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CHROMIUM(VI) REDUCTION BY A MIXED CULTURE OF SULFATE REDUCING BACTERIA DEVELOPED IN COLUMN REACTOR

(Reduksi Kromium(VI) oleh Kultur Campuran Bakteri Pereduksi Sulfat pada Reaktor Kolom)

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Abstract

A lactate enriched mixed sulfate reducing bacteria (SRB) culture was examined for the reduction of Cr(VI) in a continuous flow system. The influent was mineral salts media added with lactate and sulfate with amounts of 8 and 6 mM respectively as electron donor and electron acceptor. The SRB culture was allowed to stabilize in the column before adding the Cr(VI) to the influent. Chromium and sulfate reduction and lactate oxidation were examined by measuring the concentrations of Cr(VI), sulfate and lactate in the influent and the effluent over time. The experiment was discontinued when Cr(VI) concentration in the effluent was breakthrough. In the absence of Cr(VI), sulfate was not completely reduced in the column, although lactate was completely oxidized and acetate as an intermediate product was not often detected. Almost all of Cr(VI) loaded was reduced in the column seeded with the SRB culture at influent Cr(VI) concentrations of 192, 385 and769 mM. There was no significant Cr(VI) loss in the control column, indicating that Cr(VI) removal was due to the reduction of Cr(VI) to Cr (III) by the SRB culture. The instantaneous Cr(VI) removal decreased to a minimum of 32%, 24 days after the influent Cr(VI) concentration was increased to 1540 µM, and sulfate removal efficiency decreased to a minimum of 17%. The SRB population in the column decreased 100 days after Cr(VI) was added to the column. The total mass of Cr(VI) reduced was approximately 878 µmol out of 881 µmol of Cr(VI) loaded in 116 days. The results clearly show that our developed SRB culture could reduced Cr(VI) considerably.

Keywords: Chromium(VI), reduction, sulfate-reducing bacteria

Abstrak

Kemampuan kultur campuran bakteri pereduksi sulfat (SRB) yang menggunakan elektron donor laktat diuji untuk mereduksi Cr(VI) dalam continuous flow system. Influen merupakan media garam mineral yang ditambahkan laktat dan sulfat masing-masing dengan konsentrasi 8 dan 6 mM sebagai elektron donor dan akseptor. Setelah kultur SRB stabil di dalam kolum, Cr(VI) ditambahkan pada media influen. Reduksi kromium dan sulfat dan oksidasi laktat diamati dengan mengukur konsentrasi kromium, sulfat dan laktat di influen dan effluen sesuai waktu pengamatan. Eksperimen dihentikan setelah tidak semua Cr(VI) tereduksi yang ditandai dengan meningkatnya konsentrasi Cr(VI) di effluen. Sebelum penambahan Cr(VI), tidak semua sulfat tereduksi padahal laktat teroksidasi secara sempurna, sedangkan acetat sebagai senyawa intermediat tidak selalu terdeteksi. Hampir semua Cr(VI) tereduksi pada kolum yang diinokulasi oleh kultur SRB pada konsentrasi Cr(VI) influen 192, 385 dan 769 mM. Penyisihan Cr(VI) yang sangat insignifikan pada kolum kontrol menunjukkan bahwa penyisihan Cr(VI) disebabkan oleh reduksi Cr(VI) oleh kultur SRB. Penyisihan Cr(VI) menurun sampai 32% setelah 24 hari konsentrasi Cr(VI) influen dinaikkan menjadi 1540 µM dan reduksi sulfat menurun sampai 17%. Hal ini menunjukkan bahwa konsentrasi Cr(VI) yang tinggi dapat menghmbat reduksi sulfat. Populasi SRB juga menurun setelah 100 hari penambahan Cr(VI) pada kolom. Penyisihan

total Cr(VI) mencapai kira-kira 878 µmol dari 881 µmol Cr(VI) yang dialirkan pada kolom selama 116 hari. Hasil penelitian menunjukkan bahwa kultur campuran SRB yang dikembangkan mampu mereduksi Cr(VI) secara efektif.

Kata kunci: Chromium(VI), reduksi, bakteri pereduksi sulfat

INTRODUCTION

Because of poor waste management practices in industries that utilize chromium, chromium discharge into the environment has often occurred. Chromium contaminated waters have been reported in several studies (Calder, 1988). Chromium (Cr) exists primarily in two oxidation states in aqueous environments: hexavalent chromium [Cr(VI)] and trivalent chromium [Cr(III)]. Cr(VI) predominates under oxidizing (high pE) conditions, and is typically present as an anion, either chromate at pH>6.5 or bichromate at pH<6.5. Cr(VI) is a highly soluble form of chromium and is known to be toxic and mutagenic to organisms and is a potential carcinogen in humans (Bartlett and James, 1988; Yassi and Nieboer, 1988; Nieboer and Shaw, 1988). On the other hand, Cr(III) predominates in reducing (low pE) conditions, is relatively insoluble, and precipitates as oxides and hydroxides at pH>5, thereby it is considerably less toxic (Bartlett and James, 1988). Increasing concern on the impact of chromium in the environment and on human health requires improved techniques for the treatment of Cr(VI)-contaminated waters.

The techniques for Cr(VI)-contaminated waters involve reduction of Cr(VI) to Cr(III) followed by precipitation of Cr(III). Microbial Cr(VI) reduction is an important process for improving water quality in contaminated environments, and for the treatment of Cr(VI) containing wastes. Several studies have been reported on the reduction of Cr(VI) by aerobic and anaerobic microorganisms (pure and mixed cultures) (Wang and Shen, 1995; Wang and Xiao, 1995). Cr(VI) is known to be toxic and mutagenic to microrganisms (Petrilli and de Flora, 1977; Wong and Trevors, 1988). As a result, high concentrations of Cr(VI) may inhibit the microbial reduction of Cr(VI). The rate of Cr(VI) reduction using *Enterobacter cloaceae* declined as the concentration of Cr(VI) increased above 1 mM (Ohtake *et al.*,1990). Alternatively, acclimatization of bacterial cultures to Cr(VI) may reduce or eliminate the toxic effect of Cr(VI) (Yamamoto *et al.*, 1992); strains of chromate-reducing bacteria have been isolated from hexavalent chromium-contaminated environments (Turic *et al.*, 1996). It is proposed that bacterially mediated reduction of Cr(VI) to Cr(III) significantly reduces its toxicity.

The interest in using sulfate-reducing bacteria (SRB) to treat Cr(VI) contaminated waters has been increasing due to their demonstrated capability for reducing Cr(VI) to Cr(III), enzymatically and non-enzymatically by their metabolite products hydrogen sulfide (Fude *et al,* 1994; Lovley and Phillips, 1994; Pettine *et al*, 1994). Since both Cr(VI) and sulfide are known to be toxic to bacteria at certain levels, bacterial viability can be further enhanced by abiotic Cr(VI) reduction with sulfide and furthermore the SRB may also reduce Cr(VI) enzymatically. The use of SRB can also ensure the Cr(VI) reduction and precipitation as $Cr(OH)_3$ by providing a more reducing environment.

SRB are obligate anaerobes that are notable for their end product, hydrogen sulfide, which is produced from dissimilatory sulfate reduction. SRB are found ubiquitously in anaerobic environments where sulfate is present. Several compounds can serve as electron donors for sulfate reduction, including hydrogen, pyruvate, lactate, propionate, acetate, fatty acids and ethanol (Barton and Tomei, 1995). Many SRB can grow on more than one electron donor. Two major metabolic groups of SRB, when using organic substrates such as lactate and propionate: complete oxidizers

and incomplete oxidizers. Complete oxidizers include the group of SRB which are able to oxidize their organic substrates completely to carbon dioxide. *Desulfonema* are grouped as complete oxidizers. Incomplete oxidizers, on the other hand, are the group of SRB that oxidize their substrates incompletely to acetate. Acetate is not oxidized further by these SRB. Many species from the genus *Desulfovibrio* fall into this category but some, such as *Desulfovibrio baarsi*, are considered complete oxidizers (Widdel, 1988).

Most of previous studies on Cr(VI) reduction by SRB cultures have been reported using batch systems. However, there is no reported study using a continuous flow system. Therefore the objective of this research is to examine the capability of a developed mixed SRB culture on the reduction of Cr(VI) to Cr(III) in a continuous flow system.

EXPERIMENTAL METHOD

Stock culture. Microorganisms obtained from an anaerobic digester were used as seed for producing a lactate enriched culture of SRB in a completely-mixed flow (CMF) reactor. The reactor was maintained anaerobically at 30 °C with a 15-d hydraulic retention time by continuously pumping medium into the reactor at a flow rate of 1 L/d. Degassed and autoclaved mineral salts media adopted from a previous study by Maillacheruvu (1993) with addition of sulfate (8 mM) and lactate (6 mM) was used to grow the SRB culture for the study. The pH of the media was adjusted to 7.5. Reactor was monitored for pH, biomass, sulfate, sulfide, lactate, and acetate periodically to ensure that the stock was healthy. Ferric chloride was added to the stock culture as needed to precipitate sulfide in order to eliminate sulfide toxicity.

Column study. The columns were set up in anaerobic glove box to maintain the column anaerobic. One column containing glass beads was inoculated with the SRB enrichment culture, while another column served as a control, which was amended with autoclaved culture to account for the loss of Cr(VI) by sorption. The columns (2.25 cm i.d. x 9.2 cm an adjusted long) were fed via a syringe pump at a flow rate of 10 mL/d, resulting in a hydraulic residence time of 1.1 days. The columns were maintained in a 30 °C room. The influent sulfate and lactate concentrations were 6 and 8 mM respectively. Due to difficulty of maintaining column contamination free from unwanted bacteria, lactate was not added to the control column. The fresh influent was added every other day. The SRB culture was allowed to stabilize in the column before adding Cr(VI) to the feed solution. The Cr(VI) concentration was successively doubled when the column effluent Cr(VI) concentration reach at steady state. The influent Cr(VI) concentration to each column was initially at 192 and was increased to 385, 769 and 1540 μM. Cr(VI), sulfate, lactate, acetate and pH were analyzed every two days.

Batch study. The batch study was conducted as a control study to determine if lactate contributes the Cr(VI) reduction. Autoclaved 160-mL serum bottles were filled with 90 mL of degassed and autoclaved mineral media contained 6 mM of sulfate. Bottles were amended with 5 mL of four different stock Cr(VI) solutions to give final concentrations of 192, 385, 769 and 1540 μM. Five mL of stock lactate solution was added to give a final concentration of 8 mM. One set of bottles that were amended with Cr(VI) solution without addition of lactate served as a control. Bottles were prepared in an anaerobic glove box with an atmosphere of N_2/H_2 gas (90:10%, v:v). Bottles were sealed with teflon-coated rubber septa (West Co., Phoenixville) and crimped with aluminum crimp caps. All bottles were incubated at 30 °C on a shaker table. Cr(VI) was measured at 0, 2, and 4 days.

Analytical methods. The pH was measured using a Ross combination microelectrode (Orion Instruments) and pH meter. All samples were filtered using 0.2 mm-pore size, PTFE syringe-tip filters (Millipore) prior to analyses. Sulfate was measured using a DIONEX Model DX 500 ion chromatography equipped with a Dionex AS4 column and AG4A guard column. The MDL of sulfate was 0.31 mg/L. Lactate and

acetate were measured with a Hewlett Packard Series 1050 HPLC equipped with a PRP X300 column (Hamilton). The MDL for lactate and acetate were 2.05 and 4.47 mg/L, respectively. Cr(VI) was analyzed using the colorimetric diphenylcarbazide method described from a previous study (Phelps *et al*, 1994). The MDL of Cr(VI) was 1.35 mg/L.

Microbial Enumeration. Various microbial populations were determined using the most probable number (MPN) technique described in Sections 9221 B and C of standard Methods (APHA, 1992). Heterotrophs were enumerated using the mineral medium with the addition of the following (g/L): yeast extract (0.1), peptone (0.05), tryptone (0.05), and glucose (0.1). SRB were enumerated using mineral medium with 10 mM lactate and 40 mM sulfate. Methanogens were quantified using mineral medium with 10 mg/L of yeast extract and 10 mM of methanol. All MPN tubes were prepared in an anaerobic glove box with an atmosphere of N_2/H_2 gas (90:10%, v:v) and incubated for one month at 23 ± 1 oC.

SEM analyses. The SEM analyses for the cell suspension from the stock culture reactor and column were conducted using AMR 1000 (AMRay, Bedford, MA). The procedure used for samples fixation was adopted from the SEM Lab Procedure-University of Maine.

RESULTS AND DISCUSSION

SRB stock culture. The lactate enriched SRB stock culture was developed in a completely mixed flow reactor. The culture reactor had pH range of 6.8 to 7.8. Most of the time lactate was oxidized completely however sulfate was not always all reduced by the culture. Acetate was also often undetected. The SEM analyses showed that homogenous bacteria cells were predominant in the reactor. Total sulfides concentrations in the reactor reached as high as 6 mM. Iron chloride was added into the reactor periodically to minimize sulfide concentration to a non-toxic level. When sulfide concentrations were high sulfate concentrations were also high in the reactor before the addition of iron chloride indicating that high sulfide concentration can inhibit the sulfate reduction by the SRB. The MPN results showed that predominant bacteria present in the stock culture reactor was sulfate reducing bacteria. However, fermentative bacteria appeared to be present in a small number in the stock culture reactor.

Results in the absence of Cr(VI). The SRB culture was maintained for 100 days (phase I) in the column before adding Cr(VI) (Figure 1). During this time sulfate reduction and lactate oxidation were monitored. The fluctuation of lactate influent for the first 40 days was due to the procedure of the feeding preparation, where micro liter volume of pure lactate was added directly to the feeding media. After this time, the medium was amended with a lactate stock solution, which greatly reduced the variability of the influent lactate concentration. The lactate and acetate concentrations in the effluent for the first 100 days of the study was almost non detectable. The concentration of sulfate appeared to oscillate from about 2.5 to 5.1 mM, with an average of 3.5 mM during the first 100 days (Table 1). It is well known that SRB are inhibited by high levels of sulfide, the end product of sulfate reduction (Maillacheruvu, 1993; McCartney and Oleskiewicz, 1993; McCartney and Oleskiewicz , 1991). At the periods of high sulfate reduction activity, the sulfide level was high, which may have inhibited SRB until the sulfide was washed out. At this time, sulfate reduction would pick up again and the cycle would repeat. Based on both stoichiometric equations below sulfate should be completely reduced.

$$2\ CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2\ CH_3COO^- + HS^- + 2\ HCO_3^- + H^+ \quad (eq.\ 1)$$
$$\Delta G^o = -160.1\ kJ/mol$$

$$2\ CH_3CHOHCOO^- + 3\ SO_4^{2-} \rightarrow 3\ HS^- + 6\ HCO_3^- + H^+ \quad (eq.\ 2)$$
$$\Delta G^o = -255.3\ kJ/mol$$

However, lactate was 100 % oxidized, sulfate reduction was not incomplete and acetate as an intermediate product was not often detected. It was possible that due to the sulfide inhibition of

SRB, the condition in the system might affect the microbial community in this column. It was possible that the other type of bacteria such as heterotrophic or acetotrophic, might be present and prevailed in the system. Acetate might be consumed by acetoclastic methanogens during this time (Maillacheruvu, 1993; McCartney and Oleskiewicz, 1993; McCartney and Oleskiewicz , 1991).

Results in the Presence of Cr(VI). Cr(VI) was fed to the SRB+Lactate column on day 100. The target influent Cr(VI) concentration was 192 μM. The influent Cr(VI) concentration was maintained at this level up to day 122 when Cr(VI) reduction in the column reached at steady state.

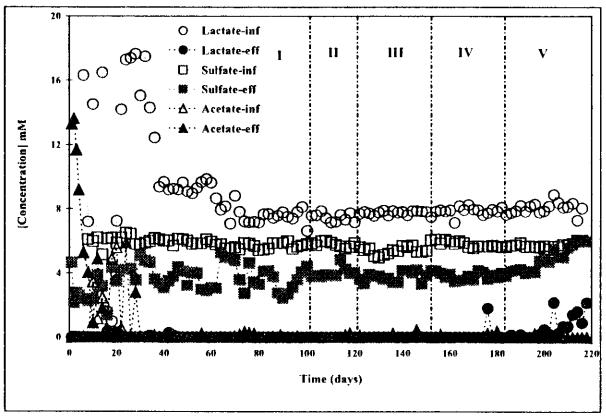

Figure 1. Influent (inf) and effluent (eff) sulfate, lactate and acetate concentrations

Table 1. Average influent and effluent Cr(VI), lactate and sulfate concentrations in the SRB+Lactate column for the long-term column study.

Phase	Days	Concentration (μM)		Concentration (mM)			
		Cr(VI)-inf	Cr(VI)-eff	Lactate-inf	Lactate-eff	Sulfate-inf	Sulfate-eff
I	0 - 100	0.00	0.00 ± 0.00	7.57 ± 0.51	0.00 ± 0.00	6.13 ± 0.24	3.63 ± 0.88
II	100 - 122	192 ± 0.00	0.00 ± 0.00	7.43 ± 0.34	0.00 ± 0.00	5.91 ± 0.21	3.99 ± 0.33
III	122 - 152	385 ± 0.00	0.00 ± 0.00	7.76 ± 0.12	0.00 ± 0.00	5.51 ± 0.29	3.80 ± 0.31
IV	152 - 186	769 ± 0.00	0.00 ± 0.00	7.85 ± 0.26	0.11 ± 0.40	5.78 ± 0.13	3.84 ± 0.23
V	186 - 216	1540 ± 0.00	0.20 ± 0.38	8.08 ± 0.32	0.55 ± 0.65	5.87 ± 0.14	4.86 ± 0.66

Phases represent influent Cr(VI) concentration added to the columns: 0 μM (I); 192 μM (II); 385 μM (III); 769 μM (IV); 1540 μM (V). SRB+Lactate: glass beads seeded with SRB cell suspension and fed with lactate.

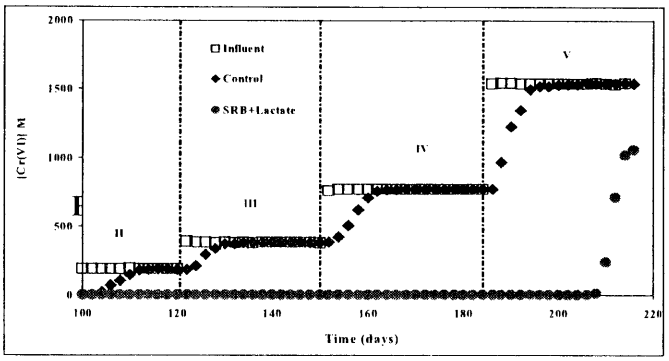

Figure 2: Cumulative and Instantaneous Cr(VI) removal

During this time, the effluent concentration of Cr(VI) from this column was nondetectable (Figure 2). All mass Cr(VI) was removed during this time (Figure 2). The instantaneous and cumulative Cr(VI) removal efficiencies of the column were 100% (Figure 3). The steady state effluent concentration of Cr(VI) from the control column was 185 μM. The average of influent concentration of Cr(VI) was 192 μM and the average effluent Cr(VI) concentration was 185 μM from control column. The lactate was not added to the column control. Batch experiment was conducted to examine the reduction of Cr(VI) by lactate. The results showed no significant loss of Cr(VI) by lactate. This indicated that the removal of Cr(VI) in the SRB+Lactate column was due to the reduction of Cr(VI) by SRB and their metabolite products, not due to reduction by lactate or to sorption of Cr(VI) onto the column or glass beads.

The addition of Cr(VI) to the influent during this time had little impact on the biological activity in this column. The effluent concentration of lactate and acetate were still nondetectable, while the effluent sulfate concentration was within 10% of the respective values for each prior to the addition of Cr(VI) (Figure 1).

During phase III (from day 122 until 152) and phase IV (from day 152 until 186) the target influent Cr(VI) concentration was 385 and 769 μM respectively. The effluent Cr(VI) concentration from this column was nondetectable, while the steady-state concentration of Cr(VI) in the effluent firm the control column was 768 μM (Figure 2). The increase of the influent of Cr(VI) concentration did not result in an increase in greater than 10% for the concentration of sulfate, lactate or acetate in the effluent form this column when compared to its respective values at the previous Cr(VI) influent concentration (Figure 1).

During phase V, from day 186 until 216, the target influent Cr(VI) concentration was 1540 μM. The average effluent Cr(VI) concentration from this column was 200 μM (Table 1). The failure of this column resulted in the instantaneous Cr(VI) removal efficiency of this column decreasing to a minimum of 32% (Figure 2). The high Cr(VI) influent concentration also impacted sulfate reduction and lactate oxidation in this column. The average effluent sulfate concentration rose to an average concentration of 4.86 mM, corresponding to a sulfate removal efficiency of 17% (Table 1). The average effluent lactate concentration increased to 0.55 mM, corresponding to a lactate removal efficiency of 93% (Table 1). High Cr(VI) concentration appeared to inhibit the bacterial activity. The toxicity effect of high level of Cr(VI) to microorganisms have been reported previously (Chirwa and Wang, 1997, Mazierski, 1994; Yamamoto, *et al.*,1993).

As previously mentioned, in the columns seeded with SRB culture, Cr(VI) might have been reduced by sulfide as by product of sulfate reduction as well as by enzymes. Two stoichiometric equations of interest to the research of Cr(VI) reduction to Cr(III) by SRB culture enzymatically and non-enzymatically by sulfide:

Cr(VI) + H2 + NADH + endogenous e- reserves → *Cr(III)* *(eq. 3)*

(Cytochromes)

(Wang and Shen, 1995)

$$2CrO_4^{2-} + 3HS^- + 7H^+ \rightarrow 2Cr(OH)_3 + 3S + 2H_2O \quad (eq.\ 4)$$

(Thornton and Amonette, 1999)

Cr(VI) reduction by sulfide can also result in sulfate instead of sulfur element (Smillie *et al.*, 1981). Therefore Cr(VI) reduction in the column might also contribute to the increase of sulfate concentration in the effluent. The loss of Cr(VI) in the column seeded with SRB culture due to reduction of Cr(VI) to Cr(III). The total chromium concentrations were not measured in the effluent to ensure the reduction of Cr(VI) to Cr(III), however it was clear that most of Cr(VI) was not detected in the effluent. The effluent pH range was 7.5 to 8.3. At this pH Cr(III) mostly precipitates as Cr(OH)3 in the column (Bartlett and James, 1988). The precipitates could be spotted in the column.

This study did not further examine the contribution of Cr(VI) reduction by enzymatic versus nonenzymatic mechanisms, e.g, by sulfide produced by SRB. However, the results from previous studies could be used to explain the mechanisms of the Cr(VI) reduction in the system used in this study. Fude *et al.* (1994) examined Cr(VI) reduction by SRB acclimated to high Cr(VI) concentrations. There was insignificant loss /reduction of Cr(VI) by dead cells, which suggested that Cr(VI) removal required active cells. Using live cells in the presence of lactate, 95 % of Cr(VI) was reduced from concentrations ranging from 0.96 to 38.45 mM. Cr(VI) reduction in bottles amended with molybdate was similar to Cr(VI) reduction in uninoculated controls. This confirmed the mechanism of Cr(VI) reduction via hydrogen sulfide and that Cr(VI) loss was not entirely due to a passive interaction with reactive sites on the bacterial surfaces. It was also confirmed in this previous study that Cr(VI) reduction was mostly due to sulfide produced by SRB, and not due to enzymatic Cr(VI) reduction. The consortium of SRB in this previous study was fed with lactate as an electron donor. The type of bacteria found in the consortium of SRB were predominated by hydrogen sulfide producers.

Based on this previous study, it can be suggested that enzymatic Cr(VI) reduction in this study might be smaller when compared to non-enzymatic Cr(VI) reduction by sulfide.

Microbial population. Microbial population quantified by MPN was sulfate reducing bacteria, fermentative bacteria (anaerobic heterotrophic bacteria) and methanogens. The MPN results showed that both SRB and non SRB populations were present in the stock culture with the SRB population being predominant. The SRB population decreased from $10^{8.2}$ in the stock culture to $10^{3.8}$ cells/100 mL 100 days after Cr(VI) was added to the column, while the non SRB population decreased from 10^{4} in the stock culture to $10^{3.4}$ cells/100 mL 100 days after Cr(VI) was added to the column. The addition of Cr(VI) had an impact on the microbial population in the column. The SEM micrographs also showed different forms of cells existed in the stock culture and in the column (Figure 3).

The cells form was more homogeneous in the stock culture, while long rod type-cells were observed in the column. The results suggest that the dominant microbial form in the columns changed with time. Methanogens were not successfully detected. However, based on that most lactate was oxidized, sulfate was not reduced completely, and acetate was often not detected, it is possible that methanogens coexist with the SRB along with the fermentative bacteria. The column was fed with excess lactate but not excess sulfate. Lactate, which was the electron donor, is a complex substrate that can be utilized via many different metabolic reactions (Widdle, 1988; Maillacheruvu, 1993; McCartney and Oleskiewicz, 1993; McCartney and Oleskiewicz, 1991). Thus lactate could stimulate the growth of other types of microbes as well. The pH of effluent ranged from 7.5 to 8.3. At this pH bacterial community quanti-

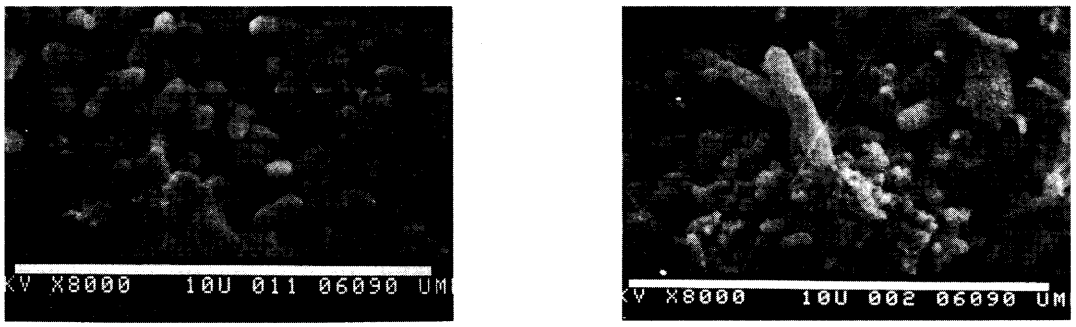

Figure 3. The SEM micrographs of bacterial community: in the stock culture reactor (a) and in the column reactor (b)

fied in this study can grow optimally (Widdle, 1988). The environmental conditions affect the pathway taken by a microbial community, and the changes in environmental conditions will change the pathway as well.

CONCLUSIONS

The results clearly show that our developed SRB culture could reduced Cr(VI) considerably in the continuous flow system at Cr(VI) concentration as high as 1500 μM. Cr (VI) removal efficiency in the column seeded with the SRB culture was nearly 100% at influent Cr(VI) concentrations ranging from 192 to 769 mM. The total mass of Cr(VI) removed was approximately 878 μmol out of 881 μmol of Cr(VI) loaded in 116 days. SRB are inhibited by high concentrations of Cr(VI). Sulfate level in the effluent increased when the influent Cr(VI) concentration was increased to 1540 μM. Our developed SRB culture might potentially be used for in situ bioremediation of chromium contaminated soils and groundwaters; or to remediate Cr(VI) contaminated waste by reduction of Cr(VI) to Cr(III) in an anaerobic system.

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