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# Layered structure of granules in upflow anaerobic sludge blanket reactor gives microbial populations resistance to metal ions

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## Abstract

Metal ions ($Cd^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Zn^{2+}$ and $Cr^{3+}$) did not affect glucose degradation or the production of methane during anaerobic digestion with intact and disintegrated granules from a UASB (Upflow Anaerobic Sludge Blanket) reactor. However, when $Cu^{2+}$ was at 500 mg $g^{-1}$ VSS (volatile suspended solids) in the media, the glucose degradation rates and methane production rates decreased by 14% and 32% in disintegrated granules, respectively, whereas, in intact granules, decreases were 3% and 14%, respectively. When various electroplating metal ions were tested, 50% inhibition of acetate degradation and methane production were produced by 210–770 mg $g^{-1}$ VSS and 120–630 mg $g^{-1}$ VSS, respectively. The relative toxicity of the electroplating metals on methane production was in the order of $Zn^{2+}$ (most toxic) $>$ $Ni^{2+}$ $>$ $Cu^{2+}$ $>$ $Cr^{3+}$ $>$ $Cd^{2+}$ (least toxic).

## Introduction

Anaerobic digestion has gained a solid position in the biological wastewater-treatment field over the past 20 years (Razoflores *et al.* 1996). During anaerobic digestion, complex organic substrates are first hydrolysed by enzymes forming soluble acids and sugars, which are then degraded by acidogenic bacteria into volatile fatty acids (VFAs). The intermediate VFAs are further degraded by acetogenic bacteria forming acetate, formate, $CO_2$ and $H_2$. These final intermediates are ultimately converted to methane by methanogenic bacteria.

The Upflow Anaerobic Sludge Blanket (UASB) reactor is an anaerobic wastewater treatment process that produces methane (De Zeeuw & Lettinga 1980). Because of its ability to retain a high biomass concentration, an UASB reactor can be operated at a high rate of digestion (Schmidt & Ahring 1996). After initial 1–6 months of UASB reactor operation, the suspended sludge is slowly flocculated and granulated. The granules of a successfully operated UASB reactor are very compact and have a high settling velocity (Quarmby & Forster 1995). This is caused by the inorganic nuclei (Fukuzaki *et al.* 1991) and the extracellular polymers produced by the acidogens, methanogens and $H_2$-consuming microorganisms (Shen *et al*. 1993). Studies on internal structure suggest that there are, essentially, three layers within a granule, each having a different bacterial composition (Macleod *et al*. 1990). The acidogens and $H_2$-producing and $H_2$-consuming organisms are located in the outer layer, and the methanogens at the center (Schmidt & Ahring 1996).

The structural characteristics of bacterial aggregates and high biomass retention increases the tolerance of anaerobic bacteria to toxic compounds (Blum *et al*. 1986, Dwyer *et al*. 1986, Fedorak & Hrudey 1986). A few reports have indicated that the high resistance of UASB granules to toxic chemicals is attributable to their layered structure (Fang 1997, Lin 1993). Our previous work (Bae & Lee 1999) observed that intact granules with a layered structure had more resistance to toxic organic chemicals than did disintegrated granules.

Metal ions as components of some wastewater inhibit the operation of the anaerobic digester (Lin 1993). Although metal ion toxicity to the conventional UASB process has been reported, there has been no report on the effect of the layered structure on the resistance of microorganisms to metal ions in the UASB reactor. In this paper, we describe the relationship between the layered structure of granules and their resistance to metal ions by comparing intact layered and disintegrated granules. This is the first comprehensive study to evaluate the effect of granular structure on microbial resistance to metal ions.

## Materials and methods

### *Source of granules and sludge*

Sludge granules were sampled from a 1000 $m^3$ UASB reactor treating beer brewery wastewater having a COD of 3000–4000 mg $l^{-1}$. The reactor has 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 diam and were brownish black. Scanning electron microscopy of granular sludge indicated that sampled granules had the layered structure that is composed of two sections.

### *Preparation of non-layered disintegrated sludge*

To obtain non-layered sludge with the same microbial populations as intact granules, the same volume and weight of granules were blended with a homogenizer (Ace Homogenizer, Nihonseiki Kaisha Ltd.) at 10 000 rpm for 2 min. The disintegrated granules were sieved with a mesh size of 0.5 mM to remove the undisintegrated granules prior to experiment. All the sludge samples prepared were washed three times with 0.1 M $KH_2PO_4$/$K_2HPO_4$ buffer (pH 7.0) before the experiments. The sludge had not been previously acclimated to any heavy metals. All the procedures were conducted in an anaerobic atmosphere (Controlled Atmosphere Chamber, Lancing, Mich.) and 0.02 mM $Na_2S$ was used as reducing agent.

### *Basal medium*

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.

### *Resistance assay of sludge to heavy metals*

Specific glucose degradation activity measurements were performed with 120 ml glass serum bottles sealed with a 12 mm-thick butyl rubber septa. The 30 ml sieved granular sludge was filled up to vials lined at 100 ml containing 70 ml basal medium and 40 mM acetate from a neutralized stock solution. Five heavy metals commonly found in electroplating effluent (Fang 1997), i.e., $Cd^{2+}$ ($CdCl_2$), $Cr^{3+}$ ($CrCl_3$), $Cu^{2+}$ ($CuCl_2$), $Ni^{2+}$ ($NiCl_2$) and $Zn^{2+}$ ($ZnCl_2$) were selected for this study. The assay medium was then adjusted at pH 7.0 $\pm$ 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 phase and liquid phase were sampled to determine microbial activity.

### *Analytical methods*

Acetate and propionate concentrations in the liquid phase was determined by isocratic HPLC with an ion exchange column (Aminex, HPX-87H, 300 $\times$ 7.8 mm) and an organic acid analysis kit (Bae & Lee 1999). Degassed 4 mM $H_2SO_4$ was used as an eluant. The acetate and propionate were detected at 210 nm.

The gas contents in the headspace of the assay bottles were determined with a pressure meter and their compositions calculated by GC. The gas contents were revised with the pressure and temperature but 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 $min^{-1}$. The samples for measuring gas content in the headspace were determined with a pressure-lock gas syringe (Pressure Lock series A-2).

## Results

To investigate the influence of a layered structure of granules on the toxicity of metal ions, disintegrated and intact granules were incubated with $Cu^{2+}$ (Figure 1). Glucose was degraded with a transient accumulation of VFAs. The VFAs identified were acetate,

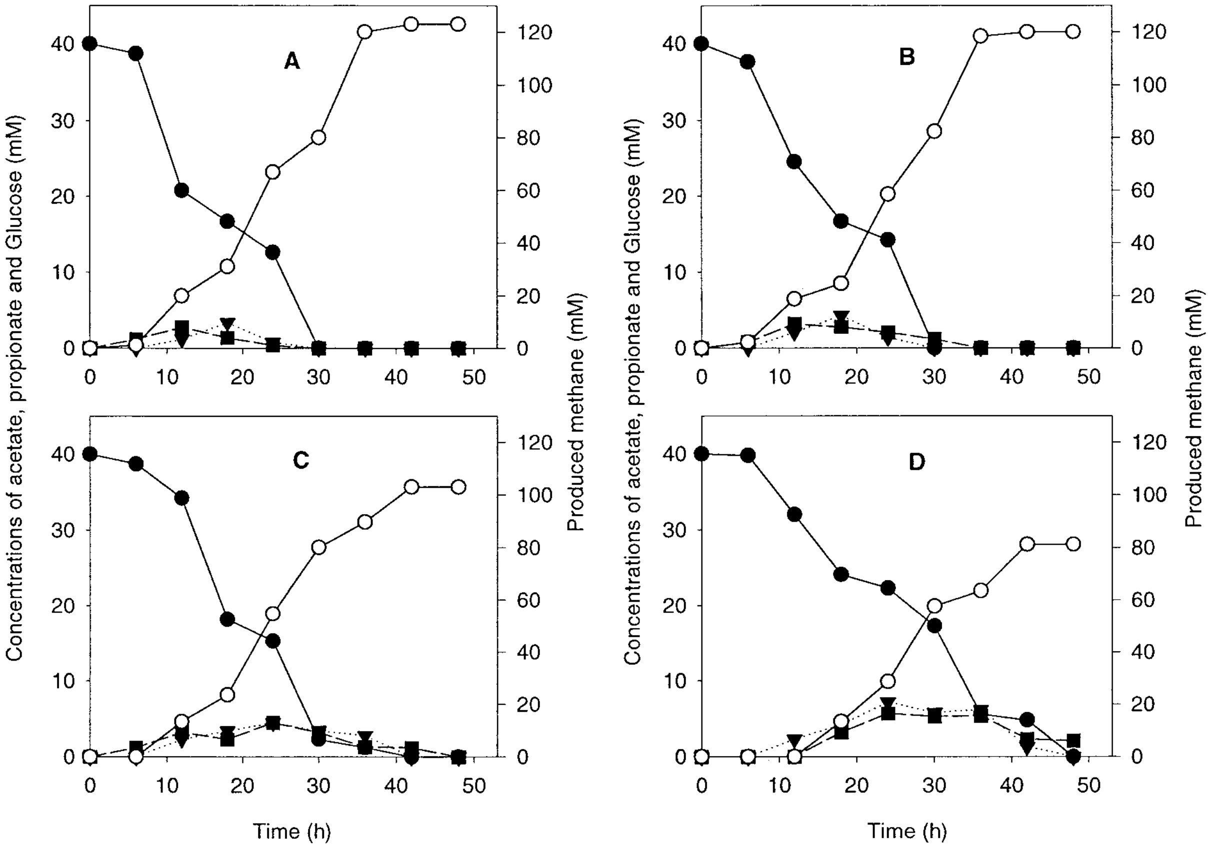


*Fig. 1.* Fermentation of glucose, the generation and degradation of propionate and acetate and the production of methane by intact and disintegrated granules in media containing 0 mg $g^{-1}$ VSS and 500 mg $g^{-1}$ VSS of $Cu^{2+}$; (A) intact granules without $Cu^{2+}$, (B) disintegrated granules without $Cu^{2+}$, (C) intact granules with $Cu^{2+}$, (D) disintegrated granules with $Cu^{2+}$; (●) glucose concentration (○) methane production, (■) acetate concentration, (▼) propionate concentration. (The data are mean value of duplicates.)

propionate, lactate, butyrate, isobutyrate and valerate. Acetate and propionate were the main products of the glucose fermentation, accounting for about 70–80% (mol/mol) of the total VFA accumulated. The glucose degradation was not significantly affected by the disintegration of the granules using a homogenizer. Both the intact and disintegrated granules completely degraded 40 mM of glucose in 30 h at the same rate in the absence of $Cu^{2+}$. However, with $Cu^{2+}$ at 500 mg $g^{-1}$ VSS in media, the glucose degradation rates and methane production rates decreased by 14% and 32% in disintegrated granules, respectively, whereas, in intact granules, decreases were 3% and 14%, respectively. Because the methanogens were more sensitive to the metals than the cells that generated acetate and propionate, acetate and propionate were accumulated up to 7.2 mM and 5.7 mM, respectively, two times higher than the concentration without $Cu^{2+}$.

To see the resistance of granules to various metal ions, electroplating metals were selected and used as toxic metal ions (Figure 2). To study the effect of $Cu^{2+}$ on methanogenesis which is the most sensitive step in anaerobic digestion, acetate was selected as the substrate among the accumulated VFAs, because acetate is directly degraded to methane by acetoclastic methanogens. The toxicity of each metal ion can be expressed by a simple $C_{L50}$ value, which represents the metal concentration at which the specific methane production rate of the granules was reduced to 50% of the control (Fang 1997). Table 1 summarizes the respective $C_{L50}$ values for intact granules and disintegrated granules. Disintegrated granules were more sensitive to the toxicity of all the metals tested than were intact granules. The inhibitory effect on the acetoclastic activity of each type of granule was in the following order: $Zn^{2+}$ (most toxic) > $Ni^{2+}$ > $Cu^{2+}$ > $Cd^{2+}$ > $Cr^{3+}$ (least toxic). However, the inhibitory

*Table 1.* $C_{L50}$ of granular activity of acetate degradation and methane production.

| Mg $g^{-1}$ VSS | Acetate degradation | | Methane production | |
|---|---|---|---|---|
| | Intact granules | Disintegrated granules | Intact granules | Distintegrated granules |
| $Zn^{2+}$ | 250 | 210 | 170 | 120 |
| $Ni^{2+}$ | 450 | 300 | 240 | 180 |
| $Cu^{2+}$ | 580 | 520 | 360 | 260 |
| $Cd^{3+}$ | 660 | 610 | 630 | 480 |
| $Cr^{2+}$ | 770 | 660 | 510 | 380 |

$C_{L50}$: The concentration (mg $g^{-1}$ VSS) at which metal ions inhibited the activity of the control by 50%.

effect on the methanogenic activity of the granules was different: $Zn^{2+}$ (most toxic) > $Ni^{2+}$ > $Cu^{2+}$ > $Cr^{3+}$ > $Cd^{2+}$ (least toxic). Furthermore, methanogenic activity was more sensitive to the toxicity of all the metal ions tested than was acetoclastic activity.

## Discussion

Glucose degradation in anaerobic conditions is a multi-step process conducted jointly by fermentative/acidogenic bacteria, acetogens and methanogens. In the comparison of intact granules with disintegrated granules, the disintegrated granules were more sensitive to copper ions toxicity than were intact granules in all the steps of glucose degradation (fermentative/acidogenic process and methanogenic process). This indicates that the microbial populations in intact granules were more resistant to copper ions than were those in the disintegrated granules. Furthermore, in our study, the VFAs accumulated increased in media containing copper ions. This result implies that methanogenic activity might be more sensitive to metal ions than fermentative activity. It was generally known that methanogens are vulnerable to the toxicity of metal ions (Hickey *et al*. 1989, Lin & Lin 1997).

To study methanogenesis as the rate-limiting step of anaerobic digestion in the presence of heavy metals, we selected acetate as the substrate for the methanogens among the major VFAs accumulated, because acetate conversion to methane is a one-step process conducted by acetoclastic methanogens alone (Fang 1997). Methane production was more significantly inhibited than was acetate consumption in the presence of copper ions. Furthermore, in the presence of heavy metals, the degradation rate of acetate and the production rate of methane decreased more significantly in disintegrated granules than they did in intact granules. This result indicated that the layered structure of the intact granules gave the resistance to copper ions to the methanogens.

Chemical toxicity was closely correlated to the sludge surface area with the bigger surface areas yielding an increased toxicity. Furthermore, it is believed that the inner part of the granules composed of methanogens was shielded from the harsh environment by the layered structure. Therefore, the methanogenic activity could have become more sensitive to metal toxicity by the disintegration of the granules. Apparently, this granular sludge phenomenon is similar to that of immobilized cells. When treating toxic chemicals with immobilized cells, the immobilized cells are more resistant to chemical toxicity, compared with suspended cells (Dwyer *et al.* 1986, Keweloh *et al.* 1989).

The toxicity of the various metal ions used in the electroplating industry to granular methanogenic activity was compared with that of copper ions. As expected from the copper ion experiment, intact granules degraded acetate faster and produced more methane than did disintegrated granules irrespective of the type of electroplating metal ions. Furthermore, acetate consumption was less sensitive than methane production. Our results on the relative toxicity of various heavy metals differed from what was previously reported by Lin & Lin (1997). In their report, the metals' relative toxicity to volatile fatty acid degradation was in the order of $Cu^{2+}$ > $Cr^{3+}$ > $Cd^{2+}$ > $Zn^{2+}$ > $Ni^{2+}$. Our results accord with the study of Fang (1997) while it was shown that the toxicity of metal ions to methane production were in the following descending order: $Zn^{2+}$ > $Ni^{2+}$ > $Cu^{2+}$ > $Cd^{2+}$ > $Cr^{3+}$. However, interestingly, the relative toxicity of various metal ions

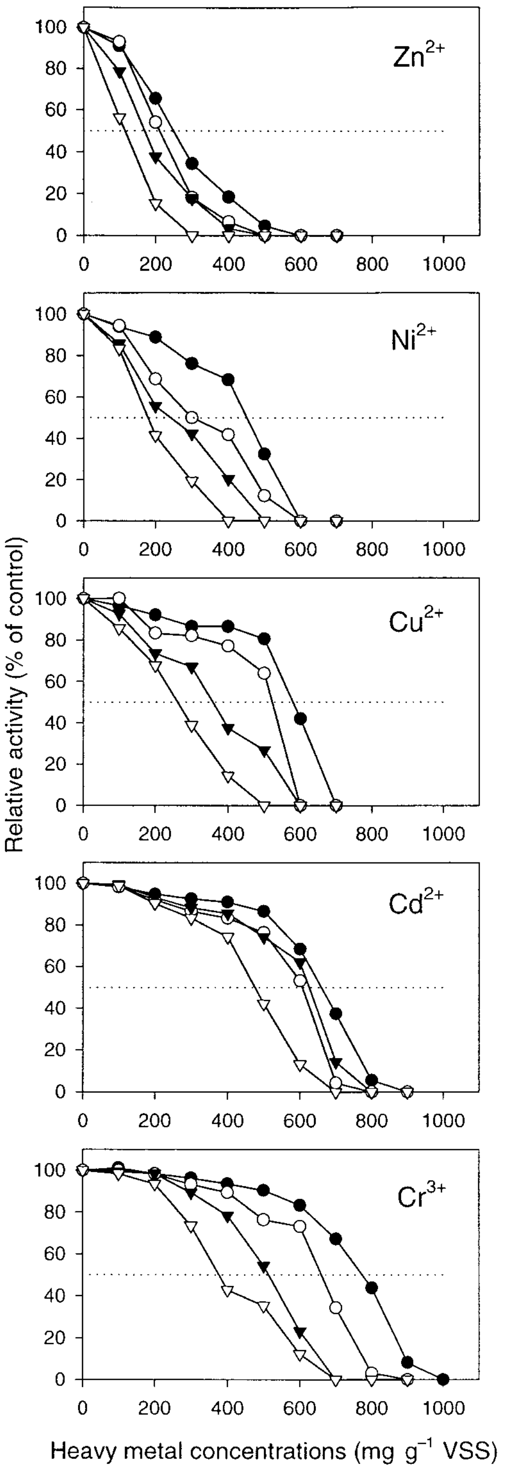


*Fig. 2.* Decrease of specific acetoclastic and methanogenic activity of granules with increased metal ion concentration; (●) acetate degradation with intact granules, (○) acetate degradation with disintegrated granules, (▼) methane production with intact granules, (▽) methane production with disintegrated granules. (Control means reaction with intact granules in the absence of metal ions effect and the data are mean value of duplicates.)

to acetate consumption and methane production was slightly different.

We also reported previously that intact granules had more resistance to toxic organic chemicals than did disintegrated granules (Bae & Lee 1999). These results indicate that the resistance of anaerobic digestion to various toxic materials improves with the granulation of sludges in UASB reactors. Therefore, the UASB reactor, in which the microbial population is in a granular state, is more feasible for the treatment of electroplating wastewater containing toxic metal ions compared to traditional anaerobic digestion.

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