Heavy Metal Resistant Anaerobic Bacterial Strains from Brewery Wastewater

Nusara Sinbuathong [1,*], Pramote Sirirote [2], Daniel Watts [3], Suphang Chulalaksananukul[4]

Abstract —This work focused on the study of the types of heavy-metal-resistant anaerobic bacteria from a brewery wastewater treatment plant exposed to high concentrations of dissolved Cd (II), Cu (II) and Zn (II). Characterizations were carried out by polymerase chain reaction of 16S rRNA gene of bacterial strain. Using special culture media, two types of strong heavy metal-resistant bacterial strains were isolated. One is a sulfate reducing bacterium identified as *Clostridium ganghwense* strain HY-42-06. This strain of sulfate reducing bacteria tolerated Cd (II), Cu (II) and Zn (II) at the tested concentration. The other type was identified as consisting of a mixture of *Micrococcus luteus*, *Wolinella Succinogenes*, *Sporosarcina* sp. PIC-C28 and *Alicycliphilus* sp. R-24604. The results found that these four dominant strains tolerated Cd (II) at 20 mg/l, only *Wolinella Succinogenes* cannot tolerate Cu (II) at 2 mg/l while none of them tolerated Zn (II) at 30 mg/l.

Keywords —Anaerobic bacteria, Brewery wastewater, Heavy metal, Sulfate reducing bacteria

1 INTRODUCTION

Major environmental problems are encountered with organic wastewaters with high sulfate (SO_4^{2-}) content, such as those from beverage industries, edible oil and potato starch processing as well as pulp and paper manufacturers [1], and with heavy metal-containing wastewaters [2]. Sulfate reducing bacteria (SRB) use SO_4^{2-} as the terminal electron acceptor during oxidation of organic matter, resulting in the production of hydrogen sulfide (H_2S). The presence of either high levels of metals [3] or of free H_2S [4] is generally regarded as toxic for anaerobic digestion. However, when both are present in the same solution, metals may precipitate as metal sulfides, eliminating the toxic effects of the individual components. The precipitation would immobilize heavy metals as well as lower sulfide and, indirectly, SO_4^{2-} and organic levels in the effluent. Biological treatment of metal-containing wastewaters with anaerobic cultures is an attractive technique for the bioremediation of this kind of medium. Numerous heavy metals are toxic to microorganisms including SRB due to their capacity to deactivate enzymes. In order to design a suitable process to address this environmental problem, identification of tolerant bacterial strains able to reduce SO_4^{2-} and consume organic substrates effectively and investigation of bacteria able to tolerate high concentrations of heavy metal are important. Previous work found that anaerobic microorganisms from brewery digester sludge were appropriate cultures for the treatment of wastewater with high SO_4^{2-} and heavy metal content due to their growth rate and their waste and SO_4^{2-} reduction rate [5]. The goal of this project was to identify the dominant culture of the SRB and any other strains that tolerate high concentrations of dissolved cadmium (Cd II), copper (Cu II), and zinc (Zn II). Enumeration of strains was carried out when the system contained SO_4^{2-} alone and in the presence of both SO_4^{2-} and high content of heavy metal.

1* *Scientific Equipment Center, Kasetsart University Research and Development Institute (KURDI), Kasetsart University, Bangkok 10900, Thailand. * E mail: rdinrs@ku.ac.th*
2 Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand. Email: fscipms@ku.ac.th
3 Otto H. York Center for Environmental Engineering and Science, New Jersey Institute of Technology, Newark, New Jersey 07102, USA. E mail: Daniel.Watts@njit.edu
4 Department of Chemical Engineering, Faculty of Engineering, Mahidol University, Salaya campus, Nakornpathom 73170, Thailand. Email: egscl@mahidol.ac.th

2 MATERIALS AND METHODS

2.1 Detection of Bacteria

The seed sludges were obtained from a brewery wastewater treatment plant and were acclimated with glucose based substrate in five liter reactors for more than 2 months at 30±1^{0}C. The hydraulic retention time (HRT) for the fill-and-draw operation type seed sludge reactors was 20 days. This was

used as a parent culture. The synthetic glucose substrate contained sufficient inorganic nutrients based on the formulations reported by Leighton and Forster [2] to facilitate growth (Table 1).

Table 1 Glucose synthetic wastewater composition.

Constituent in 10 liters	Weight or Concentration
Glucose	300 g
Urea	45.76 g
$NaHCO_3$	128.16 g
KH_2PO_4	13.856 g
K_2HPO_4	10.656 g
$MgCl_2$	18.112 g
$FeCl_2.6H_2O$	0.1376 g
$NiSO_4.6H_2O$	0.1056 g
$MnCl_2.4H_2O$	0.1056 g
$ZnSO_4.7H_2O$	0.1056 g
H_3BO_3	0.0224 g
$COCl_2.6H_2O$	0.0112 g
H_3PO_4 12 MoO.24 H_2O	0.00832 g
$CuSO_4.5H_2O$	0.001056 g
COD of this glucose synthetic waste	32000 mg/l

Experiments were carried out in 120 ml serum bottles by batch tests. These bottles were placed in a shaking water-bath with temperature controlled at 35±1^{0}C. The bottles were initially purged with N_2 and then seeded with 80 ml acclimated steady-state seed sludge. The quantity of biomass in each bottle was measured by the content of mixed liquor volatile suspended solids (MLVSS), following the standard methods [6]. The initial biomass concentration was around 10000 mg/l.

Proper amounts of glucose synthetic waste and distilled water were added into the bottles to make the initial COD around 3000 mg/l in order to mimic industrial wastewater. The initial loading factor (COD/MLVSS) in each bottle was similarly controlled. SO_4^{2-} was added to bottles (in the form of Na_2SO_4 solution) to give the optimum ratio of biomass in contact with SO_4^{2-} at 1:0.114 by weight in order to balance the population size of the methane producing bacteria (MPB) and that of the SRB [7]. The total working volume of each bottle was 100 ml. Optimum growth conditions were provided in this operation. A single heavy metal to be investigated from the set of Cd (II), Cu (II) and Zn (II) was added to the serum bottles as an aqueous nitrate form. These metallic ions were selected because they are present in a real contaminated effluent. The tested concentration of each heavy metal used in this study was determined from the solubility of that heavy metal under the expected hydroxide concentrations in the wastewater at the operating pH (7.0 ± 0.5). The metal concentrations used in the test were selected based on the differing levels of inhibition shown by different metals and to be soluble under the experimental conditions. Hence, the concentrations of dissolved Cd (II), Cu (II) and Zn (II) used in this study were 20, 2 and 30 mg/l, respectively. A serum bottle that contained only the optimum amount of SO_4^{2-} served as a no-metal control. Gases were vented through a tube in the rubber cap that was connected to a tube containing water. The experiments were conducted in batch mode after inoculation with the parent acclimated culture as previously described. The study lasted about 120 hours, by which time the gas production normally had leveled off.

The enumeration, isolation and identification of SRB and any other anaerobic bacteria that tolerated these heavy metals were performed when the assays were finished in order to demonstrate the number and type of bacteria that tolerated each heavy metal compared with the no-metal control. Based on the enumeration, the number of bacteria was reported in colony forming unit per gram of mixed liquor volatile suspended solids (CFU/gMLVSS). All experiments were conducted in duplicate and the results were calculated using the mean of the experimental values.

2.2 Isolation of Bacteria

Aliquots (1 ml) of each suspension were spread on the surface of nutrient rich melt agar medium containing 0.02% $Fe(SO_4)_2$ $(NH_4)_2.6H_2O$ as a primary enrichment for SRB. After 3 days of incubation at room temperature (30^{0}C), FeS was observed. The culture showed colony formation in medium containing 0.7% agar and 0.02% $Fe(SO_4)_2$ $(NH_4)_2.6H_2O$ in a plate, after 3 days, and a colony was transferred to the melted agar medium. For examination of the physiological characteristics, sodium lactate (20mM) was evaluated as electron donor. Growth was examined at room temperature, and pH 7 in the medium.

Aliquots (1 ml) of each suspension were spread on nutrient rich solidified agar medium (phosphate buffer basal medium, PBBM) that was designed for anaerobic strains. Streak plates in the anaerobic glove box were inspected daily for bacterial colonies. After 7 days of incubation at room temperature (30 ^{0}C), several different colonies were observed. The culture showed colony formation in medium in a plate. Growth was examined at room temperature, and pH 7 in the medium. The bacterial types that formed colonies with visually different morphologies were picked and sub-cultured to obtain

pure colonies. Each colony was transferred to new agar medium in streak tubes. Pure culture isolates of anaerobes were identified with 16S rRNA gene analysis.

2.3 Identification of Bacteria

Cells were harvested from 1.2 ml of culture on agar medium. The DNA extraction was carried out using a combination of the ISOPLANT II kit Nippon Gene Co., Tokyo, Japan) and DNeasy Tissue kit (QIAGEN K.K., Tokyo, Japan). The cell was suspended in 400 µl Tris-EDTA buffer (pH 8.0). 8 µl of 50 mg/ml lysozyme was added and then incubated at 37^0 C for 30 minutes. Then 4 µl of the Proteinase K (20 mg/ml) and 20 µl of sodium dodecyl sulfate (SDS 10%) and 4 µl of RNase A (100 mg/ml) were added and then incubated at 37^0 C for 30 minutes. The purification of DNA was carried out using phenol/chloroform extraction. The 16S rRNA gene of the isolates was amplified using the following primers: forward primer 27F 5'-AGAGTTTGATCMTGGCTCAG-3' (*E. coli* positions) and reverse primer 1389R 5'-ACGGGCGGTGTGTACAAG-3' (*E. coli* positions where M is A or C). The reaction mixture (15 µl) contained: 10 pmol of each primer, 2.5 mM of each dNTP, PCR buffer containing 15 mM $MgCl_2$ and 1.25 U *Ex-Tag* (TAKARA, Japan). PCR was performed as follows: 95^0C for 1 minute, followed by 35 cycles consisting of 95^0C for 20 seconds, 50^0C for 30 seconds and 72 ^{0}C for 2 minutes, with a final 4 minute extraction at 72^0 C. The PCR products were sequenced using an ABI 377 automated sequencer (Applied Biosystems). The primer 520F 5' CAGCMGCCGCGGTAAT(A/T) C-3' was used for sequencing. Approximately 500 bp of the 16S rRNA genes were initially sequenced using the PCR primers. Sequences were compared using the BLAST program of the National Center for Biotechnology Information (NCBI) [8].

3 RESULTS AND DISCUSSION

Significant differences in bacterial growth were observed during the addition of different metals (Fig. 1). A significant decrease in the amount of SRB was observed with the addition of 20 mg/l Cd (II) as well as 30 mg/l Zn (II) (compared with a no-metal control), indicating that the concentrations in the test exert a significant inhibitory effect on SRB culture. However there was not a significant difference in SRB growth observed between the serum bottle containing Cu (II) and a no-metal control. The reported toxic concentrations of heavy metals to SRB range from a few mg/l to as much as 100 mg/l [9]. Cabrera et al. studied the SRB that tolerated heavy metal and found that dissolved Cu (II) 4 mg/l and Zn (II) 20 mg/l were the maximum tolerable concentrations (MTC), defined as the highest concentration of a metal in the medium that does not cause death of microorganism, for *Desulfovibrio vulgaris* and *Desulfovibrio sp.* [10].

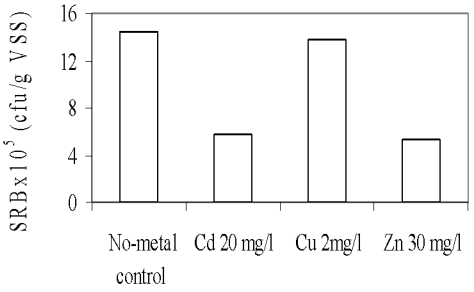

Fig. 1. Evolution of SRB population in the presence of 20 mg/l Cd (II), 2 mg/l Cu(II) and 30 mg/l Zn(II) compared with a no-metal control.

In this study, amplification of 16S rRNA was used to investigate the community composition in the consortium. Microbiological analysis showed that *Clostridium ganghwense* strain HY-42-06 was the dominant species of SRB originated from a brewery wastewater treatment plant. The strain had a similarity of 99%. Liu et al. reported that clostridial species were predominantly found on the surface layer of brewery-degrading anaerobic sludge granules [11].

Table 2 reports all strains and the number in colony forming unit per gram of mixed liquor volatile suspended solids (CFU/gMLVSS) from this study in the presence of 20 mg/l Cd (II), 2 mg/l Cu (II) and 30 mg/l Zn (II) compared with the no-metal control. In this study, according to the PCR technique, distinct differences were found in microbial community structure. The four dominant strains, which were revealed by the analysis based on 16S rRNA gene sequences, were all closely related to a member of *Micrococcus luteus* (AB 079788), *Wolinella succinogenes* (BX 571660), *Sporosarcina* sp. PIC-C28 (DQ 227790) and *Alicycliphilus* sp. R-24604 (AM 084015), and the sequence strain exhibited 94%, 100%, 100% and 100 % similarity, respectively.

The four highly resilient Cd-resistant strains were identified as *Micrococcus luteus, Wolinella succinogenes, Sporosarcina* sp. PIC-C28 and *Alicycliphilus* sp. R-24604, according to the analysis of 16S rRNA gene sequences (Table 2). On the other hand, there were no strains that could tolerate 30 mg/l Zn (II). Cu showed an adverse effect

to *Wolinella succinogenes*, but it seemed to stimulate *Sporosarcina* sp. PIC-C28 (Table 2). Moreover *Micrococcus luteus* and *Alicycliphilus* sp. R-24604 were found to tolerate Cu (II) at the tested concentration. None of these are methane producing bacteria (MPB).

Table 2 Evolution of anaerobic bacterial population in the presence of 20 mg/l Cd (II), 2 mg/l Cu (II) and 30 mg/l Zn (II) compared with the no-metal control.

Serum bottles	**Isolate (CFU/gMLVSS)**			
	Micro-coccus Luteus	***Woli-nella Succino-genes***	***Sporo-sarcina* sp. PIC-C28**	***Ali cyclip-hilus* sp. R-24604**
SO_4^{2-} (no-metal control)	1.04x 10^4	1.25x 10^5	5.75x 10^5	8.97x 10^5
SO_4^{2-}, Cd (II) 20mg/l	1.00x 10^4	1.22x 10^5	5.09x 10^5	5.13x 10^5
SO_4^{2-}, Cu (II) 2mg/l	0.98x 10^4	nd	8.74x 10^5	5.86x 10^5
SO_4^{2-}, Zn (II) 30 mg/l	nd	nd	nd	nd

nd = not detectable

Although there was not a significant decrease observed when SRB were exposed to 2 mg/l Cu (II) (Fig. 1), but *Wolinella Succinogenes* did not appear (Table 2). A significant decrease in the enumeration of SRB and no other strains of bacteria were observed in the experiment with 30 mg/l of Zn (II) (Fig. 1 and Table 2), meaning that this concentration is extremely toxic for all cultures. The values obtained indicated that the exposure to 30 mg/l of Zn (II) results in a greater inhibition for SRB (Fig. 1) and there were not any strains found in the system (Table 2).

4 CONCLUSION

From brewery digester sludge, *Clostridium ganghwense* strain HY-42-06 was found to be the prevalent species of SRB and this strain can tolerate dissolved metals at 20 mg/l Cd (II), 2 mg/l Cu (II) and 30 mg/l Zn (II) added separately. The other type present was the culture that was identified as *Micrococcus luteus*, *Wolinella Succinogenes*, *Sporosarcina* sp. PIC-C28 and *Alicycliphilus* sp. R-24604. The results found that these four dominant strains tolerated Cd (II) at 20 mg/l, only *Wolinella Succinogenes* cannot tolerate to Cu (II) at 2 mg/l while none of them tolerated Zn (II) at 30 mg/l. These strains are the tolerate microorganisms that require a further study to apply to the treatment plant of wastewater with high sulfate and heavy metal content for metal reduction processes of industrial wastewater.

ACKNOWLEDGEMENT

This research was supported by Kasetsart University Research and Development Institute (KURDI), Kasetsart University, Bangkok, Thailand.

REFERENCES

[1] M.Yoda, M. Kitagawa, Y. Miyaji, "Long term competition between sulfate-reducing and methane-producing bacteria for acetate in aerobic biofilm," *Water Research*, 21:1547–1556, 1987.

[2] I.R. Leighton, C.F. Forster, "The adsorption of heavy metals in an acidogenic thermophillic anaerobic reactor," *Water Research*, 31:2969- 2972. 1997.

[3] C.Y. Lin, "Effect of heavy metals on volatile fatty acid degradation in anaerobic digestion," *Water Research*, 26:177-183, 1992.

[4] S.Okabe, P.H. Nielsen, W.L. Jones, W.G. Charackklis, "Sulfide product inhibition of *Desulfovibrio desulfuricans* in batch and continuous cultures," *Water Research*, 29:571-578, 1995.

[5] N. Sinbuathong, P. Sirirote, W. Liengcharernsit, S. Khaodhiar, D.J. Watts, "Kinetic comparison of microbial assemblages for the anaerobic treatment of wastewater with high sulfate and heavy metal contents," *Journal of Environmental Biology*, 30:11-15. 2009.

[6] APHA.: Standard methods for examination of water and wastewater. 21st Edn. APHA, AWWA, WPCF, Washington DC, USA, 2005.

[7] N. Sinbuathong, S. Khaodhiar, W. Liengcharernsit, P. Sirirote, D.J.Watts, "Effect of sulfate on the methanogenic activity of a bacterial culture from a brewery wastewater during glucose degradation," *Journal of Environmental Sciences*, 19:1025-1027, 2007.

[8] M. Ohkuma, T. Kudo, "Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*," *Applied and Environmental Microbiology*, 62: 461-468, 1996.

[9] V.P. Utgikar, S.M. Harmon, N. Chaudhary, H.H.Tabak, R. Goving, J.R.Haines, "Inhibition of sulphate-reducing bacteria by metal sulphide formation in bioremediation of acid mine drainage," *Environmental Toxicology*, 17:40-48, 2002.

[10] G. Cabrera, R. Perez, J.M. Gomez, A. Abalos, D. Cantero, "Toxic effect of dissolved heavy metals on *Desulfovibrio vulgaris* and *Desulfovibrio sp. strains.*, " *Journal of Hazardous Materials*, 135:40-46, 2006.

[11] W.T . Liu, O.C. Chanc and H.H.P. Fang, "Characterization of microbial community in granular sludge treating brewery wastewater," *Water Research*, 36:1767–1775, 2002.

Nomenclature

CFU	=	Colony forming unit per gram of mixed liquor volatile suspended solids
COD	=	Chemical oxygen demand
PCR	=	Polymerase chain reaction
MLVSS	=	Mixed liquor volatile suspended solid
MTC	=	Maximum tolerable concentration
SRB	=	Sulfate reducing bacteria