CHAPTER V

DISCUSSION AND CONCLUSION

The present studies document the removal of soluble cadmium by cadmium-resistance bacterial strains isolated from local sources (polluted and non-polluted). Cadmium accumulation by exopolysaccharide from those selected bacterial isolates was demonstrated and originated in Thailand.

Five of strains of Cd-resistant bacterial isolates were selected, named CdR-87, CdR-98, CdR-205, CdR-207, and CdR-273, and resisted to Cd, e.g., 500, 400, 750, 400 and 750 mg/l, respectively, and also they were able to produce exopolysaccharide. They were characterized and chosen as the test organisms. All were gram-negative, rod-shaped and identified by some biochemical tests, indicated that they should be classified in family ***Enterobactericeae*** genus ***Enterobacter sp.***

The advantages of metal resistance test by multi-inoculation method are ; facilitate testing of a large volume of culture, low-cost and each plate can be subjected to strict quality control using three or more test organisms with known reactivity and etc.,(Koneman, Allen, Dowell and Sommer, 1994). However, the procedure were tested with carefulness. Many factors should be controlled for constancy, e.g., the quantity of cell density in each

strain, volume of medium in each plate that affect to metal concentration, incubation period, pH adjustment (metal precipitation) and the component of media. In 1989, Nieto and Colleagues commented that in moderately halophilic eubacteria, the salinity and yeast extract concentration of the culture medium have influence on the toxicity of the test metal. Lowering the salinity led to enhance Cd or Cd and Cu sensitivities of the test organism. However, increased salinity resulted in only a slight reduction of toxicity of Cd, Cu and Ni. Reduction of yeast extract concentration resulted in an increased sensitivity of many metals, e.g., Ag, Co, Hg, Ni, Pb, Cd, Cr and Cu especially, when this component was lowered to 0.01% (w/v). Also, use of gelatin as a gel agent decreased the apparent toxicity of $SnCl_4$ (Hallus, Thayer and Cooney, 1982). To prevent other factors that affect the toxicity of any test metal, the minimal medium was chosen for metal resistance tests (see **Appendix B**). The pH factor to solubility of Cd in culture medium is acidic (see **Appendix H**). Possibly, selected bacterial isolates in this study were resistant to certain amount of Cd and also acid tolerant.

Effects of pH and temperature on growth and EPS production on all of the selected bacterial strains were similar. Optimum pH was found to be slightly alkaline (pH 8-9), and optimum temperature was found to be mesophilic range (30-40 C). Hence, EPS production by these selected bacterial strains (proposed ***Enterobacter*** **sp.**) may be dependent of the same growth rate of other bacteria, e.g., ***Pseudomonas aeruginosa***, ***Alcaligenes vinelandii*** and ***Rhizobium trifolii***, etc. (Margaristis and Pace, 1985).

In addition, incubation period also affected EPS formation in all isolates. From long-incubated culture (72 hr.), the amount of EPS was harvested higher than from short-incubated culture (24-48 hr). Bacterial EPS synthesis was initiated in the exponential phase of growth, but the rate of production increased rapidly after the cessation of growth. EPS was produced continuously until unsuitable condition of medium occurred, e.g., less in nutrient and oxygen contents, pH reduction and increase in waste and dead cell contents.

Some metal ions may affect the EPS forming in certain bacteria, e.g., in the culture of ***Enterobacter aerogenes***. The production of EPS was stimulated by Mg, K and Ca ions (Beveridge, 1989b). By contrary, EPS production in all five strains of the selected bacterial strains was decreased in the medium added with Cd. It is possible to say that EPS formation is not induced by Cd ion (in this study) and also by Cu ions (Charnnarong, T, personal communication).

There are several methods used in extraction of EPS, e.g., high-speed centrifugation, steaming, sodium hydroxide treatment, EDTA* extraction and ultrasonication. In the present studies, high-speed centrifugation was considered because it has been the most effective extraction method and also widely used. By this method, small amount of cell disruption and relatively high extracellular polymer yield were obtained for ***Klebsiella aerogenes*** (Brown and Lester, 1980).

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*ethylenediaminetetraacetic acid

Therefore, high-speed centrifugation was chosen in the experiments and optimum speed for Enterobacter EPS extraction was investigated to be 20,000 g. At this speed, high carbohydrate and a little of protein contents were obtained.

Initial Cd concentration may affect the uptake and adsorption ratio of cadmium by whole or living cells of the selected bacterial strains. Amount of Cd uptake by living cells increased follow as the external Cd concentration increased (see **Figure 4.23**). In Cd uptakes by the selected bacterial strains, e.g., CdR-87, CdR-98, CdR-205, CdR-207, and CdR-273 were 310.91, 258.87, 205.32, 245.26 and 200.6 nM/mg dried cells, respectively. in the solution containing 102.5 mg/l or 0.925 mM Cd. Comparing with others (see **Table 5.1**), uptake of Cd by those selected strains seems to be quite high, especially, strain CdR-87.

Those Cd-resistant bacterial isolates ***Enterobacter sp.*** exhibited a high activity but non specificity with respect to the uptake of other metals, e.g., Cu, Mn and Zn. Uptake of Cu and Mn seems to be less than of Cd, and the amount of Cd uptake by EPS, living cells and dead cells was similar. It suggested that metabolic uptake of Cd was low and indicated that Cd adsorption to the cell surface should be the major mechanism of uptake. It was similar to the metal uptake by activated sludge presented by Iourdon, Rus, Bhen de and Sofer (1990). However metal uptakes by active and non-active (sterilized by autoclaving) sludge were performed, the result showed that the uptake efficiency of non-active sludge was lower than that of the original viable sludge

possible, no second phase of metal removal occurred in the non-active sludge.

There were at least 2 steps involving in Cd accumulation by cells; the first step was metabolic-independent, rapid uptake of metal ions due to attachment onto the cell surface and then, followed by the metabolic-dependent step, a relatively slow uptake due to membrane transport of the metal into the cell (Ting, Lawson and Prince, 1989 ; Norris and Kelly, 1977 ; and Blackwell, Singleton and Tobin, 1995).

In the study, Mn and Cu accumulations in those Cd-resistant isolates were found to be higher in dead cells of each strain than in living cells or EPS of the same strain, the evidence may be depended on the size of atom. Generally, carboxylated polysaccharides exhibit preferential binding to cations with larger ionic radii (Geesey and Jang, 1989). The radius of Mn ion is smaller than Cu ion and Cd ion (26 A° for Mn, 28 A° for Cu and 54 A° for Cd). Hence, EPS has less affinity in binding to Mn and Cu than Cd. Another experiment showed that ***Rhizopus arrhizus*** biomass was able to adsorb larger ions more. Strongly than the smaller ones. It can be explained by a complexation mechanism involving active site in the biomass contained carboxylates, phosphates and other functional groups (Tobin, Cooper and Neufeld, 1984). Bond formation between carboxyl group and metal ion is much stronger than hydrogen bonding between neutral polysaccharide and metal ions (Brown and Lester, 1979).

Viable cells of bacteria can accumulate essential metal ions, e.g., Mn ion, by transport system (Gadd, 1990a), then, certain amount of Mn is removed by viable cell.

EPS or exopolysaccharide, natural substance produced by cell, seem to be an important part of Cd adsorption. Similar results were shown that bacterial exopolysaccharide was able to significantly enhance biosorption of Cd, e.g., EPS of ***Arthrobacter viscocus*** exhibited 2.3 times greater accumulation capacity than the cells of ***A. globiformis*** EPS non-producer (Scott and Palmer, 1988). And also, Brown and Lester (1982a and 1982b) found that the metal adsorption capacities of ***Klebsiella aerogenes*** and activated sludge that EPS had been limited formerly, were reduced comparing to the capacity of the viable of ***K. aerogenes*** and the floc.

Contract time for Cd uptake in immobilized cell of CdR-205 was observed (see **Figure 4.27**). The amount of adsorbed Cd was very rapid during the first 10 minutes and gradually increased until 30 minutes. It is similar to the previous investigation, e.g., Cd uptake by ***Zoogloea ramigera*** (Park, Jin and Chang, 1999) and by ***Ascophyllum nodusum***, brown marine algae (Voleskey and Passetyo, 1994).

From the experiment, it showed that after exposure to 10.39 mg/l Cd for 10 minutes, alginate adsorbed about 40% of Cd from the surrounding, whereas immobilized cells of CdR-205 and CdR-273 adsorbed about 90% of the metal and the amount of adsorbed Cd was raised at increasing level of Cd concentration. Alginate, a

mixture polygaluronic and polymannuronic acids contained abundant amount of hydroxy group that concern metal binding capacity of the molecule (Kuhn and Pfister, 1989) the binding site of cation in alginate was shown in **Figure 2.9.**

High efficiency for Cd removal was found in unregenerate immobilized cell. After first and second regeneration the Cd removal efficiency of the regenerated immobilized cell were decreases rapidly. Possibly, nitrilotriacetic acid, washing agent, might affect the bead by suspending the exopolysaccharide out of the beads (Park, Jin and Chang, 1999).

In conclusion, research on metal-resistant bacteria and their EPS had made a significant contribution to the understanding of metal biosorption, like in case of cadmium. Thus, the isolation of selected new metal-resistant bacterial species could provide valuable material for future genetic or other biological investigation. The selected cadmium-resistant bacteria found in the present studies might be used as a guideline and applied to other metal adsorption system.

Table 5.1 Comparison of Cd uptake by microorganisms.

Organisms	Cd conc.	Cd uptake (nM/mg dry wt.)	Ref.
YEAST			
Saccharomyces cerevisiae			
101N, sensitive	0.5 mM	4.9	Joho and others, 1985
301N, resistance		3.6	
NCYC 431	0.2 mM	80	Norris and Kelly, 1977
S. cerevisiae	0.2 mM	110	Brandy and Duncan, 1994
FUNGI			
Aureobasidium pullulans			
IMI 45533-yeastlike cells	0.5mM	27	Mowell and Gadd, 1984
-mycelium		29	
-chlamydospores		38	
Mucor racemosus	0.089mM	55	Kurek and others, 1982
Penicillium chrysogenum		26	
Trichoderma viride		20	
Aspergillus niger		33	
A.niger	0.712mM	1377.10	Doyle, Marshall and Pfander, 1975
Pleurotus astreatus Tohoku H67	1.0mM	293.57	Sanglimsuwan and others, 1993
Streptomyces pimprina	0.89mM	36.83	Puranik, Chabukswar and Paknikar, 1995
GREEN MICROALGAE			
Chlorella regularis	8.896 µM	29.43	Sakaguchi, Tsuji, Nakajima and Horikoshi, 1979.
Chlamydomonas angulosa		17.62	
C. reinbardtii		31.48	
Scenedesmus bijuga		23.69	
S. chlorelloides		16.43	
S. obliquus		21.85	

Table 5.1 (continuous)

Organisms	Cd Conc.	Cd uptake (nM/mgdry wt.)	Ref.
BACTERIA			
Straphylococcus aureus	0.089mM	249.09	Dole. Marshall, and Pfander,
	0.178 mM	358.51	1975
Bacillus cereus	0.178mM	464.37	
	0.356mM	588.91	
	0.712mM	792.63	
Streptococcus faecalis	0.178mM	6.23	
Lactobacillus acidophilus	0.178mM	25.79	
Eschericher coli	0.178mM	26.69	
	0.356mM	47.15	
	0.712mM	87.18	
E. coli	0.222mM	58.71	Kefera and Ekaterniadou,
Bacillus subtilis	0.222mM	120.10	Zouboulis, Matisand and
Xanthomonas campestris	0.222mM	120.98	Kyriakidis, 1994
Leuconostoc mesenteroides	0.222mM	100.52	
Arthrobacter viscosus	8.896μM	12.45	Scott and Palmer, 1988
(EPS producing)			
A. globiformis	8.896μM	2.05	
(non EPS producing)			
A.viscosus EPS	8.896μM	29.18	
Streptococcus faecalis	50μM	0.84	Bhattacherjee, 1986
	250μM	3.35	
	500μM	4.40	
CdR-87	**0.925mM**	**310.91**	**This study**
CdR-98	**0.925mM**	**258.87**	**This study**
CdR-205	**0.925mM**	**205.32**	**This study**
CdR-207	**0.925mM**	**245.26**	**This study**
CdR-273	**0.925mM**	**200.60**	**This study**