CHAPTER V

DISCUSSON AND CONCLUSION

The present studies document the removal of soluble copper by copper–resistant bacterial strains isolated in Thai environment. Copper accumulation by those selected bacterial isolates was demonstrated and originated in Thailand.

Two of 350 strains of Cu-resistant bacterial isolates were selected and named: CuR-38 and CuR-40. Both of them resisted to 700 μg/ml Cu and also were able to produce exopolysaccharide (EPS). They were characterized and chosen as the test organisms. CuR-38 was gram–negative and rod shape but CuR-40 was gram–positive, endospore forming and rod shape, and identified as ***Zoogloea*** **sp.** for CuR-38 and ***Bacillus*** **sp.** for CuR-40.

Five of 350 strains of Cu-resistant bacterial isolates were selected and named, CuR-4, CuR-14, CuR-24, CuR-25 and CuR-32. All of them also resisted to 700 μg/ml Cu but were unable to produced EPS. They were characterized and chosen as the test organisms during the initial phase of the studies. All were gram–negative, rod shape and selected from soil and sludge collected from industrial sites.

The study indicated that certain industrial sites might be a habitat of copper-resistant bacteria because high concentration of Cu was possibly found and also promoted by acidic condition, and copper-resistant bacteria might be selected naturally. Activity (low pH) is one of the factors, which affects the solubility of heavy metals. In a solution, the concentration of heavy metals increased where as the pH value decreased see **APPENDIX D**. So, capability of heavy metal resistance in bacteria may be closed relationships to be acidic tolerant, not acidophilic. Therefore, proliferation of microorganisms in the environment containing high concentration of Cu should be possible.

Copper resistant in bacteria was found to be stable after, at least, 20 times repeated subculturing in stock culture containing small amount of Cu. Now, it is not known that plasmid or chromosomal mediated is genetically associated with resistance capability of high Cu concentration among those organisms.

The bacterial community was also found to be resistant to a number of other heavy metals (**Table 4.4**). Copper-resistant bacterial isolates were also resistant to Cu, Zn, Mn and Cd, but not resistant to Ag, Ni and Cr. However, mechanisms of Cu resistance in Cu-resistant bacterial isolates may be different in resistance to other metals. This implies that the copper-resistant mechanisms were partially successful in improving resistant toxicity of the other metals. There are specific copper–resistant mechanism and a nonspecific metal-resistant mechanism. Metal resistance mechanism is different in community. The

present studies also showed that resistant to one metal in bacterial sometimes does confer resistant to other metals.

In some experiments, resistance to copper and other heavy metals was found in the selected bacterial isolates. Uses of growth in metal-resistance tests are also important. However, it is recommended that some growth media for metal-resistance test should be avoid (Bird et. al., 1985). Many components of microbiological media, for example, agar, peptones and yeast extract are capable of binding significant amount of metal ions and there are some examples of metal toxicity being reduced by such binding (MacLeod et. al., 1967). Metal resistant is markedly dependent on medium composition due to differences in the chelating property of medium components and specific effects. Thus, in these experiments with regarded to metal ions and growth medium. The growth medium is used only 1/3 strength to reduce those effects. But the effect after reduced amount of medium was not evaluated in this research.

Clearly, the growth rate of CuR-38 and CuR-40 used in this study, the presence of copper ions (700 μg/ml; pH 4) was related to growth rate. By, there was a decreased in growth rate compared in the normal TSB (pH 7) and TSB (pH 4), due to the toxicity of Cu ions and acid condition (**Figure 4.10-4.11**).

The results of experiments examining the effect of pH and temperature on growth rate can be summarized as follows. The optimal pH that they could proliferate at neutral pH (7), the acid condition

copper-resistant can growth more than base condition, but at the extreme pH (2, 3, 11 and 12) bacteria can not growth. The result from this study shown that temperature at 37°C was suitable for growth of them, but at 30°C or 40°C is not more different. From preliminary study indicated at high temperature (50°C) the bacteria can not growth and the rate of growth at 35°C and 37°C is not different (data not shown).

The results of the present study, the effects of pH, temperature and incubation time on EPS production by CuR-38 and CuR-40 can be summarized as follows. The potential for EPS production, the maximum of production was obtain when the cells were inoculated at pH 7, but there was not synthesis of EPS when they grown in TSB medium which adjusted pH to 4 and 10. The response of CuR-38 and CuR-40 to an acidic pH on EPS production, they can produce EPS more than base condition. The pH optimum for EPS production depends on the individual species, but is near neutrality for most bacteria (Wilkinson, 1958).

Table 4.7 was shown a comparison of the different temperature on the EPS production. The potential for EPS production during the growth period at 37°C, a maximum of EPS production was obtained. At the temperature of 30°C and 40°C, the production was decreased, but did not more different. The results of the preliminary study indicated that at 35°C and 37°C the EPS production is not different (data not shown).

The incubation time on EPS production by selected bacteria (CuR-38 and CuR-40). The EPS production appeared to increase per unit

weight of cells well or production continued maximally during the stationary phase of selected bacteria. Maximum production for 48 hr. before the rate of production decreased for 72 hr. However, the production of EPS by selected bacteria were production continues after the stationary phase, although the rate of production decreased. Consequently, the incubation time of EPS-production was differed from that many species, in the EPS production was not concurrent with growth. By general EPS production continued maximally during the stationary phase. For instances, the EPS production by ***P. fluorescens*** (Eagon, 1956) and ***Zoogloea*** **MP6** (Unz and Farrah, 1976) occurred in the late exponential and stationary phase of growth. Williams and Wimpenny (1977) summarized that the EPS production by ***Pseudomonas*** **NCIB 11264** grown in batch culture, the EPS production it production continued maximally during the stationary phase.

Our results indicated that copper is not a signal for EPS production in CuR-38 and CuR-40 (**Table 4.9**). From the results reported by Chao and Chen (1991), the growth of the ***Pseudomonas*** **sp.** used in this study, in the presence of copper ion were not related to either the amount of exopolymer produced or to the copper ion binding ability of these exopolymer. However, metal have been shown to influence the production of EPS in some bacterial strains. For instances, ***Enterobacter aerogenes*** that EPS was stimulated by Mg, K and Ca ions (Wilkinson and Stark, 1956) and EPS production increased as more Cr (III) was added to the medium in coryneform bacterium (Bremer and Loutit, 1986).

The results from study the contact time of the accumulation copper by EPS, indicated that the equilibrium was completely within 15 min. (**Figure 4.12**) and prolonged exposure time did not increase accumulation of copper from solution. This implies that the bound metal ions were start adsorbed within the first minute after metal addition.

Table 4.10 was shown the effect of copper concentration on the accumulation of copper by EPS. The results indicated at low concentration the percentage of accumulation was high, but the percentage of this decreased as the concentration increased. Whether, the concentration of EPS could not be determined the effect on accumulation. The amount of metal bound also depended on the concentration of EPS in the solution. The interaction and relation of this requires further study.

A comparison of the accumulation capacities between EPS and whole cells, the results show that biosorbent reached similar accumulation rate (not more different). EPS and whole cells show the high efficiently for accumulation (**Table 4.11**). Therefore, the results indicated continuous process is that likely to be efficient method for copper recovery by used whole cells. Clearly, showed that the EPS contributed significantly to copper adsorption in strains CuR-38 and CuR-40 (**Table 4.12**). However, the results showed that remain part (intracellular and/or cell walls) of cell were contributed to metal adsorption.

The results presented in this study demonstrate that the capability of EPS, which isolation from strains CuR-38 and CuR-40 to bind heavy metal is not rather specific. The EPS exhibit metal-complexing capacity, which could be used to adsorb other as well as copper. Previous study indicated that each metals is not interfere on accumulate of heavy metals by EPS when mixed the metals in the solution. The efficiency of accumulation for each metals and mixed metals was the same (not more different) and high efficiency except Mn (**Table 4.13-4.14**).

Clearly, the results from all experiments showed that the EPS is very important to metal adsorption. Due to a correlation between highly anionic charged polymers and metal-complexing capacity was found that in extensive studied. It seem that the negative charge namely carboxyl, hydroxyl groups (contributed by uronic acid) and hydroxyl groups (contributed by neutral sugar) of EPS play a major role in metal-complexing (Geesey and Jang, 1989). However, more information is required concerning EPS structure and composition in relation to binding properties.

The release of metals from EPS achieved by an inexpensive and simple reagent: HCl. The amount of metal desorbed with HCl varied from 26.3% for Mn to 98.3% for Zn of CuR-38 and varied from 25.8% for Mn to 88.7% for Zn of CuR-40 (**Table 4.15**). In mixed metal solution, the percentage of recovery is not more different (except Cd) in **Table 4.16**. Copper in the presence of Zn, Mn and Cd were as readily recovered as copper alone in solution. But, Cd in the presence of mixed

metal was not as readily recovered as Cd alone in solution. The comparison between time on percentage of metal recovery in each metal solution and mixed metal solution were shown in **Figure 4.17**, **4.18**, **4.19** and **4.20**. However, metal recovery and concentration using HCl was not fully evaluated. The interaction of HCl with the EPS requires future study.

Results from the study about efficiency of EPS after regeneration to accumulation copper. The copper accumulation obtained upon reexposure of used and regenerated EPS to a copper solution revealed that the acid treatment did not more effect the metal binding capacity of the EPS. After first regeneration, the percentage of accumulation was 76.5 and 63.0 (CuR-38 and CuR-40; respectively) which it very high accumulation rate (**Table 4.17**). Whether, other metals are not study, though the latter seems more likely.

Although the result from this study show the ability of EPS for accumulation metals. Freely suspended EPS has disadvantage that include small particle size, low mechanical strength and low density (Gadd, 1992). Immobilized systems appears of greater potential in reactor systems with benefit including control of particle size, better capacity of regeneration, easy separation of biomass and effluent and re-circulation, high biomass loading and minimal clogging under continuous flow (Tsezos, 1990; Macaskie and Dean, 1989 and White and Gadd, 1990). So, immobilization of EPS, biomass and viable cell could be used in further study to improve the capacity of EPS by base on primary data was found in this research.

From the present study, the copper-resistant bacterial isolates can resistant to 700 μg/ml copper and high stability of resistant. Compared to the former investigations, the resistant higher than those of many researches done so far, although less than some research (**Table 5.1**). The suitable growth and EPS-production was the same (pH 7 and 37°C), which easy to cultivation and operate. The contact time of copper accumulation by EPS was reached within short time (15 min.). The percentage of copper accumulation in this research was greater than 80% in each and mixed metal solution. Simple and inexpensive reagents were used to release metals from EPS in high efficiency. The percentage of accumulation after regeneration was high rate. To enhance the efficiency of biosorbent. The immobilized system should be used. I fell that after immobilized, were comparable to other metal removal system and the immobilized cell systems has potential industrial application value.

In conclusion, research on removal of soluble copper by copper-resistant bacterial isolates has made a significant contribution to reduce and protect environmental problem, which extremely and difficult to solve in the future. Due to research demand in country to find out the best method to remove copper which release in high concentration. Thus, the isolation of selected bacterial strain could provide valuable material for further genetic and other biological investigation at the molecular level. A general conclusion which this study can be drawn from data reported here and some characteristics of selected bacterial isolates CuR-38 (***Zoogloea* sp.**) and CuR-40 (***Bacillus* sp.**) were summarized in **Table 5.2**.

Table 5.1 The percentage of copper accumulation by bacteria compared to the former investigations

Organisms	Cu resistant (μg/ml)	% of Cu Accumulation	Initial Cu Conc. (μg/ml)	Remarks	Ref.
***Pseudomonas* sp.** (AS 1)	127.14	-	-	-	Dunn and
P. putida (AS 4)	317.85	-	-	-	Bull, 1983
Alcaligenes faecalis (AS 5)	953.55	-	-	-	
P. aeruginosa (AS 6-AS 9)	127.14	-	-	-	
P. aeruginosa (AS 10)	63.57	-	-	-	
P. fluorescens (AS 11)	63.57	-	-	-	
Whole community (Activated Bacteria)	953.55	98.39	620	Viable cells (for 15 days)	
***Z. ramigera* 115** (ATCC 25935)	-	83.33	480	-	Norberg and Persson, 1983
Z. ramigera	-	99.90	117	Immobilized in Ca - alginate	Kuhn and Pfister, 1989
***P. putida* 3**	0.01	-	-	-	Chao and
***P. aeruginosa* 7**	3	-	-	-	Chen, 1991
***P. pseudomalle* 13-1**	4	-	-	-	
***E. coli* HB 101**	100	-	-	-	
P. syringae* pv. *syringae (FF 5.22, F 5.31 and FF 5.32)	800	-	-	-	Kidambi et. al., 1995
P. syringae* pv. *syringae (FF 5.21)	< 600	-	-	-	
Z. ramigera (CuR-38)	**700**	**80.63**	**112**	**Whole cells**	**This Study**
		84.73		**EPS**	
B. licheniformis (CuR-40)	**700**	**76.96**	**112**	**Whole cells**	
		80.00		**EPS**	
Z. ramigera (CuR-38)	**700**	**77.23**	**112**	**Whole cells**	**This Study**
		82.41		**EPS**	
		15.39		**Cells**	
B. licheniformis (CuR-40)	**700**	**72.77**	**112**	**Whole cells**	
		80.45		**EPS**	
		21.13		**Cells**	

Table 5.2 Some characteristics of the selected bacterial isolates CuR-38 (***Zoogloea* sp.**) and CuR-40 (***Bacillus* sp.**)

Some Characteristics		Selected Bacterial Strains	
		CuR-38	**CuR-40**
1.	Maximum resistance to copper (μg/ml)	700	700
2.	Maximum resistance to other metal (μg/ml)		
	2.1 Zn	300	300
	2.2 Mn	300	300
	2.3 Cd	300	300
3.	EPS production	positive	positive
4.	Optimum condition for growth and EPS production		
	4.1 Optimum pH, temperature for growth	7 and 37°C	7 and 37°C
	4.2 Optimum pH for EPS production	7	7
	- dry weight of EPS (mg)	64.39	59.25
	- dry weight of cells without EPS (mg)	18.85	17.09
	4.3 Optimum temperature (°C) for EPS production (pH 7)	37°C	37°C
	- dry weight of EPS (mg)	64.39	59.25
	- dry weight of cells without EPS (mg)	18.85	17.09
	4.4 Optimum incubation period (hr.) for EPS production (pH 7 and 37°C)	48	48
	- dry weight of EPS (mg)	69.86	66.33
	- dry weight of cells without EPS (mg)	18.59	17.88
	4.5 Maximum EPS production (not depend on Cu induction) pH 7, 37°C for 48 hr.		
	- dry weight of EPS (mg)	67.86	56.75
	- dry weight of cells without EPS (mg)	13.11	13.47
5.	Accumulation of Copper by EPS		
	5.1 Contact time of copper accumulation (min.)	15	15
	- Maximum copper accumulation from 500 μg/ml Cu solution	270 μg/ml	248 μg/ml
	5.2 Effect of initial copper on biosorption of EPS (μg/ml)		
	- Highest percentage of copper biosorption	93	92
6.	Copper accumulation by EPS and whole cells (112 μg/ml as initial copper concentration in solution)		
	6.1 Copper accumulation (μg/ml) by		
	- 5 g (wet wt.) of EPS	94.9	89.6

- percentage of copper accumulation	84.73	80.00
6.2 Copper accumulation (µg/ml) by		
- 5 g (wet wt.) of whole cells	90.3	86.2
- percentage of copper accumulation	80.63	76.96
7. Copper accumulation by whole cells, EPS and cells with out EPS (112 µg/ml as initial copper concentration in solution)		
7.1 Copper accumulation (µg/ml) by		
- 1 g (dry wt.) of whole cells	86.5	81.5
- Percentage of copper accumulation	77.23	72.77
7.2 Copper accumulation (µg/ml) by		
- 1 g (dry wt.) of EPS	92.3	90.1
- Percentage of copper accumulation	82.41	80.45
7.3 Copper accumulation (µg/ml) by		
- 1 g (dry wt.) of cells without EPS	17.2	23.9
- Percentage of copper accumulation	15.39	21.34
8. Other heavy metal accumulation by EPS		
8.1 Each metal solution (112, 114, 109 and 111 µg/ml as initial Cu, Zn, Mn, Cd, respectively, concentration)		
- Cu		
• 1 g (dry wt.) of EPS	97.5	92.7
• Percentage of Cu accumualtion	87.05	82.77
- Zn		
• 1 g (dry wt.)of EPS	94.2	83.5
• Percentage of Zn accumualtion	82.63	73.25
- Mn		
• 1 g (dry wt.) of EPS	83.6	73.00
• Percentage of Mn accumualtion	76.70	66.96
- Cd		
• 1 g (dry wt.) of EPS	95.9	89.50
• Percentage of Cd accumualtion	86.40	80.63
8.2 Mixed metal solution (112, 114, 109 and 111 µg/ml as initial Cu, Zn, Mn, Cd, respectively, concentration)		
- Cu		

• 1 g (dry wt.) of EPS	24.6	23.28
• Percentage of Cu accumualtion	21.97	20.78
- Zn		
• 1 g (dry wt.)of EPS	23.38	20.90
• Percentage of Zn accumualtion	20.50	18.33
- Mn		
• 1 g (dry wt.) of EPS	20.38	17.13
• Percentage of Mn accumualtion	18.69	15.71
- Cd		
• 1 g (dry wt.) of EPS	23.18	22.30
• Percentage of Cd accumulation	20.88	20.09
9. Recovery		
9.1 Maximum percentage of recovery	120 min.	120 min.
9.2 Percentage of recovery (each metal solution)		
- Cu	77.8	68.0
- Zn	98.3	88.7
- Mn	26.3	25.8
- Cd	77.1	72.6
9.3 Percentage of recovery (mixed metal solution)		
- Cu	77.7	65.3
- Zn	98.2	89.7
- Mn	25.2	26.3
- Cd	45.2	27.3
10. Percentage of accumulation after regeneration efficiency		
- No. of regeneration		
• 1	76.5	63.0
• 2	73.5	59.5
• 3	73.5	55.5
• 4	73.0	55.0
• 5	65.0	54.0
11. Expected species	***Zoogloea ramigera***	***Bacillus licheniformis***