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EXTRACELLULAR POLYMERS OF HYDROGEN-UTILIZING METHANOGENIC AND SULFATE-REDUCING SLUDGES

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Abstract—Extracellular polymers (ECP) play an important role in biological wastewater treatment. They are believed to be responsible for the formations of flocculent activated sludge as well as anaerobic granules. In this study, the ECP characteristics of two types of enrichment cultures were examined. One enriched culture was composed of hydrogenotrophic methanogens (HM) alone while the other was composed of HM and hydrogenotrophic sulfate-reducing bacteria (HSR). Both cultures were enriched through a series of 107 repeated batches using H_2/CO_2 plus, in the HM/HSR series, sulfate and nutrients. At various stages of the enrichment process, the ECP were extracted from the sludge samples and analyzed for their protein (ECP_p) and carbohydrate (ECP_c) contents. Results showed that in the mixed culture HM consumed 67% of hydrogen and HSR 33%. The net yields of biomass for HM and HSR cultures were 0.046 g-VSS/g-COD and 0.059 g-VSS/g-COD, respectively. Productions of ECP were dependent upon substrate. Glucose-degrading sludge produced more ECP than the HM culture, which in turn produced more than the HSR culture. The net ECP yields for HM culture were 1.06 mg-ECP_p/g-COD and 0.64 mg-ECP_c/g-COD, respectively; the corresponding yields for HSR culture were 0.74 mg-ECP_p/g-COD and 0.52 mg-ECP_c/g-COD. For the enriched HM culture, more ECP_p, as well as ECP_c but to a lesser degree, were produced at the beginning of each batch when high concentration of hydrogen was available. This was, however, not noticeable for the HSR culture. Copyright © 1996 Elsevier Science Ltd

Key words—anaerobes, carbohydrates, ECP, extracellular polymers, hydrogenotrophs, methanogenic, protein, sulfate-reducing

INTRODUCTION

In biological wastewater treatment, bacteria tend to aggregate forming flocs, biofilms and even granules. This allows the reactor to retain more biomass and to reduce the tendency of bacteria washout. Thus, it is critical for an activated sludge treatment plant to produce flocculent sludges. However, the precise mechanisms involved in this bioflocculation process have not been fully understood, even though extracellular polymers (ECP) have been believed to play a critical role. ECP are metabolic products of bacteria which excreted from the cell onto the cell surface (Morgan *et al.*, 1990). Tenney and Stumm (1968) believed that interactions of these polymers between cells allows adjacent bacteria to aggregate and to form flocs. Many mechanisms have been proposed for such a bioflocculation process (Tenney and Stumm, 1968; Busch and Stumm, 1968; Henning Ryssov-Nielson, 1975; Sutherland, 1985) by analyzing the complex electrostatic and chemical interactions between ECP of adjacent cells. However, studies of bioflocculation have been mostly limited to aerobic processes.

Anaerobic technology, on the other hand, has matured in the past two decades as a viable alternative to the aerobic processes for the treatment of concentrated industrial wastewaters. Anaerobic processes have two intrinsic advantages. They not only save the energy required for aeration but also convert wastes into a useful fuel, methane; furthermore, they produce less sludge than the aerobic counterparts and, thus, significantly reduce the cost related to sludge handling and disposal. Among the high-rate anaerobic reactors developed in recent years, the upflow anaerobic sludge blanket (UASB) reactor (Lettinga *et al.*, 1980; Fang and Chui, 1993; Fang *et al.*, 1995) has probably received most commercial interests, especially in Europe and, more recently, in Asia. In the UASB reactor, bacteria aggregate to form biogranules which have high activity and also settle well.

Ross (1984) suggested that the bioflocculation mechanisms proposed for the aerobic processes may also be applicable to the formation of anaerobic granules. However, research on the ECP production

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of anaerobic sludge is still very limited. Harada *et al.* (1988) examined the average size and mechanical strength of two types of UASB granules: one degraded carbohydrates and the other degraded short chain fatty acids. They found that the former was larger in size and have higher mechanical strength than the latter; more ECP produced by the former granules was believed the reason. Sam-Soon *et al.* (1987, 1991), on the other hand, speculated that high hydrogen pressure would induce hydrogen-utilizing methanogens to produce ECP, thus boosting sludge granulation. Morgan *et al.* (1990) showed that the ECP concentrations, as well as the electric charge, on the bacterial cell surface affected the structure stability of the anaerobic granules. Nevertheless, the precise role of ECP in granulation of anaerobic sludge is still not fully understood.

This study was conducted to examine the formation of ECP for two types of enrichment cultures: one was composed of hydrogenotrophic methongens (HM) alone while the other was a mixed culture comprising HM and hydrogenotrophic sulfate-reducing bacteria (HSR). Results from this study, hopefully, would lead to a better understanding of the granulation mechanism of anaerobic sludge.

MATERIALS AND METHODS

In this study, two series of culture enrichment experiments were conducted in parallel in 135 ml glass serum vials. Both series were run in duplicate to demonstrate the reproducibility. Runs 1 and 1a were conducted for the enrichment of HM, while Runs 2 and 2a were for mixed culture of HM and HSR. Each vial was seeded with 100 ml of flocculent sludge obtained from the blanket zone of a UASB reactor, which was operated at 5.0 g-COD/l d using glucose as substrate. The gas mixture of hydrogen and carbon dioxide was used as substrates in both series, in each of which the batch operation was repeated 107 times. In each batch, 5.0 ml of mixed liquor was removed from the vial, and was replenished with an equal volume of aqueous solution of pH 6.9–7.3 containing nutrients and trace metals using the formulation described in previous studies (Kuba *et al.*, 1990; Jia *et al.*, 1991). For Runs 2 and 2a, an extra 80 mg of sulfate was also added in all batches. Meanwhile, the vapour phase in each vial was replenished with a fresh mixture of hydrogen and carbon dioxide at the ratio of H_2:CO_2 = 4:1. A total 82 ml of gas mixture at room temperature (20°C) and atmospheric pressure was injected into each vial using a syringe, the amount of hydrogen added corresponding to 50 mg of COD.

Samples taken from the vapour phase of each vial were periodically checked for the hydrogen and methane contents using a gas chromatograph (GC; Shimadzu GC-8APT) equipped with a thermal conductivity detector using nitrogen as the carrier gas. A batch was assumed being completed when hydrogen was depleted from the vapour phase. One batch cycle would take seven hours in the early batches, but less than three hours in the later batches as hydrogen-utilizing cultures were gradually enriched. Throughout the experiment, all vials were submerged in a 35°C water batch sitting on a reciprocal shaker table (35 mm × 125 strokes min^{-1}); the vigorous shaking motion enhanced the dissolution of hydrogen and carbon dioxide from the vapour phase to the mixed liquor.

Throughout the 107-batch experiments, mixed liquor samples taken at the end of the batch were analyzed for the contents of volatile suspended solids (VSS), proteinaceous ECP (ECP_p) and carbohydrate ECP (ECP_c). The rates of hydrogen depletion as well as methane production during the batch operation were also closely monitored in a number of selected batches. For these batches, in order to obtain accurate methane and hydrogen concentration data, the residual vapour in each vial was flushed off by nitrogen before a fresh mixture of hydrogen and carbon dioxide was injected. The partial pressures of hydrogen and methane were then closely monitored by GC analysis. Based on these data, the specific hydrogen-utilizing and methanogenic activities of the enriched cultures were calculated.

The VSS contents were measured according to APHA (1985). ECP in the mixed liquor samples were extracted using the cold aqueous extraction techniques (Sutherland and Wilkinson, 1971; Jia *et al.*, 1991). Sludge in each mixed liquor sample was washed twice with de-ionized water followed by low speed (3500 rpm) centrifugation. The centrifuged biomass was then re-suspended in a 5 ml 8.5% sodium chloride solution containing 0.22% formaldehyde. The solution was then chilled in ice and mixed with an ultrasonic homogenizer at 40 W for 3 min, during which time the ECP of bacteria were extracted into the solution. After removing the residual solids by high speed centrifugation (12,000 rpm for 30 min), the carbohydrate content of the ECP (ECP_c) in the extracted solution was measured using the phenol/sulphuric acid method and the protein content (ECP_p) using the Folin method (Lowry *et al.*, 1951).

RESULTS AND DISCUSSION

Enrichments for hydrogen-utilizing anaerobes

A total of 107 batch cycles were repeated in both series. As the number of batch cycle increased, HM culture was enriched in Runs 1 and 1a while a mixed HM/HSR culture was enriched in Runs 2 and 2a. Figure 1 illustrates the changes in VSS content and specific methanogenic activity (SMA) in Runs 1 and 1a. Figure 2 illustrates the corresponding plots for Runs 2 and 2a. In all runs, the VSS content decreased as the batch proceeded, and it gradually levelled off after 80 batches to an average of 520 mg/l for Runs 1 and 1a, and 505 mg/l for Runs 2 and 2a. This indicates that those bacteria unable to use H_2/CO_2 as substrate were gradually washed out, while the hydrogen-utilizing culture were gradually enriched. The enrichment process became steady after about 80 batch cycles. This was confirmed by the SMA plots in Figs 1 and 2, which illustrate that for each gram of VSS the methanogenic activity increased with batch cycles, and reached maximum levels after about 80 batches.

Figures 1 and 2 further illustrate that the SMA of the enriched HM culture average 7.35 g-COD/g-VSS d, whereas the SMA of the enriched mixed HM/HSR culture was 4.94 g-COD/g-VSS d. In Runs 1 and 1a, hydrogen was utilized by HM alone, generating methane as a product; whereas in Runs 2 and 2a, HM and HSR competed for hydrogen producing methane and hydrogen sulfide, respectively. This is based on the assumption that there was little homoacetogenic activity which is thermodynamically less favorable (Li and Noike, 1989). Thus, the SMA of mixed HM/HSR culture was only 67% (100% × 4.94/7.35) of that of the "pure" HM culture,

suggesting that in the mixed HM/HSR culture 67% of hydrogen was utilized by the HM and the remaining 33% presumably by the HSR.

Based on these results, the net yields of both HM and HM/HSR mixed cultures could be estimated. Figure 3 illustrates that the SMA of HM increased linearly with the specific hydrogen utilizing activity with a slope of 0.943 (with $r = 0.993$). This means that HM culture consistently converted 94.3% of hydrogen to methane. The remaining 5.7% of hydrogen (as measured in COD) presumably could only be converted to biomass. Since each gram of HM was found to have 1.24 grams of COD equivalent, the net yield of HM was estimated at 0.046 g-VSS/g-COD-removed ($0.046 = 0.057/1.24$). This value could be checked by the mass balance. The total amount of hydrogen added in the last 30 batches equalled 1.50 g of COD, which would produce 69 mg of biomass assuming a net yield of 0.046 g-VSS/g-COD. By further assuming that the last 30 batches were operated under steady-state condition with an average VSS content of 520 mg/l, as illustrated in Fig. 1, the total amount of biomass removed from the vials in the last 30 × 5.0-ml samples was calculated as 78 mg (30 × 0.005 l × 520 mg-VSS/l). The 13% deviation from the estimated 69 mg was acceptable judging from the accuracies of the data used for the mass balance.

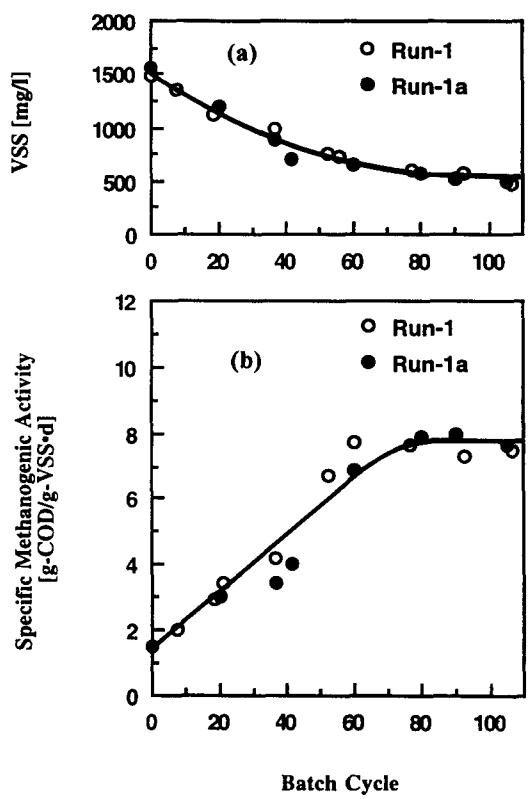

Fig. 1. (a) Reduction of VSS, and (b) increase of SMA during the enrichment of HM culture in Runs 1 and 1a.

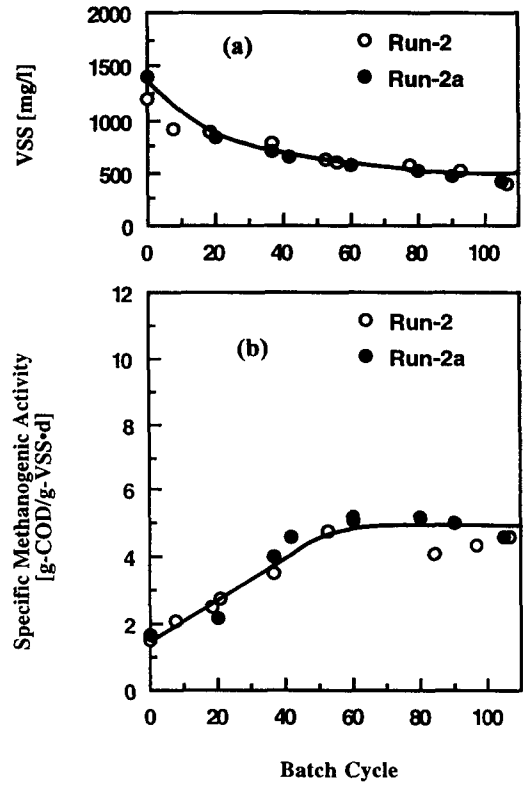

Fig. 2. (a) Reduction of VSS, and (b) increase of SMA during the enrichment of mixed HM/HSR culture in Runs 2 and 2a.

The net yield of 0.046 g-VSS/g-COD for HM culture observed in this study is comparable to the reported values of 0.043 g-VSS/g-COD (Shea *et al.*, 1968), 0.04–0.10 g-VSS/g-COD (Schauer and Ferry, 1980) and 0.04 g-VSS/g-COD (Gujer and Zehnder,

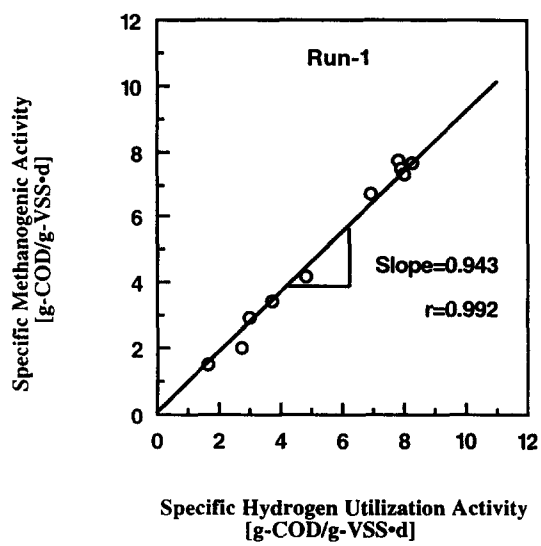

Fig. 3. Relation between SMA and specific hydrogen utilization activity for the HM culture.

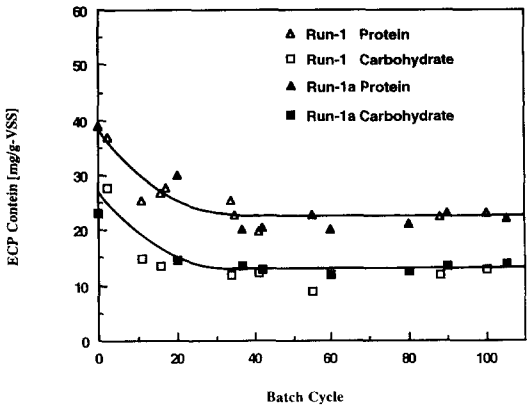

Fig. 4. Changes of ECP_p and ECP_c contents in VSS during the enrichment of HM culture in Runs 1 and 1a.

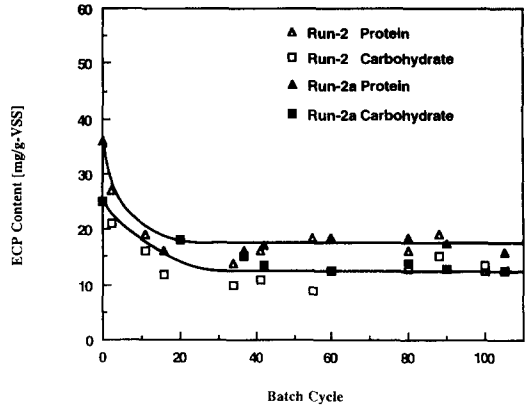

Fig. 5. Changes of ECP_p and ECP_c contents in VSS during the enrichment of mixed HM/HSR culture in Runs 2 and 2a.

1983) for the hydrogen-utilizing bacteria. It is known that all the hydrogen utilizing methanogens identified so far are also able to use formate as substrate producing similar amount of Gibbs free energy per reaction (Vogels *et al.*, 1988). It is, thus, of interest to note that the net yield of HM culture is also comparable to that of 0.050 g-VSS/g-COD for the formate-degrading sludge (Chui *et al.*, 1994).

Moreover, the net yield value of the HSR culture in Runs 2 and 2a could also be estimated from mass balance, based on the estimated yield of the HM culture. Assuming steady state condition and an average VSS of 505 mg/l in the last 30 batches in Runs 2 and 2a, as illustrated in Fig. 2, the total amount of hydrogen added equalled 1.50 g of COD of which 67% was converted to methane producing 46 mg HM culture. However, a total of 75 mg biomass of mixed HM/HSR culture was produced (30 × 0.005 l × 505 mg-VSS/l). The net yield of HSR culture was, thus, estimated as 0.059 g-VSS/g-COD, [75–46 mg-VSS/(1500 mg-COD × 0.33)], which is also comparable with the 0.053 g-VSS/g-COD reported by Robinson and Tiedje (1984).

Formation of ECP by hydrogen-utilizing anaerobes

All runs were started with flocculent glucose-degrading sludge, each gram of which contained 35–38 mg of ECP_p and 23–25 mg of ECP_c. Figures 4 and 5 illustrate that in all runs, both ECP_p and ECP_c contents decreased from these initial values and gradually levelled off to steady concentrations as hydrogen-utilizing cultures became enriched. The average ECP_p and ECP_c contents were 23 and 14 mg/g-VSS, respectively, for the HM culture, and the corresponding values for the enriched HM/HSR mixed culture were 19 and 12 mg/g-VSS, respectively. These results indicate that the glucose-degrading sludge contained more ECP than the two enriched hydrogenotrophic anaerobes, between which the HM culture contained more ECP than the HSR culture.

Moreover, results of mass balance calculations, using data of the last 30 batches and assuming steady state condition, show that ECP yields for the HM culture were 1.06 mg-ECP_p/g-COD and 0.64 mg-ECP_c/g-COD, whereas the corresponding yields for the HSR culture were 0.74 mg-ECP_p/g-COD and 0.52 mg-ECP_c/g-COD. Each gram of HSR culture contained 12.5 mg-ECP_p and 8.8 mg-ECP_c/g-VSS.

At the last batch of each run, the changes of ECP_p and ECP_c contents in the mixed liquor were closely monitored. Figure 6 illustrates that, in Runs 1 and 1a, the ECP_p and ECP_c contents of enriched HM culture increased readily as soon as the H_2/CO_2 mixture was

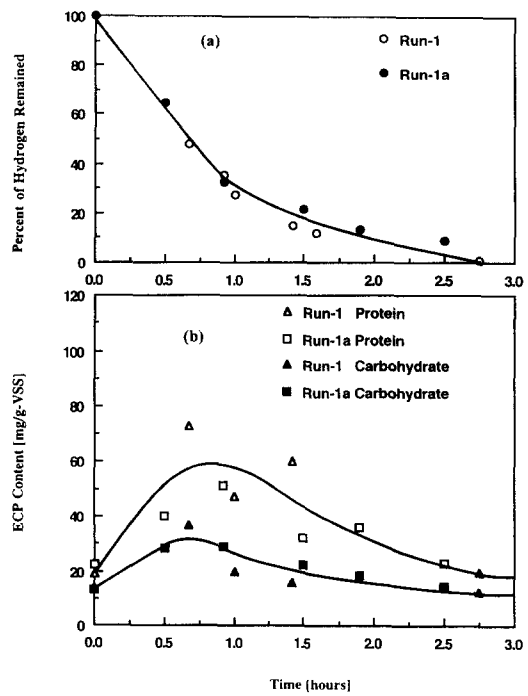

Fig. 6. Changes of (a) hydrogen in gas phase, and (b) ECP_p and ECP_c contents in VSS in a batch cycle for the enrichment of HM culture.

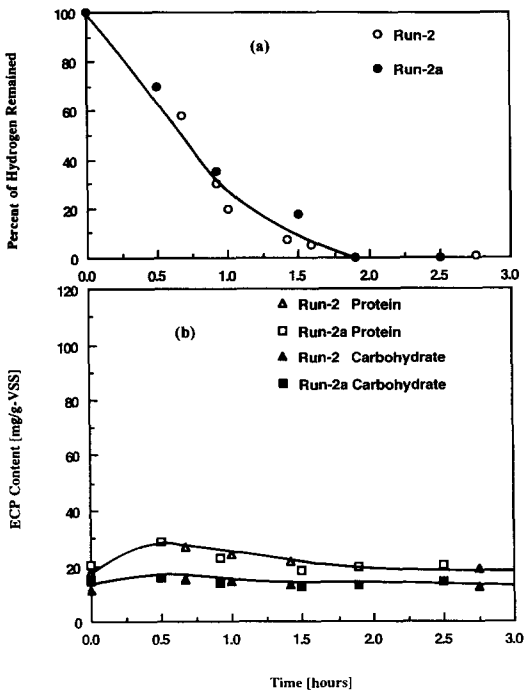

Fig. 7. Changes of (a) hydrogen in gas phase, and (b) ECP_p and ECP_c contents in VSS in a batch cycle for the enrichment of mixed HM/HSR culture.

added. The initial ECP increases are likely resulting from the high concentration of hydrogen. In two previous studies (Jia *et al.*, 1991; Furumai *et al.*, 1993), it was noted that the concentration of dissolved hydrogen in the mixed liquor of a UASB reactor treating glucose was the highest at the bottom, and became lower along the height of the reactor. Results illustrated in Fig. 6 suggest that it is likely the ECP produced at the bottom of the UASB reactor by the hydrogen-utilizing methanogens could play an important role in the granulation of sludge.

Figure 6 also illustrates that more ECP_p than ECP_c was produced at the beginning of the batch when the concentration of hydrogen remained high. It further illustrates that, as hydrogen became depleted, both types of ECP gradually returned to their original levels. The reason for this is not clear. On the other hand, Fig. 7 illustrates that for the enriched HM/HSR mixed culture, the increase of ECP at high concentrations of hydrogen was not as noticeable as the enriched HM culture. This is probably because the production of ECP by HSR culture was not as dependent on the initial hydrogen concentration.

CONCLUSIONS

From two series of hydrogenotrophic culture enrichment experiments conducted in this study, the following conclusion may be drawn: The net biomass yields were 0.046 g-VSS/g-COD and 0.059 g-VSS/g-COD, respectively, for HM and HSR cultures. In the mixed HM/HSR culture, 67% of hydrogen was utilized by the HM and 33% by HSR bacteria. Productions of ECP_p and ECP_c depended upon substrates. Glucose-degrading sludge produced more ECP_p and ECP_c than the HM culture, which in turn produced more ECP_p and ECP_c than the HSR culture. The net ECP yields for HM culture were 1.06 mg-ECP_p/g-COD and 0.64 mg-ECP_c/g-COD, respectively; the corresponding yield for HSR cultures were 0.74 mg-ECP_p/g-COD and 0.52 mg-ECP_c/g-COD. For the enriched HM culture, more ECP_p, as well as ECP_c but to a lesser degree, were produced at the beginning of each batch when more hydrogen was available. This effect was not noticeable to the HSR culture.

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