Copper Ion Toxicity Causes Discrepancy between Acetate Degradation and Methane Production in Granular Sludge

BAE, JIN-WOO, SUNG-KEUN RHEE[1], AM JANG[2], IN S. KIM[2], AND SUNG-TAIK LEE*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon, Korea
[1]Oak Ridge National Laboratory, Oak Ridge, TN 37831, U.S.A.
[2]Kwangju Institute of Science & Technology, Kwangju, Korea

Abstract Metal ions have an adverse effect on anaerobic digestion. In an acetate degradation test of upflow of anaerobic sludge blanket granules with Cu^{2+}, not all of the acetate that disappeared was stoichiometrically converted to methane. In the presence of 400 mg/g-VSS (volatile suspended solids) Cu^{2+}, only 26% of the acetate consumed was converted to methane. To study acetate conversion by other anaerobic microorganisms, sulfate and nitrate reductions were investigated in the presence of Cu^{2+}. Sulfate and nitrate reductions exhibited more resistance to Cu^{2+} than methanogenesis, and the granules reduced 2.2 mM and 5.4 mM of nitrate and sulfate, respectively, in the presence of 400 mg/g-VSS copper ion. However, the acetate degraded by sulfate and nitrate reductions was only 24% of the missing acetate that could have been stoichiometrically converted to CO_2. Accordingly, 76% of the acetate consumed appeared to have been converted to other unknown compounds.

Key words: Bacterial resistance, granules, metal ions, nitrate reduction, sulfate reduction

Acetate is an important intermediate in the anaerobic breakdown of organic matter in methanogenic bioreactors [8, 11, 14, 19]. The two genera of methanogenic Archaea known to use acetate as a sole energy source are *Methanosarcina* and *Methanosaeta* ("*Methanothrix*") [23]. *Methanosaeta* is the dominant acetoclastic methanogen in anaerobic habitats with low acetate concentrations (e.g., anaerobic bioreactors), because of its high affinity and low-threshold value for acetate [12]. In anaerobic reactors treating sulfate-rich wastewaters, such as paper-mill wastewaters or food oil industry wastewaters, sulfate-reducing bacteria compete with methanogens for acetate. The outcome of this competition is not yet clear. In many studies with freshwater or low-salt

*Corresponding author
Phone: 82-42-869-2617; Fax: 82-42-863-5617;
E-mail: stlee@sorak.kaist.ac.kr

systems, acetate conversion via methanogenesis is predominant, even with excess sulfate [10, 15, 21]. However, after the long-term operation of such reactors, acetate degradation mainly by sulfate reducers has also been reported [1, 9, 22]. Factors that affect the outcome of the competition between methanogens and sulfate reducers have already been reviewed [17]. Among these factors, metal ions appear to be one of the most crucial factors [13]. The effect of metal ions and toxic chemicals on anaerobic digestion was studied previously using intact and disintegrated granules from an upflow anaerobic sludge blanket (UASB) reactor [3, 4]. The degradation of glucose and production of methane were not affected by the disintegration of the granules. However, when Cu^{2+} ions were added to the media, the disintegrated granules were more sensitive to the toxicity of Cu^{2+} than the intact granules in the glucose conversion to methane. This indicates that the layered structures of UASB granules make the microorganisms resistant to the metal ions. A previous study also revealed that Cu^{2+} inhibits methane production more significantly than acetate degradation [3]. In the presence of copper ions, not all the acetate that disappears is stoichiometrically converted to methane. Since Bhatti *et al.* [5] reported that a large fraction of anaerobic sludge consists of sulfate reducing bacteria (SRB) and nitrate reducing bacteria (NRB), these bacteria could also degrade the acetate into CO_2. Accordingly, in this study, we measured the activity of SRB and NRB in the presence of metal ions. The results provide an estimate of the proportion of acetate not converted to methane during acetate degradation, and the reasons for the granular sensitivities to acetate degradation and methane production with toxic metal ions were also investigated.

Sludge granules were sampled from a 1,000 m^3 UASB reactor treating beer brewery wastewater with a COD of 3,000–4,000 mg/l. The reactor had been in operation for 3 years with a hydraulic retention time of 10 h while

removing 95% of influx COD. The granules were about 2 mm in diameter and brownish black. Scanning electron microscopy of the granular sludge indicated that the sampled granules had a layered structure composed of two sections. To obtain non-layered sludge with the same microbial populations as the intact granules, equal volume and weight of granules were blended using a homogenizer (Ace Homogenizer, Nihonseiki Kaisha Ltd.) at 10,000 rpm for 2 min. The disintegrated granules were sieved using a 0.5 mm mesh to remove any undisintegrated granules prior to the experiment. All the sludge samples prepared were washed three times with a 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 7.0) before the experiments. The sludge had not been previously acclimated to metal ions. All the procedures were conducted in an anaerobic atmosphere (Controlled Atmosphere Chamber, Lansing, Mich., U.S.A.) and 0.02 mM Na_2S was used as the reducing agent. The basal medium used in the anaerobic resistance assay contained the following (in milligrams per liter): KH_2PO_4, 30; K_2HPO_4, 70; $CaCl_2$, 100; $MgCl_2$, 100; KCl, 70; NH_4Cl, 260; Na_2SO_3, 50; sodium acetate, 40 mM. In the sulfate and nitrate reduction experiments, only 40 mM sodium acetate was used for the test.

The measurements of specific acetate degradation activity were performed using 120-ml glass serum bottles sealed with 12 mm-thick butyl rubber septa. The vials were filled with 30 ml of the sieved granular sludge and 70 ml of the basal medium containing 40 mM acetate from a neutralized stock solution up to a line at 100 ml. Copper ions ($CuCl_2$) were selected as the metal ions in the current study. The assay medium was then adjusted to pH 7.0 ± 0.1 and flushed for 2 min with argon. The mixtures were incubated at 37°C with shaking at 150 rpm. During the incubation periods, the gas and liquid phases were sampled to determine the microbial activity. The acetate concentrations in the liquid phase were determined by an isocratic HPLC using an ion exchange column (Aminex, HPX-87H, 300× 7.8 mm) and organic acid analysis kit [2]. Degassed 4 mM H_2SO_4 was used as the eluant. Acetate was detected at 210 nm. The gas contents in the headspace of the assay bottles were determined using a pressure meter and their compositions calculated by GC. The gas contents were revised according to the pressure and temperature, yet the dissolved contents were not estimated. The gas chromatograph was equipped with a steel column (1.83 m, 2.125 cm) packed with a Poropak Q (80/100 mesh size: Millipore). The temperatures of the column, the injection port, and thermal conductivity detector were 35°C, 60°C, and 100°C, respectively. The carrier gas was argon at 30 ml/min. The samples used to measure the gas content in the headspace were determined with a pressure-lock gas syringe (Pressure Lock series A-2). Sulfate was determined using the turbidometric method in Standard Methods; that is, the samples were mixed with an acetate-containing buffer, $BaCl_2$ was then added, and the optical density was finally

measured at 420 nm. Nitrate was determined with Szechrome NAS reagent (Polysciences, Inc) [7]: A 0.5 ml aqueous nitrate sample was gently mixed with 5 ml of the NAS reagent, nitrate-free concentrated phosphoric acid, and sulfuric acid. The violet color intensity was read at 570 nm in a 1-cm cell after 10–60 min.

To study the effect of Cu^{2+} on methanogenesis, acetate degradations were tested at various Cu^{2+} concentrations (Fig. 1). Acetate was selected as the substrate among the major volatile fatty acids accumulated, because its conversion to methane is a one-step process conducted by acetoclastic methanogens [6]. The degradation of acetate and production of methane were basically unaffected by the disintegration of the granules by homogenization. In the absence of Cu^{2+}, both the intact and disintegrated granules completely degraded 40 mM of acetate in 12 h (3.3 mM/h), although the methane production rate was slightly decreased with granule homogenization (2.88 mM/h) (Fig. 1A). However, in the presence of Cu^{2+}, the methane production was more significantly inhibited than the acetate consumption. When the Cu^{2+} concentration was increased to 300 mg/g-VSS, the methane production rates of the intact and disintegrated granules decreased remarkably to 73% and 43% of the control, respectively, whereas the acetate degradation rates were not significantly affected (87% and 82% of the control, respectively). The methane production rates of the disintegrated granules were about one-half of the rates with the intact granules. With a concentration of 400 mg/g-VSS Cu^{2+}, the acetate degradation rates of the intact and disintegrated granules maintained at about 75% of the control. However, the methane production of the intact and disintegrated granules was 38% and 14% of the control, respectively. A further increase of the Cu^{2+} concentration to 700 mg/g-VSS resulted in no acetate degradation by the granules. This result indicates that the layered structure of the normal granules rendered the copper resistance to the methanogens.

In the absence of copper, the granules stoichiometrically converted the acetate to methane with an 80% ratio. The remaining 20% would appear to have been used by the other populations and for cell proliferation. However, when greater concentrations of metal ions were added to the media, more acetate were stoichiometrically missing. When 400 mg/g-VSS of Cu^{2+} was added to the media, only 27% of the consumed acetate appeared as methane.

In the previous study, the proportion of SRB in granular sludge was 13–16% according to 16S rRNA hybridization [16]. As shown in previous acetate degradation tests with UASB sludge [20], methanogens would appear to be more sensitive to metal ions than SRB, within a certain range of metal ion concentrations, and would seem to be severely inhibited while SRB and NRB were relatively active. Therefore, it likely means that the missing acetate was consumed by SRB and NRB without producing methane.
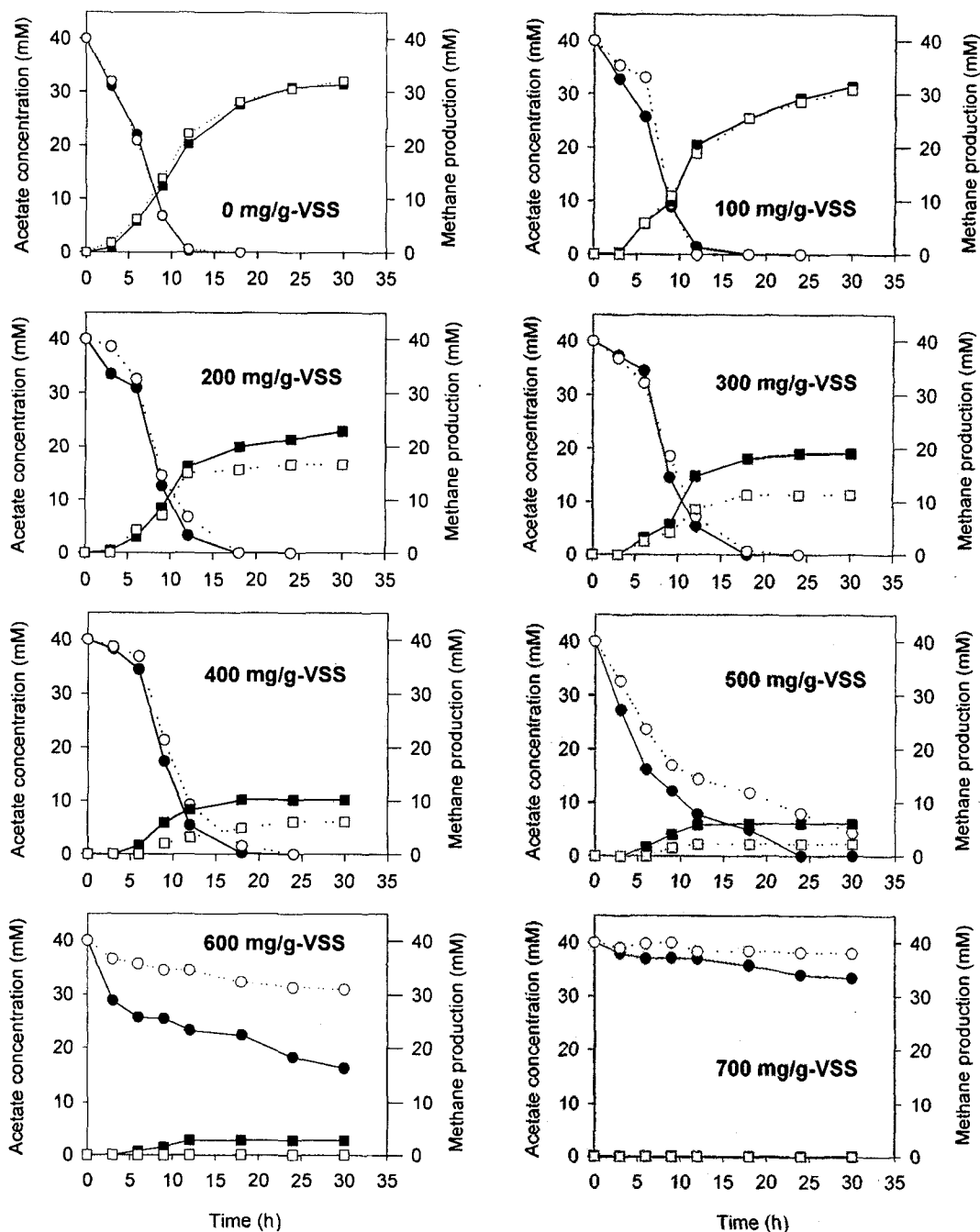
Fig. 1. Acetate degradation and methane production by intact and disintegrated granules in media containing gradually increased concentrations of copper.

($\bullet$) Acetate degradation with intact granules, ($\bigcirc$) acetate degradation with disintegrated granules, ($\blacksquare$) methane production with intact granules, ($\square$) methane production with disintegrated granules.

After washing the granules with the buffer, it was found that the granules still contained 250 mg/l (4.0 mM) nitrate and 727.5 mg/l (7.6 mM) sulfate. Therefore, according to the following chemical equation, it was theoretically assumed that 9.6 mM acetate was consumed with sulfate and nitrate present in the granules.

$$SO_4^{2-}+C_2H_4O_2+2H^+\rightarrow H_2S+2CO_2+2H_2O$$
$$2NO_3^{2-}+C_2H_4O_2\rightarrow N_2+2CO_2+4H_2O$$
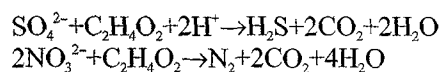
To determine how much acetate was consumed by SRB and NRB, the reduction of nitrate and sulfate was investigated with various concentrations of Cu^{2+} (Fig. 2).
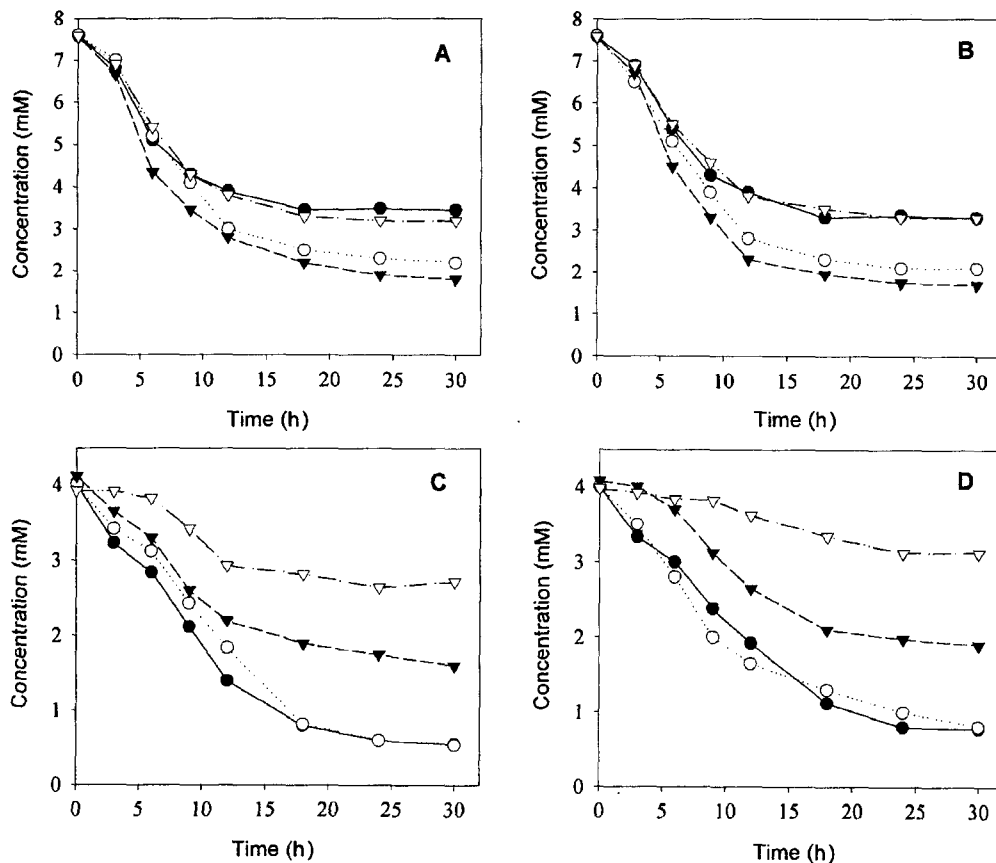
Fig. 2. Effect of copper on reduction of sulfate and nitrate by intact and disintegrated granules.
(A) Sulfate reduction by intact granules, (B) sulfate reduction by disintegrated granules, (C) nitrate reduction by intact granu les, (D) nitrate reduction by disintegrated granules; (●) 0 mg/g-VSS of copper, (○) 200 mg/g-VSS of copper, (▼) 400 mg/g-VSS of copper, (▽) 600 mg/g-VSS of copper.

In the absence of copper, 4.1 mM of sulfate was reduced. However, when more Cu^{2+} was added to the media, more sulfate was reduced, up to a concentration of 400 mg/g-VSS, where 5.4 mM of sulfate was reduced corresponding to the same molar acetate. This result implies that Cu^{2+} turned the tide in the competition between SRB and methanogens for acetate. A further increase in the Cu^{2+} concentration to 600 mg/g-VSS resulted in a decrease of sulfate reduction. Nitrate reduction was also unaffected by the Cu^{2+} toxicity up to a concentration of 200 mg/g-VSS of Cu^{2+} (Fig. 2C). In the presence of 0–200 mg of Cu^{2+}, 3.3 mM of nitrate was completely degraded within about 24 h. However, a further increase in the Cu^{2+} concentration to 400 mg/g-VSS resulted in a decrease in the nitrate reduction rate. These results indicate that SRB and NRB were more resistant to Cu^{2+} than the methanogens. As such, in the presence of copper, the acetate was degraded without active methane production.

To demonstrate clearly the effect of Cu^{2+} on sulfate reduction and denitrification in granular sludge, the effect of the initial concentration of Cu^{2+} was plotted relative to the reduction rate (Fig. 3). The inhibitory effect of Cu^{2+}

was greater on the denitrification activities than the sulfate reduction. However, there was no significant difference in the sulfate and nitrate reduction ratios between the intact and disintegrated granules. This result differed from those related to acetate and glucose degradation [3]. This means that UASB granulation was not helpful in regards to the resistance of SRB and NRB to Cu^{2+} toxicity under the current experimental conditions.

It is generally known that anaerobic SRB and NRB make up minor portions of granular sludges [5]. Thus, when considering that the granules reduced the nitrate and sulfate present (2.2 mM and 5.4 mM, respectively) in the presence of 400 mM copper, about 18% of the initial acetate was stoichiometrically converted to CO_2 by anaerobic SRB and NRB. This indicates that respiratory nitrate and sulfate reduction had a significant effect on the acetate degradation of the UASB granules. However, since this value only corresponded to 24% of the missing acetate, the SRB and NRB activity did not account for all the missing acetate. It would seem that the rest of the missing acetate might have been consumed by other populations, accumulated as intermediates during the methanogenesis, or stored as
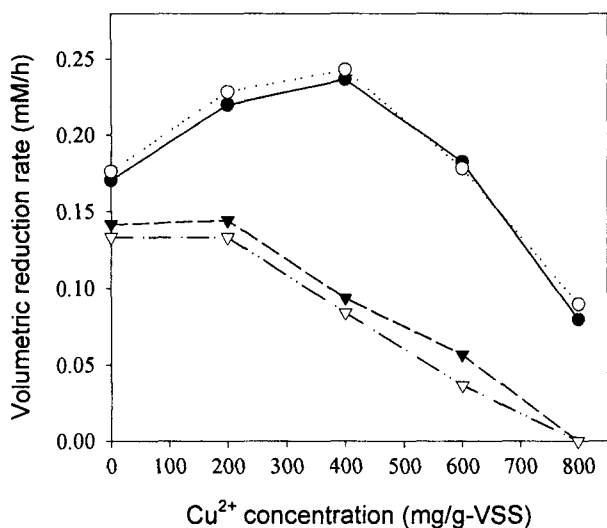
Fig. 3. Change of rate constant of sulfate reduction and denitrification by increasing the concentration of Cu^{2+}.

(●) Sulfate reduction by intact granules, (○) sulfate reduction by disintegrated granules, (▼) nitrate reduction by intact granules, (▽) nitrate reduction by disintegrated granules.

storage materials such as poly-β-hydroxybutyrate [4, 17]. Further elucidation of this discrepancy in the stoichiometry is under investigation.

REFERENCES

1. Alphenaar, P. A., A. Visser, and G. Lettinga. 1993. The effect of liquid upward velocity and hydraulic retention time on granulation in UASB reactors treating wastewater with a high sulphate content. *Bioresource Technol.* **43:** 249–258

2. Bae, J.-W. and S.-T. Lee. 1999. Layered structure of UASB granules gives microbial population resistance to toxic chemicals. *Biotech. Lett.* **21:** 159–162.

3. Bae, J.-W., S.-K. Rhee, S.-H. Hyun, I. S. Kim, and S.-T. Lee. 2000. Layered structure of granules in upflow anaerobic sludge blanket reactor gives microbial populations resistance to metal ions. *Biotech. Lett.* **22:** 1935–1940.

4. Beun, J. J., K. Dircks, M. C. M. van Loosdrecht, and J. J. Heijnen. 2002. Poly-β-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures. *Water Res.* **36:** 1167–1180.

5. Bhatti, Z. I., K. Furukawa, and M. Fujita. 1997. Microbial diversity in UASB reactors. *Pure Appl. Chem.* **69:** 2431–2438.

6. Fang, H. H. P. 1997. Inhibition of bioactivity of UASB biogranules by electroplating metals. *Pure Appl. Chem.* **69:** 2425–2429.

7. Friedmann, E. I. and A. P. Kibler. 1980. Nitrogen economy of endolithic microbial communities in hot and cold deserts. *Microb. Ecol.* **6:** 95–108.

8. Gujer, W. and A. J. B. Zehnder. 1983. Conversion processes in anaerobic digestion. *Water Sci. Technol.* **15:** 127–167.

9. Harada, H., S. Uemura, and K. Momonoi. 1994. Interaction between sulfate-reducing and methane-producing bacteria in UASB reactors fed with low strength wastes containing different levels of sulfate. *Water Res.* **28:** 355–367.

10. Isa, Z., S. Grusenmeyer, and W. Verstraete. 1986. Sulfate reduction relative to methane production in high-rate anaerobic digestion: Technical aspects. *Appl. Environ. Microbiol.* **51:** 572–579.

11. Jeris, J. S. and P. L. McCarty. 1965. The biochemistry of methane fermentation using C14 tracers. *J. Water Pollut. Control Fed.* **37:** 178–192.

12. Jetten, M. S. M., A. J. M. Stams, and A. J. B. Zehnder. 1992. Methanogenesis from acetate: A comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* sp. *FEMS Microbiol. Rev.* **88:** 181–198.

13. Kim, H. Y., T. S. Kim, and B. H. Kim. 1991. Isolation and characterization of a dibenzothiophene degrading sulfate-reducing soil bacterium. *J. Microbiol. Biotechnol.* **1:** 1–5.

14. Lim, J. G. and D. H. Park. 2001. Degradation of polyrinyl alcohol by *Brewbacillus laterosporus*: Metabolic Pathway of Polyrinyl alcohol to acetate. *J. Microbiol. Biotechnol.* **11:** 928–933.

15. McCartney, D. M. and J. A. Oleszkiewicz. 1991. Sulfide inhibition of anaerobic degradation of lactate and acetate. *Water Res.* **25:** 203–209.

16. Omil, F., S. J. W. H. Oude Elferink, P. Lens, L. W. Hulshoff Pol, and G. Lettinga. 1997. Effect of the inoculation with *Desulforhabdus amnigenus* and pH or O_2 shocks on the competition between sulphate reducing and methanogenic bacteria in an acetate fed UASB reactor. *Biores. Technol.* **60:** 113–122.

17. Oude Elferink, S. J. W. H., A. Visser, L. W. Hulshoff Pol, and A. J. M. Stams. 1994. Sulfate reduction in methanogenic bioreactors. *FEMS Microbiol. Rev.* **15:** 119–136.

18. Park, E.-J., J.-K. Seo, J.-K. Kim, K.-H. Suh, and S.-K. Kim. 2000. Denitrification characteristics and microorganisms composition of acclimated denitrifier consortium. *J. Microbiol. Biotechnol.* **10:** 410–414.

19. Smith, P. H. and R. A. Mah. 1966. Kinetics of acetate metabolism during sludge digestion. *Appl. Microbiol.* **14:** 368–371.

20. Uberoi, V. and S. K. Bhattacharya. 1997. Effects of chlorophenols and nitrophenols on the kinetics of propionate degradation in sulfate reducing anaerobic systems. *Environ. Sci. Technol.* **31:** 1607–1614.

21. Visser, A., I. Beeksma, F. Van der Zee, A. J. M. Stams, and G. Lettinga. 1993a. Anaerobic degradation of volatile fatty acids at different sulphate concentrations. *Appl. Microbiol. Biotechnol.* **40:** 549–556.

22. Visser, A., P. A. Alphenaar, Y. Gao, G. Van Rossem, and G. Lettinga. 1993b. Granulation and immobilisation of methanogenic and sulfate-reducing bacteria in high-rate anaerobic reactors. *Appl. Microbiol. Biotechnol.* **40:** 575–581.

23. Whitman, W. B., T. L. Bowen, and D. R. Boone. 1992. The methanogenic bacteria. *In* Balows A., H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (eds.) *The Prokaryotes*, Springer, Berlin Heidelberg New York, **2:** 719–767.