 without CaCl₂ using the 24 h culture of strain AT1 was 160 units/ml and was defined as 100%. The optimum incubating temperature for the protease production in the strain AT1 culture was 65 °C in the presence of 2 mM CaCl₂ (Fig. 5). The relative protease activity of strain AT1 increased by 210% with and 160% without 2 mM CaCl₂ at 65 °C compared with that at 35 °C. On the other hand, it decreased to 14% at 35 °C without CaCl₂ (Fig. 5). Ghorbel *et al.* (2003) reported that 2 mM CaCl₂ stimulated protease activity by 500%.¹⁵⁾ Mabrouk *et al.* (1999) optimized the protease production from *Bacillus licheniformis* by adding CaCl₂ to the culture medium.²⁰⁾ The protease activity in the strain AT1 culture was activated by the presence of 2 mM CaCl₂.

The protease activity of the strain AT1 culture grown at 65 °C was higher than that grown at 35 °C (Fig. 6). Protease activity was rapidly reduced at 85 °C after 30 min (Fig. 6). The protease enzyme from *B. cereus* BG1 was rapidly denatured at 70 °C after 4 min of incubation.¹⁴⁾ The protease produced by strain AT1 could be a thermostable enzyme.

Table 1 VS reduction with the addition of different incubation periods of strain AT1 culture to the methanogenic sludge

Different incubation periods of strain AT1 culture	Initial VS (%)	Final VS (%)	VS reduction (%)
6 h culture	0.62	0.57	8
12 h culture	0.65	0.55	15
24 h culture	0.65	0.55	15
48 h culture	0.57	0.52	9
FJ-X medium	0.59	0.55	7
Distilled water	0.65	0.59	9

Fig. 2 Growth curve of *Geobacillus* sp. strain AT1 at different temperatures. The strain AT1 culture was incubated with aerobic shaking. A cell concentration of the AT1 culture was measured by optical density at 660 nm. Bars indicate standard error.

Fig. 3 Growth curve of *Geobacillus* sp. strain AT1 at different pH. The strain AT1 culture was incubated at 65°C with aerobic shaking. A cell concentration of the AT1 culture was measured by optical density at 660 nm. Bars indicate standard error.

Fig. 4 Growth curve of *Geobacillus* sp. strain AT1 under anaerobic and aerobic conditions. The strain AT1 culture was incubated at 65°C. A cell concentration of the AT1 culture was measured by optical density at 660 nm. Bars indicate standard error.

Fig. 5 Change in relative protease activity of *Geobacillus* sp. strain AT1 culture at different temperatures with or without 2 mM CaCl₂. The strain AT1 culture was incubated with aerobic shaking. Symbols: square; with 2 mM CaCl₂, circle; without 2 mM CaCl₂.

Protease inhibitor The highest volume of methane produced was 32 ml/reactor with the addition of the strain AT1 culture without protease inhibitor. Biogas production was not enhanced with the addition of protease inhibitor mixture and strain AT1 culture (Fig. 7). On the other hand, DMSO ($2 \mu\text{l}/40 \text{ ml}$) did not inhibit the enhancement of the biogas production with the addition of the strain AT1 culture. These results suggest that the enhancement by strain AT1 was caused by protease activity. The methane composition of the biogas (70%) was not affected by the protease inhibitor addition.

Fig. 6 Change in relative protease activity in *Geobacillus* sp. strain AT1 culture at different incubating temperature during protease activity measurement with 2 mM CaCl_2

Biogas production did not increase with the addition of F-JX medium or of autoclaved strain AT1 culture to the methanogenic sludge. This result indicates that the enzymatic activity of strain AT1 is destroyed at 120°C . F-JX medium without the strain AT1 cells did not enhance biogas production.

Table 2 shows the VS reduction with the addition of protease inhibitor mixture with the strain AT1 culture to the methanogenic sludge. The highest VS reduction (14%) occurred with the addition of the strain AT1 culture to the methanogenic sludge. The high VS reduction was directly correlated with the

Fig. 7 Biogas yield with the addition of protease inhibitor and strain AT1 culture to the anaerobic digestion of sewage sludge

Table 2 Effect of the addition of protease inhibitor and strain AT1 culture to the methanogenic sludge on the anaerobic digestion

Added samples to the methanogenic sludge	Initial VS (%)	Final VS (%)	VS reduction (%)
Strain AT1 culture	0.58	0.50	14
Strain AT1 culture + Protease inhibitor mixture solution ^{a)}	0.63	0.58	7.9
Strain AT1 culture + DMSO ^{b)}	0.63	0.56	11
Autoclaved strain AT1 culture	0.58	0.53	8.6
F-JX medium	0.56	0.52	7.1

^{a)} Protease inhibitor mixture solution (1 ml) contains AEBSF hydrochloride (final concentration $200 \mu\text{mol/l}$), bestatin ($17 \mu\text{mol/l}$), E-64 protease inhibitor ($2 \mu\text{mol/l}$), EDTA disodium ($850 \mu\text{mol/l}$), pepstatin A ($20 \mu\text{mol/l}$), $2 \mu\text{l}$ dimethyl sulfoxide (DMSO) and distilled water. The active volume of the bioreactors was 40 ml, made up of methanogenic sludge 37 ml, AT1 culture 2 ml, and inhibitor mixture solution 1 ml.

^{b)} DMSO $2 \mu\text{l}$ was added to the 40 ml methanogenic sludge.

relatively high methane production. DMSO did not affect VS reduction. These results suggest that protease activity in the strain AT1 culture improves sludge degradation and methanogenesis.

Effect of authentic protease Biogas production was markedly enhanced with the addition of both authentic protease (50 ml/reactor) and serine protease S (48 ml/reactor) to the methanogenic sludge at a final concentration of 20 units/ml (Fig. 8). Methane production was enhanced by 2.1 times with the addition of the strain AT1 culture (final protease activity 8 units/ml) compared with F-JX medium alone, and was enhanced by 2.8 times with the additions of authentic protease and serine protease S (final protease activity 20 units/ml). Biogas production did not increase with the addition of distilled water or 25 mM Tris-HCl with 40% ethanol to the methanogenic sludge. The methane compositions were not different among these treatments. These results suggest that protease can improve sludge degradation and methanogenesis.

We have reported that AT bacteria can be grown with sewage sludge and anaerobically digested sewage sludge, and that AT bacterial sludge enhances degradation and biogas production from anaerobic digestion of

sewage sludge.⁶⁾ The present results show that this enhancement is caused by protease activity released from *Geobacillus* sp. We propose that a small addition of AT bacterial sludge to methanogenic sludge will improve biogasification of sewage sludge.

CONCLUSIONS

The present results suggest that the enhancement of biogas production from anaerobic digestion of sewage sludge was caused by protease activity of thermophilic *Geobacillus* sp. strain AT1. Strain AT1 grew aerobically in F-JX synthetic medium, and optimum growth occurred at pH 7.0 and 65°C with shaking at 140 rpm. The relative protease activity in the strain AT1 culture reached 210% in the presence of 2 mM CaCl₂ at 65 °C. The protease activity was stable from 35 to 65°C, but rapidly decreased within 30 min of incubation at 85 °C. The protease inhibitor mixture inhibited the biogas enhancement by strain AT1. Methane production was enhanced by 2.1 times with the addition of the strain AT1 culture compared with F-JX medium alone, and was enhanced by 2.8 times with the additions of authentic protease and serine protease S. Addition of a small amount of AT bacteria to methanogenic sludge could improve biogasification of sewage sludge.

REFERENCE

- 1) Fannin, F. F., Conrad, J. R., Srivastava, V. J., Jerger, D. E., and Chynoweth, D. P.: Anaerobic processes, J. Water Poll. Cont. Fed., 55, 623-632 (1983)
- 2) Haug, R. T.: Sludge processing to optimize digestibility and energy production, J. Water Poll. Control Fed., 49, 1713-1721 (1977)
- 3) Sawayama, S., Inoue S., Yagishita, T., Ogi T., and Yokoyama, S. Y.: Thermochemical liquidization and anaerobic treatment of dewatered sewage sludge, J. Ferment. Bioeng., 79, 300-302 (1995)
- 4) Hasegawa, S., Shiota, N., Katsura, K., and Akashi, A.: Solubilization of organic sludge by thermophilic aerobic bacteria as a pretreatment for anaerobic digestion, Water Sci. Technol., 41, 163-

Fig. 8 Biogas yield with the addition of authentic protease or serine protease S and strain AT1 culture to the anaerobic digestion of sewage sludge

- 169 (2000)
- 5) **Cheunbarn, T. and Pagilla, K. R.:** Aerobic thermophilic and anaerobic mesophilic treatment of sludge, *J. Environ. Eng.* 129, 790-795 (2000)
- 6) **Miah, M. D., Tada, C., Tsukahara, K., Yagishita, T., and Sawayama, S.:** Effect of aerobic thermophilic pretreatment of anaerobically digested sewage sludge on anaerobic re-digestion, *Proceedings of Asian Waterqual*, 20033Q4I05 p1-9 (2003)
- 7) **Taguchi, F., Guofu, S., and Guanglei, Z.:** Microbial treatment of kitchen refuse with enzyme-producing thermophilic bacteria from Giant Panda feces, *J. Biosci. Bioeng.*, 92, 602-608 (2001)
- 8) **Aoki, K., Miyamoto, K., Murakami, S., and Shinke, R.:** Anaerobic synthesis of extracellular proteases by the soil bacterium *Bacillus* sp, AM-23: purification and characterization of the enzymes, *Soil. Biol. Biochem.*, 27, 1377-1382 (1995)
- 9) **Yang, J., Shih, I., Tzeng, Y., and Wang, S.:** Production and purification of protease from *Bacillus subtilis* that can deproteinize crustaceans wastes, *Enzyme Microbiol. Technol.*, 26, 406-413 (2000)
- 10) **Kim, Y. K., Bae, J. H., Oh, B. K., Lee, W. H., and Choi, J. W.:** Enhancement of proteolytic enzyme activity excreted from *Bacillus stearothermophilus* for a thermophilic aerobic digestion process, *Biores. Technol.*, 82, 157-164 (2002)
- 11) **Cowan, D. A. and Daniel, R. M.:** Purification and some properties of an extracellular protease (caldolysin) from an extreme thermophile, *Biochem. Biophys. Acta*, 705, 293-305 (1982)
- 12) **Hutadilok, T. N., Painupong, A., and Suntainalert, P.:** Purification and characterization of an extracellular protease from alkaliphilic and thermophilic *Bacillus* sp. PS719, *J. Biosci. Bioeng.*, 87, 581-587 (1999)
- 13) **Vanugopal, V., Ahr, M., and Nerkar, D. P.:** Solubilization of fish proteins using immobilized microbial cells, *Biotechnol. Bioeng.*, 33, 1098-1103 (1989)
- 14) **Nomura, T., Akihara, S., Takai, K., and Sako, Y.:** *Thermaerobacter nagasakiensis* sp., a novel aerobic and extremely thermophilic marine bacterium, *Arch. Microbiol.*, 177, 339-344 (2002)
- 15) **Ghorbel, B., Sellami-Kamoun, A. and Nasri, M.:** Stability studies of protease from *Bacillus cereus* BG1, *Enzyme Microbiol. Technol.*, 32, 513-518 (2003)
- 16) **Bomio, M., Sonleitner, B., and Fiechter, A.:** Growth and biocatalytic activities of aerobic thermophilic populations in sewage sludge, *Appl. Microbiol. Biotechnol.*, 32, 356-362 (1989)
- 17) **Kume, S. and Fujio, Y.:** Digestion of municipal sewage sludge by a mixture of thermophilic *Bacilli* and their culture extract, *J. Gen. Appl. Microbiol.*, 36, 189-194 (1990)
- 18) **Kole, M. M., Draper, I., and Gerson, D. F.:** Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentration, *J. Chem. Tech. Biotech.*, 41, 197-206 (1987)
- 19) **Fox, P. and Pohland, F. G.:** Anaerobic treatment applications and fundamentals: substrate specificity during phase separation, *Water Environ. Res.*, 66, 716-724 (1994)
- 20) **Mabrouk, S. S., Hashem, A. M., El-Shayeb, N. M. A., Ismail, A.-M. S., and Abdel-Fattah, A. F.:** Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC21415, *Biores. Technol.*, 69, 155-159 (1999)

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