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PRECIPITATES IN ANAEROBIC GRANULES TREATING SULPHATE-BEARING WASTEWATER

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Abstract—Three types of biogranules treating wastewater containing various sulphate concentrations were examined using scanning and transmission electron microscopies. The two biogranules treating wastewater containing sulphate up to 2000 mg·l^{-1} showed high degrees of activities. However, activities of the third biogranules, which contained 102 mg-S·(g-VSS)$^{-1}$ after treating wastewater containing sulphate up to 7500 mg·l^{-1} for over 286 days, dropped drastically. Microscopic examinations showed that precipitates accumulated in the biogranules treating sulphate-bearing wastewater; the degrees of precipitation increased with sulphate concentration in wastewater and the duration of treatment. Excessive precipitation was found throughout the entire biogranule cross-section of the third biogranules. Analyses by X-ray spectrometry further showed that the precipitates were mainly composed of sulphur, plus copper, iron and nickel presumably in the form of metal sulphides. These observations suggest that the inhibition of bioactivities of anaerobic granules was likely resulted from the accumulated sulphureous precipitates on the bacterial surface. This point was overlooked by previous investigators who tended to attribute the inhibition to the toxic effects of either sulphate, sulphide or un-dissociated H_2S.

Key words—anaerobic, granule, inhibition, precipitate, SEM, sulphate, sulphide, TEM, X-ray

INTRODUCTION

Anaerobic technology has been applied to the full-scale treatment of high-strength industrial wastewater in the recent decade. However, many wastewaters, such as those from pharmaceutical, pulp/paper, fermentation, petrochemical and mining industries, also contain high concentration of sulphate. It has been widely reported that the bioactivity of anaerobic sludge could be detrimentally affected when treating sulphate-bearing wastewater (Hilton and Archer, 1988). The conventional hypothesis has been that bioactivity was inhibited by the increased concentrations of sulphidogeneous products, particularly the un-dissociated hydrogen sulphide (Speece, 1983), or even sulphate itself (Fang *et al.*, 1997). However, the inhibition concentrations of sulphide and sulphate reported in literature varied considerably.

Soluble sulphide can be present, depending upon the pH, in three forms: S^{2-}, HS^- and H_2S. The reported inhibition concentrations for total dissolved sulphide varied from 200 mg-S·l^{-1} (Oonge and Parkin, 1990), 150–200 mg-S·l^{-1} (Maillacheruvu *et al.*, 1993), 260 mg-S·l^{-1} (Li *et al.*, 1996) to 600 mg-S·l^{-1} (Rinzema and Lettinga, 1988). On the other hand, the reported inhibition concentrations for the un-dissociated H_2S varied from 50 mg-S·l^{-1} (Li *et al.*, 1996), 60 mg-S·l^{-1} (Oonge and Parkin, 1990; Maillacheruvu *et al.*, 1993), 70 mg-S·l^{-1} (Rinzema and Lettinga, 1988), to 100–150 mg-S·l^{-1} (Speece, 1983). It is difficult to compare these results, because the experimental conditions were often not mentioned in details. But, it is interesting to note that, in some cases, inhibition occurred at the time neither total dissolved sulphide nor un-dissociated H_2S was at the highest concentration (Rinzema and Lettinga, 1988; Maillacheruvu *et al.*, 1993; Fox and Venkatasubbiah, 1996; Fang *et al.*, 1997).

Fang *et al.* (1997) recently treated wastewater containing sulphate and benzoate in two UASB (upflow anaerobic sludge blanket) reactors (Lettinga *et al.*, 1980). They found that there was no inhibition of bioactivities in one reactor when the mixed liquor contained up to 769 mg-S·l^{-1} of dissolved total-sulphide and 234 mg-S·l^{-1} of H_2S, while in the other reactor both activities were inhibited after 270 days of operation when dissolved total-sulphide and H_2S were at much lower concentrations. In another study treating leachate plus sulphate at concentrations up to 16800 mg·l^{-1}, Nedwell and Reynolds (1996) reported that inhibition of bioactivity did not occur until the system had been operated for 265 days. These results in literature seem to suggest that there may be factors,

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other than concentrations of sulphidogeneous products in the mixed liquor, which affect the inhibition of bioactivity. This study was thus conducted to further examine this issue.

MATERIALS AND METHODS

Three types of biogranules were sampled from two UASB reactors for TEM (transmission electron microscopy), SEM (scanning electron microscopy) and X-ray spectrophotometry analyses. Both UASB reactors were treating wastewaters at 37°C containing benzoate as the sole organic substrate, plus different concentrations of sulphate. Details of experimental conditions and performance of these two reactors have been reported elsewhere (Li *et al.*, 1995; Fang *et al.*, 1997).

The biogranules were examined using a scanning electron microscope (Cambridge Stereoscan 360) and a transmission electron microscope (Jeol JEM 2000FX) in conjunction with an X-ray microanalyzer (Link Analytical, Model eXL). For SEM examinations, the biogranule samples were washed three times in a 0.1 M phosphate buffer solution at pH 7.2, and then fixed in the phosphate buffer solution with 2.5% glutaraldehyde for 24 h. The fixed granules were then sliced in the phosphate buffer solution exposing its interior. The sliced granules were dehydrated stepwise by immersing in a graded series of ethanol/water solutions, and then critical-point dried with carbon dioxide (Fang *et al.*, 1994). The dried granules were mounted on a stud with colloidal carbon followed by sputter coating with gold and palladium, or vacuum coating with carbon. Under SEM and TEM, the granule sample was bombarded with electrons, which caused X-ray to be emitted from the sample surface (Fang and Liu, 1995). Using an electron probe pointing at a specific location of the granule interior, individual elements at the pointed location could be determined by an energy-dispersive X-ray spectrometer qualitatively (from the energy of emission) and semi-quantitatively (from the intensity). In the X-ray dot-mapping analysis, the electron probe scanned the cross-section of the sliced granule and the emitted X-ray was used to produce a micrograph depicting the distribution of the corresponding element on the whole cross-section. A number of elements could be detected, according to the energy levels selected. The relative concentration of the element corresponding the specific energy level was indicated by the degree of brightness (Goldstein *et al.*, 1981).

For TEM examinations, granules were first fixed overnight at 4°C in a 0.1 M cacodylate buffer solution (pH 7.4) containing 2.5% glutaraldehyde and 4% paraformaldehyde. After washing three times in the cacodylate buffer, the granules were then washed with cacodylate buffer and dehydrated in a graded series of ethanol. Ethanol was again cleared with two exposures of propylene oxide for 15 min. The samples were then transferred to a mixture (1:1) of propylene oxide and resin (TAAB 812 Resin, U.K.) for 3 h and in the pure resin for 12 h for infiltration. Finally, the granules were embedded in fresh resin and polymerized at 60°C for 24 hr. Ultrathin sections were cut with an ultramicrotome (Ultracut E, Reichert–Jung, Austria) by using a diamond knife and collected on 200-mesh formvar-coated copper grids. Ultrasections were stained with uranyl acetate for 20 min and lead citrate for 10 min and finally examined by the TEM at an accelerating voltage of 80 kV.

The gross contents of sulphur and metals in the three biogranules were also analyzed. The total-sulphur content was analyzed following the procedures recommended by the British Standards Institution (1990). Metal contents were analyzed by an Atomic Absorption Analyzer (Perkin Elmer, model *AA*nalyst 300) at 248.3, 232.0 and 324.8 nm for Fe, Ni, and Cu, respectively. The corresponding slit widths were 0.2, 0.2 and 0.7 nm. Prior to the measurements, biogranule samples were digested in a mixed solution of HNO_3 and H_2O_2 following the procedures recommended by Perkin (1994). The volatile suspended solids (VSS), which is a measurement of biomass content, were analyzed following the *Standard Methods* (APHA, 1989).

RESULTS AND DISCUSSION

Three types of biogranules were sampled from two separate UASB reactors. Type-A biogranules (Li *et al.*, 1995) treated wastewater containing 2675 $mg \cdot l^{-1}$ of benzoate, at a COD (chemical oxygen demand) loading rate of 13.1 $g \cdot (l \cdot day)^{-1}$, plus a small quantity (280 $mg \cdot l^{-1}$) of sulphate as sulphur source for bacterial growth. These biogranules, which consistently removed 98–99% of benzoate for 150 days.

Types-B and -C biogranules were sampled from another reactor treating wastewater containing 2520 $mg \cdot l^{-1}$ of benzoate, at a loading rate of 10 $g\text{-}COD \cdot (l \cdot day)^{-1}$, plus sulphate at step-increased concentrations (Fang *et al.*, 1997). Sampled on day 141 while treating wastewater containing up to 2000 $mg \cdot l^{-1}$ of sulphate, Type-B biogranules were able to remove 100% of soluble COD and reduce up to 56% of sulphate. The methanogenic and sulphidogenic activities of biogranules in the UASB reactor remained unaffected when the sulphate concentration was step-increased up to 6000 $mg \cdot l^{-1}$.

However, both activities drastically decreased at a rapid pace after the sulphate concentration was increased to 7500 $mg \cdot l^{-1}$ on day 270. Type-C biogranules were sampled on day 286 after the reactor had treated wastewater containing 7500 $mg \cdot l^{-1}$ of sulphate for 16 days; at that time the biogranules removed only 80% of COD and reduced 12% of sulphate from the wastewater. By day 320 when the experiment was terminated, the reactor COD removal was further lowered to 28% and sulphate reduction to nearly nil. The drastic inhibition of methanogenic and sulphidogenic activities was unlikely due to the sulphate concentration in wastewater, because Nedwell and Reynolds (1996) had treated leachate containing up to 16800 $mg \cdot l^{-1}$ of sulphate. On the other hand, during this period, the mixed liquor on average contained 147 $mg\text{-}S \cdot l^{-1}$ of total-sulphide and 8.5 $mg\text{-}S \cdot l^{-1}$ of un-dissociated H_2S. The drastic inhibition of bioactivities was, thus, also unlikely resulted from toxicity of either total-sulphide or H_2S, because a parallel study showed that there was no inhibition of bioactivities of biogranules when the mixed liquor contained up to 769 $mg\text{-}S \cdot l^{-1}$ of total-sulphide and 234 $mg\text{-}S \cdot l^{-1}$ of H_2S.

Figure 1 comprises three X-ray dot-mapping micrographs illustrating the distribution of sulphur element (S) in the cross-section of biogranules: (a)

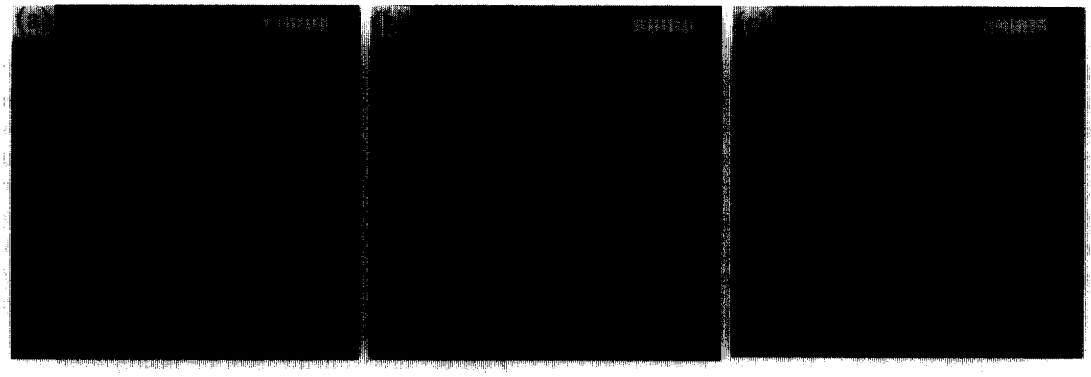

Fig. 1. X-ray dot-mapping micrographs of sulphur precipitates in biogranules treating wastewater for various durations and sulphate concentrations: (a) 150 days and 280 mg·l^{-1} of sulphate, (b) 141 days and up to 2000 mg·l^{-1} of sulphate, (c) 286 days and up to 7500 mg·l^{-1} of sulphate. (bar = 500 μm).

Type-A, (b) Type-B, and (c) Type-C. The brightness of a given spot reflects the S concentration at that location. Figure 1(a) illustrates that there was little S precipitates in Type-A biogranules, obviously due to the low concentration of sulphate in the wastewater. Figure 1(b) illustrates that there were substantial S precipitates in Type-B biogranules. One would normally expect more S would precipitate near the granule surface because of the concentrated sulphate in the mixed liquor. However, it is of interest to note that the precipitation was rather uniform throughout the biogranule cross-section. This indicates that sulphate diffused rapidly into the biogranules, and was reduced by bacteria throughout the biogranule. Figure 1(b) further illustrates that a significant fraction of S were precipitated in layers. Since most of the S would precipitate in areas with high population of sulphate-reducing bacteria, Fig. 1(b) seems to suggest that sulphate-reducing bacteria were distributed in layers inside the biogranules. Although it has been reported (Fang *et al.*, 1994) that benzoate-degrading biogranules had a two-layer microstructure with syntrophic acetogens concentrated in the outer layer and methanogens in the interior, there is no prior report on the layered distribution of S precipitates and thus sulphate-reducing bacteria, as illustrated in Fig. 1(b). This phenomenon is intriguing and warrants further investigation which might lead to a better understanding on the granulation mechanism of anaerobic sludge.

Figure 1(c) illustrates that S was densely precipitated throughout the whole biogranules. This was probably due to the large quantity of additional S precipitation after the biogranules had treated the sulphate-bearing wastewater for a prolonged period (286 days) with increased sulphate concentration (up to 7500 mg·l^{-1}). The pattern of layered precipitation disappeared because of the excessive production of S precipitates, some of which spilled to areas where the sulphate-reducing bacteria were less populated.

Figure 2(a) is a typical X-ray spectrum of precipitates, as marked by an arrow in Fig. 2(b), inside a Type-B biogranule. It illustrates that the precipi-

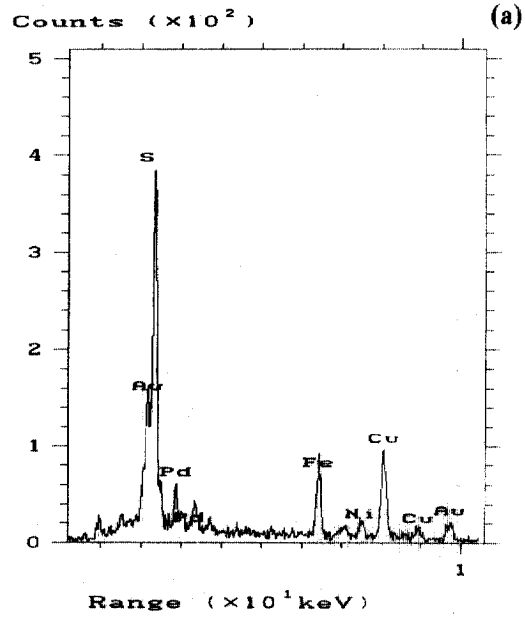

Fig. 2. X-ray spectrum analysis (a) of precipitates on bacterial surface (b) of a sulphate-reducing biogranule. (bar = 1.0 μm).

tates were composed of mostly S, plus copper, iron and nickel; presumably, the metals were in the form of metal sulphides. The presence of gold and palladium in Fig. 2(a) was because they were used to coat the sample surface during the preparation process. Sulphate reduction in the presence of benzoate is a complex process, involving a number of bacteria and steps. Sulphate was reduced to sulphide and possibly elemental S. However, X-ray analysis could not differentiate these two forms of S.

Sulphate can be reduced by bacteria competing with syntrophic acetogens for benzoate as substrate, converting benzoate either into acetate or directly into bicarbonate (Holt *et al.*, 1994). Sulphate can also be reduced by another group of bacteria competing with methanogens for acetate and hydrogen (Holt *et al.*, 1994). Thus, in the biogranules, the former sulphate-reducing bacteria tend to be syntrophically associated with acetotrophic methanogens whereas the latter sulphate-reducing bacteria tend to be syntrophically associated with other bacteria producing acetate and/or hydrogen.

Figure 3 are scanning electron micrographs illustrating syntrophic colonies composing of *Desulfovibrio*-like bacteria in (a) Type-A, (b) Type-B, and (c) Type-C biogranules, respectively. Figure 3(a) illustrates that there were few *Desulfovibrio*-like bacteria and little precipitates in Type-A biogranules, as expected for treating wastewater containing little sulphate. Figure 3(b) illustrates an increase of *Desulfovibrio*-like bacteria population and scattered S precipitates on the bacteria surface, as the sulphate concentration in the wastewater was increased up to 2000 $mg \cdot l^{-1}$. Figure 3(c) further illustrates that, after treating wastewater for 286 days containing sulphate up to 7500 $mg \cdot l^{-1}$, sulphate-

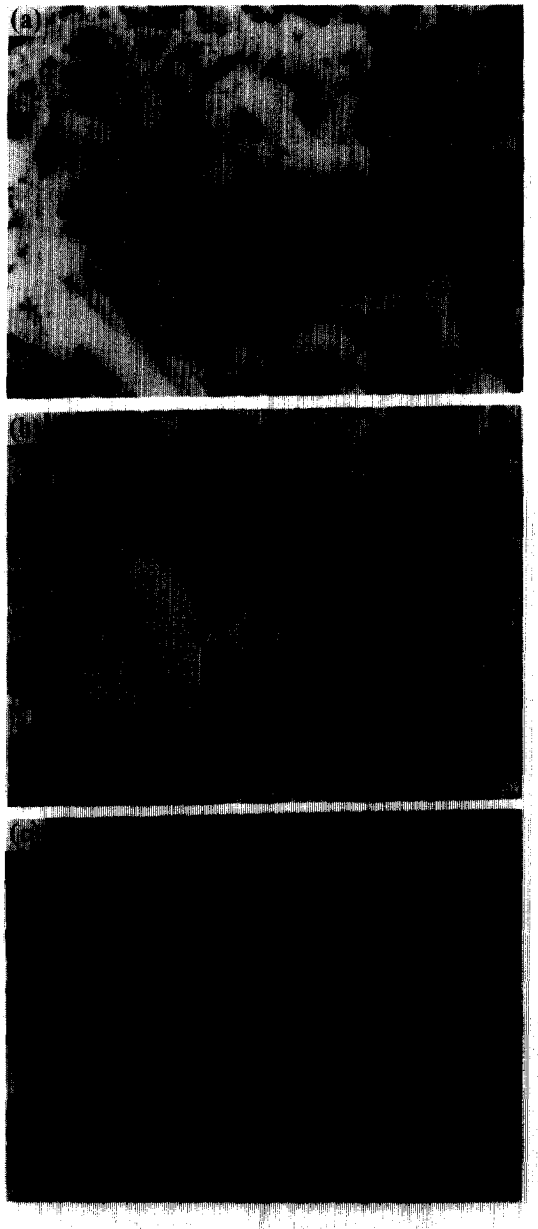

Fig. 3. Syntrophic bacteria in biogranules treating wastewater for various durations and sulphate concentrations: (a) 150 days and 280 $mg \cdot l^{-1}$ of sulphate, (b) 141 days and up to 2000 $mg \cdot l^{-1}$ of sulphate, (c) 286 days and up to 7500 $mg \cdot l^{-1}$ of sulphate. (*Desulfovibrio*-like bacteria are marked with an arrow). (bar = 2.0 μm).

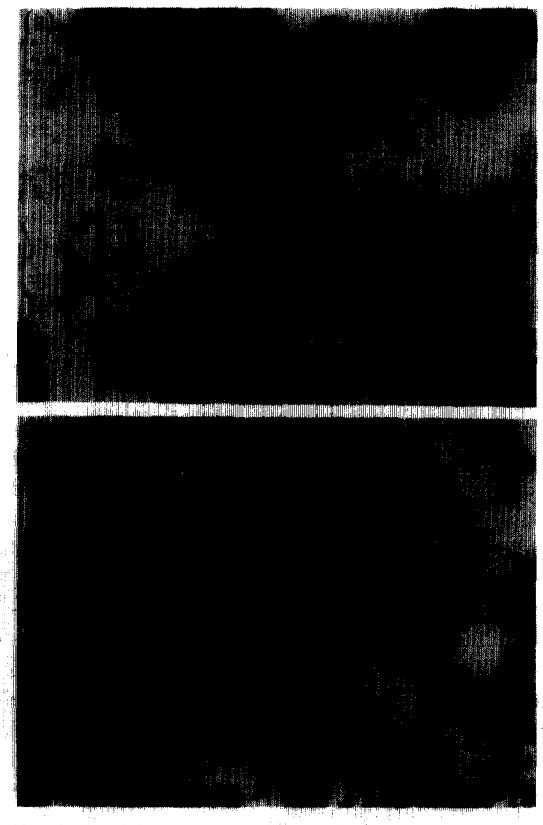

Fig. 4. Precipitates on sulphate-reducing bacteria in biogranules treating wastewaters: (a) 141 days and up to 2000 $mg \cdot l^{-1}$ of sulphate, and (b) 286 days and up to 7500 $mg \cdot l^{-1}$ of sulphate. (bar = 1.0 μm).

reducing bacteria are coated by heavy S precipitates. Similar phenomena are also illustrated in transmission electron micrographs. Figure 4(a) illustrates the scattered S precipitates on bacteria in Type-B biogranules, whereas Fig. 4(b) illustrates that bacteria in Type-C biogranules were completed coated by precipitates. It is very likely that these heavy precipitates, as clearly illustrated in Fig. 3(c) and Fig. 4(b), resulted in the loss of bioactivities of the Type-C biogranules.

Acetate is a key intermediate produced by both sulphate-reducing and syntrophic acetogenic bacteria. It can be further converted to methane by a number of bacteria, among them *Methanothrix* (Zehnder *et al.*, 1980) was found most abundant in UASB biogranules (Li *et al.*, 1995). Figure 5(a), (b) are scanning electron micrographs illustrating that *Methanothrix*-like bacteria in Types-A and -B biogranules, respectively, were free of precipitates. However, considerable precipitates were found in colonies of *Methanothrix*-like bacteria in Type-C biogranules, as illustrated in Fig. 5(c). Similar phenomena were also shown in transmission electron micrographs. Figure 6(a) illustrates colonies of *Methanothrix*-like bacteria were free of precipitates in Type-B biogranules, but Fig. 6(b) illustrates that they were coated with scattered precipitates in Type-C biogranules. The latter was likely due to the spill of excessive S precipitation from the neighboring sulphate-reducing colonies.

Results of total-sulphur and metal analyses confirmed the TEM and SEM observations. The total-S content in biomass (as measured by VSS) in the precipitates-free Type A biogranules was 40.5 mg·(g-VSS)$^{-1}$; but it increased to

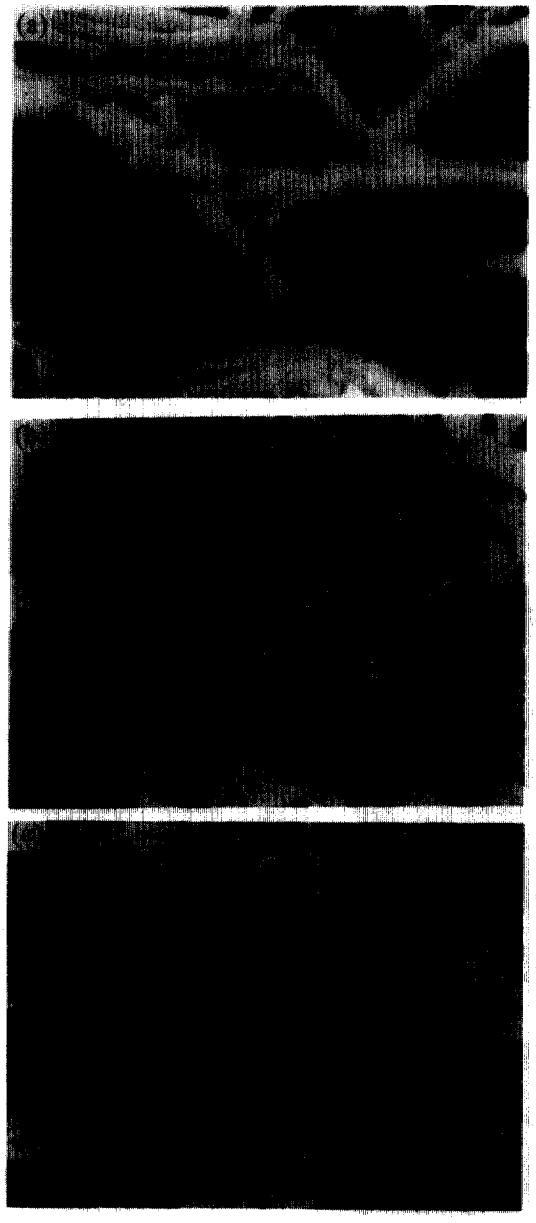

Fig. 5. *Methanothrix*-like bacteria in biogranules treating wastewater for various durations and sulphate concentrations: (a) 150 days and 280 mg·l^{-1} of sulphate, (b) 141 days and up to 2000 mg·l^{-1} of sulphate, (c) 286 days and up to 7500 mg·l^{-1} of sulphate. (bar = 2.0 μm).

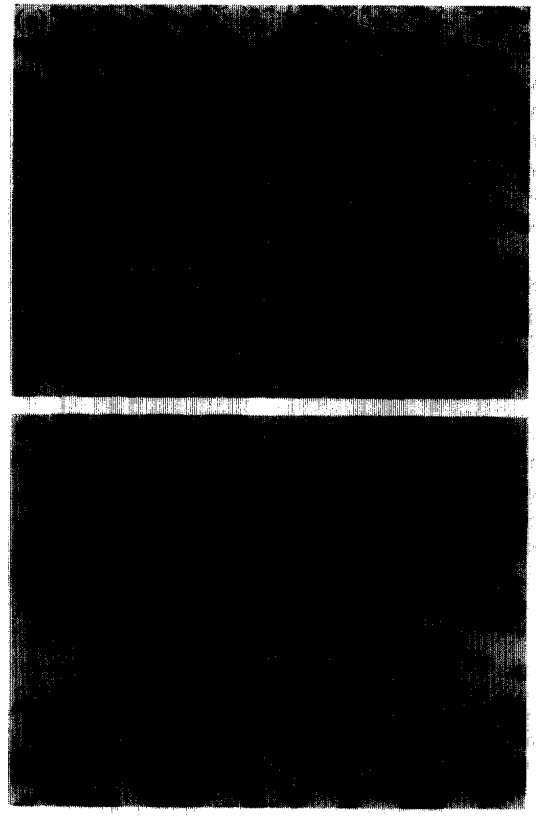

Fig. 6. Precipitates on *Methanothrix*-like bacteria in biogranules treating wastewaters: (a) 141 days and up to 2000 mg·l^{-1} of sulphate, and (b) 286 days and up to 7500 mg·l^{-1} of sulphate. (bar = 1.0 μm).

58.3 mg·(g-VSS)$^{-1}$ in Type B, and 102.4 mg·(g-VSS)$^{-1}$ in Type C. The extra sulphur contents in Types B and C biogranules were presumably attributed to the precipitation. These results suggest that bioactivities were inhibited when the total-S content in biogranules reached the level of 100 mg·(g-VSS)$^{-1}$. Metal contents in Type C biogranules were 25 mg-Fe·(g-VSS)$^{-1}$, 169 mg-Ni·(g-VSS)$^{-1}$ and 165 mg-Cu·(g-VSS)$^{-1}$. They were also considerably higher than those in Type B (18 mg-Fe·(g-VSS)$^{-1}$, 107 mg-Ni·(g-VSS)$^{-1}$ and 14 mg-Cu·(g-VSS)$^{-1}$) and Type C (16 mg-Fe·(g-VSS)$^{-1}$, 75 mg-Ni·(g-VSS)$^{-1}$ and 10 mg-Cu·(g-VSS)$^{-1}$).

Results of this study, based on microscopic observations, X-ray spectrometry and elemental analyses, suggest that the inhibition of methanogenic and sulphidogenic activities of biogranules in treating sulphate-bearing wastewater could likely be resulted from the excessive S precipitation on the bacterial cell surface throughout the entire cross-section of biogranules. The degrees of S precipitation depend not only upon the concentration of sulphate in wastewater, but also upon the duration of the treatment process.

CONCLUSION

SEM and TEM observations showed that precipitates accumulated in biogranules treating sulphate-bearing wastewater. The degrees of precipitation increased with sulphate concentration in wastewater and the duration of treatment. Excessive precipitation was found throughout the entire biogranule cross-section. The total sulphur content in biomass reached 102 mg·(g-VSS)$^{-1}$ after treating wastewater containing sulphate up to 7500 mg·l^{-1} for over 286 days. Analyses by X-ray spectrometry further showed that the precipitates were mainly composed of sulphur, plus copper, iron and nickel presumably in the form of metal sulphides. These observations suggest that the inhibition of bioactivities of anaerobic granules was likely resulted from the accumulated sulphureous precipitates on the bacterial surface. This point was overlooked by previous investigators who tended to attribute the inhibition to the toxic effects of either sulphate, sulphide or undissociated H_2S.

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REFERENCES

APHA (1989) *Standard methods — for the examination of water and wastewater*, 17th edn. American Public Health Association, Washington, DC.

British Standards Institution (1990) Methods for analysis of coal and coke, Determination of total sulphur content. BS1016: 106.4.1.

Fang H. H. P., Chui H. K. and Li Y. Y. (1994) Microbial structure and activity of UASB granules treating different wastewaters. *Water Sci. Tech.* **30**, 87–96.

Fang H. H. P. and Liu Y. (1995) X-ray analysis of anaerobic granules. *Biotech. Tech.* **9**, 513–518.

Fang H. H. P., Liu Y. and Chen T. (1997) Effect of sulphate on anaerobic degradation of benzoate in UASB reactors. *J. Environ. Eng.* **123**(4). 320–328.

Fox P. and Venkatasubbiah V. (1996) Coupled anaerobic/aerobic treatment of high-sulphate wastewater with sulphate reduction and biological sulphide oxidation. *Water Sci. Tech.* **34**, 359–366.

Goldstein J. I., Newbury D. E., Echlin P., Joy D. C., Fiori C. and Lifshin E. (1981). *Scanning electron microscopy and X-ray microanalysis — A text for biologists, materials scientists, and geologists*, pp. 299–231. Plenum Press, New York.

Hilton M. G. and Archer D. B. (1988) Anaerobic digestion of a sulphate-rich molasses wastewater: inhibition of hydrogen sulphide production. *Biotech. Bioeng.* **31**, 885–888.

Holt J. G., Krieg N. R., Sneath P. H. A., Staley J. T. and Williams S. T. (Eds.) (1994) *Bergey's Manual of Determinative Bacteriology*, 9th edn., pp. 335–346, 719–737. Williams and Wilkins, Baltimore, U.S.A.

Lettinga G., van Velsen A. F. M., Hobma S. M., de Zeeuw W. and Klapwijk A. (1980) Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment. *Biotech. Bioeng.* **22**, 699–734.

Li Y. Y., Fang H. H. P., Chui H. K. and Chen T. (1995) UASB treatment of wastewater with concentrated benzoate. *J. Environ. Eng.* **121**(10), 748–751.

Li Y. Y., Lam S. and Fang H. H. P. (1996) Effect of sulphate on anaerobic benzoate degradation. *Water Res.* **30**, 1555–1562.

Maillacheruvu K. Y., Parkin G. F., Chen Y. P., Kou W. C., Oonge Z. I. and Lebduschka V. (1993) Sulphide toxicity in anaerobic systems fed sulphate and various organics. *Wat. Environ. Res.* **65**, 100–108.

Nedwell D. B. and Reynolds P. J. (1996) Treatment of landfill leachate by methanogenic and sulphate-reducing digestion. *Water Res.* **30**, 21–28.

Oonge Z., and Parkin G. F. (1990) Poisoning of sulphate reduction with molybdenum in anaerobic reactors fed glucose, *45th Purdue Industrial Waste Conference Proceedings*, pp. 441–450. Lewis Publisher, Michigan, U.S.A.

Perkin E. (1994) *Analytical methods for atomic absorption spectrometry*, Sect. AY-3, pp. 136–137. Perkin Elmer, Norwalk, CT, U.S.A.

Rinzema A. and Lettinga G. (1988) The effect of sulphide on the anaerobic degradation of propionate. *Environ. Technol. Letters* **9**, 83–88.

Speece R. E. (1983) Anaerobic biotechnology for the industrial wastewater treatment. *Environ. Sci. Tech.* **17**(9), 416A–427A.

Zehnder A. J. B., Huser B. A., Brock T. D. and Wuhrmann K. (1980) Characterization of acetate-decarboxylating, non-hydrogen-oxidizing methane bacterium. *Arch. Microbiol.* **124**, 1–11.