# CHAPTER 5

# DISCUSSION AND CONCLUSION

The present studies document the detoxification of chromium by chromium-resistant bacterial strains and the degradation of phenol by phenol-resistant bacterial strains isolated in Thai environment. Chromium detoxification and phenol degradation by those selected bacterial isolates was demonstrated and originated in Thailand.

All 495 strains, three of 150 strains of chromium-resistant bacterial isolates were selected and named: CrR-2, CrR-14 and CrR-15. All of them resisted to 2400 μg/ml chromium and also were able to resist to 1000 μg/ml phenol. All were gram-negative, rod shape and identified as ***Escherichia*** **sp.**, ***Pseudomonas*** **sp.** and ***Enterobacter*** **sp.** for CrR-2, CrR-14 and CrR-15, respectively.

Three of 225 strains of phenol-resistant bacterial isolates were selected and named, PhR-26, PhR-33 and PhR-64. All of them also resisted to 2000 μg/ml phenol and also were able to resist to 500 μg/ml Cr. All were gram-negative, rod shape and identified as ***Klebsiella*** **sp.**, ***Pseudomonas*** **sp.** and ***Escherichia*** **sp.** for PhR-26, PhR-33 and PhR-64, respectively, and selected from soils and sludges collected from industrial sites.

Three of 120 strains of chromium/phenol-resistant bacterial isolates were selected and named, CPR-4, CPR-16 and CPR-17. All of them also resisted to 1200 μg/ml chromium and also were able to resist to 1200

μg/ml phenol. All were gram-negative, rod shape and identified as ***Pseudomonas* sp.**, ***Proteus* sp.** and ***Escherichia* sp.** for CPR-4, CPR-16 and CPR-17, respectively, and also selected from soils and sludges collected from industrial sites.

The study indicated that certain industrial sites might be habitats of chromium-resistant bacteria, phenol-resistant bacteria and chromium/phenol-resistant bacteria. It might me said that high concentration of those substances was possibly found and also the organisms might be selected naturally.

Chromium resistance in bacteria was found to be stable after, at least, 18 times repeated subculturing in stock culture containing small amount of chromium, as also in chromium/phenol resistant bacteria, containing small amount of chromium/phenol. But phenol-resistant bacteria were found to be stable after, at least 15 times repeated subculturing, containing small amount of phenol. Now, it is not known that plasmid or chromosomal mediated is genetically associated with resistance capability of high concentration among those organisms. Possibly, blocking of Cr(VI) penetration through a membrane into the cells may be a mechanism of chromium resistance in those selected bacterial strains (Horitsu et. al., 1987).

The bacterial community was also found to be resistant to a number of other heavy metals (**Table 4.4**). Chromium-resistant bacterial isolates were also resistant to phenol, arsenic and zinc but not resistant to silver, cadmium, copper, nickel and manganese, and also in phenol-resistant bacterial isolates and chromium/phenol-resistant bacterial isolates, but difference in the resistance concentration. However, mechanisms of

resistance in the selected bacterial isolates may be different in resistance to other metals. This implies that the resistance mechanisms were partially successful in improving tolerance to other metals. Two possible mechanisms were proposed, i. e., a specific chromium- and phenol-resistance and a nonspecific metal-resistance ones. Difference of metal contamination may make different metal resistance mechanism in various communities and does confer resistant to other metals because of metal-metal interactions. The present studies seems to confirm these evident that resistance to one metal in bacterial isolates (Luli et.al., 1983 and Corn, 1993).

In some experiments, resistance to chromium, phenol and other heavy metals were found in the selected bacterial isolates. Uses of phenol or other chemicals as nutrient 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 ½ strength to reduce those effects. But the effect after reduced amount of medium was not evaluated in this research.

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

bacterial isolates can growth more than acid conditions, but at the extreme pH (2, 3, 11 and 12) bacteria can not growth. The result from the study showed 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).

Effects of pH and temperature on chromium detoxification and phenol degradation by three cocultures (CrR-+PhR-) and three chromium/phenol-resistant bacteria (CPR-) can be summarized as follows. The potential of chromium detoxification and phenol degradation, the maximum of efficiency was obtained when the cells were inoculated at pH 7. The response of bacterial isolates to base condition on chromium detoxification and phenol degradation, they properly function in alkaline more than acidic pH. The comparison of the chromium detoxification and phenol degradation on the different temperature levels, the potential of both reactions during the growth period at 37°C, a maximum efficiency was obtained. At the temperature of 30°C and 40°C, the production was decreased, but did not more different. The cocultures, compared with chromium/phenol-resistant bacterial strains, found that can also grow, so the results implied two bacterial isolates (CrR-+PhR-) was not competitive to grow in the same medium or may be synergism (Alexander, 1994).

**Table 4.6** was shown the culture (normal saline and phosphate buffer plus 300 μg/ml) increased growth of bacterial isolates, compared with control. This result implies phenol as carbon and energy sources (Alexander, 1994 and Pavikov, 1995).

The incubation period on Cr(VI) detoxification, Cr(III) production and phenol degradation by selected bacterial strains appeared to increase and continued maximally efficiency during the exponential phase of selected bacterial strains (6 hr.) as shown in **Table 4.7**. Consequently, the incubation period of chromium and phenol degradation was differed from that many species, was not concurrent with growth. The results from this study the contact time of the Cr(VI) detoxification, Cr(III) production and phenol degradation, indicated that the equilibrium was completely within 15 min. (**Figure 4.13-4.14**) and prolonged exposure time did not increase. This implies that the bound metal ions were initial detoxified and degraded within the first minute after metal addition. The shortest incubation and the shortest contact time were shown. The Cr(III) formation is less than the Cr(VI) detoxification. It was possible that Cr(VI) passed easily through cellular membranes, and then was reduced to Cr(III) in the cytoplasm. Biological membranes are impermeable to Cr(III), but the Cr(III) generated inside the cell stable binds to protein and interacts with nucleic acids (Ohtake et. al., 1990). Uptake of Cr(VI) are involved in two steps : (i) the diffusion of Cr(VI) through a transport system and (ii) the intracellular reduction of Cr(VI) to Cr(III), keeping the cytoplasmic concentration of Cr(VI) low, accumulation of Cr(VI) from extracellular medium into the cell (Arslan et. al., 1987). Cr(III) may be bound to the protein inside the cell or formed complexes with nutrient and elements in culture medium. It was possible that intracellular deposition which occurred in the inner and outer organisms. Inner mechanism may be bound to protein (like siderophore) and outer mechanism may be detoxify at the surface of the cell (like exopolysaccharide).

**Table 4.8** was shown the effects of low concentration on the Cr(VI) detoxification, Cr(III) production and phenol degradation. The

results indicated that at low concentration the efficiency was high and continued maximally as the concentration increased. So the concentration could not be determined the potential of Cr(VI) detoxification, Cr(III) production and phenol degradation, as shown in **Figure 4.15**. The higher concentration (500, 1000, 1500 and 2000 μg/ml) was also shown the same results (**Table 4.9**), as shown in **Figure 4.16**.

Efficiency of phenol derivative degradation; p-cresol, p-chlorophenol and p-nitrophenol (50 μg/ml) by three phenol-resistant bacterial strains, compared with chromium/phenol-resistant bacterial strains found maximum efficiency (100%) occurred in p-cresol degradation within 2-3 weeks. But p-chlorophenol and p-nitrophenol needed continually degrade more than 3 weeks (Tabak et.al., 1964).

The result from this study was possibly similar to Shen and Wang (1994-1995), chromium detoxification and phenol degradation by cocultures; ***Escherichia coli*** **ATCC33456** and ***Pseudomonas putida*** **DMP-1** was studied under aerobic condition, as shown in equation :

$$C_6H_6O + 9.3CrO_4^{2-} + 40.7H^+ \rightarrow 9.3Cr^{3+} + 6HCO_3^- + 20.3H_2O$$

From the present study, the chromium-resistant bacterial isolates can resistant to 2400 μg/ml chromium and high stability of resistant (18 times). The phenol-resistant bacteria can resistant to 2000 μg/ml phenol and stability of resistant (15 times) and the chromium/phenol-resistant bacterial isolates can resistant to 1200 μg/ml chromium, 1200 μg/ml phenol and high stability of resistant (18 times). 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, chromium detoxification and phenol degradation was the same (pH 7 and 37°C), which is easy to cultivation and operate. The contact time of chromium detoxification and phenol degradation was reached within short time (15 min.). The percentage of both chromium detoxification and phenol degradation in this research was greater than 80% in each bacteria and mixed culture. To enhance the efficiency of biosorbent, the immobilized system should be used. Comparison between immobilization of the selected bacterial strains may be potentially applied to other metal removal systems and the immobilized cell systems.

In conclusion, research on detoxification of chromium by chromium-resistant bacterial isolates and degradation of phenol by phenol-resistant bacterial isolates have 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 chromium and phenol which release in high concentration. Thus, the isolation of the selected bacterial strains 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 the selected bacterial isolates were summarized in **Appendix E**.

**Table 5.1** The percentage of chromium detoxification and phenol degradation by bacterial strains compared with the former investigations

| Organisms | Max. resistance (μg/ml) of | | Test concentration and percentage detoxification (%) | | Condition | Enzymes / Bacteria | References |
|---|---|---|---|---|---|---|---|
| | Cr(VI) | Phenol | Cr(VI) | Phenol | | | |
| *Pseudomonas* sp. | - | - | - | 100 (99) | aerobic | - | Tabak et.al., 1964 |
| *P putida* | - | - | - | 235 (82.8) | - aerobic | 3 enz.: (1) catechol 2,3-oxygenase (2) 2-hydroxymuconic-semialdehyde hydrolase (3) 2-keto-4-hydroxyvalerate aldolase (catechol – intermediate) | Feist and Hegeman, 1969 |
| *P. putida* | - | - | - | 235 | Aerobic | 2 enz. : (1) $NAD^+$ -dependent aldehyde dehydrogenase (2) Hydrolase | Bayly and Wigmore, 1973 |
| *Salmonella typhimurium* | - | - | | - | aerobic | - | Petrilli and De Flora, 1977 |
| *Pseudomonas* sp. | - | - | 250 (29) | - | - aerobic - PYE medium - $K_2Cr_2O_7$ | - | Luli et.al., 1983 |
| Methanogenic consortium | - | - | - | 2000 (40%) | aerobic | 3 bacteria : (1) phenol-oxidizing (2) Methanothrix (3) $H_2$-utilizing | Dwyer et.al., 1986 |
| *P. fluorescens* LB300 (pLHB1) | - | - | 104 | - | aerobic | $SO_4^{2-}$ active transport system | Ohtake etal., 1987 |

| Organisms | Max. resistance (μg/ml) of | | Test concentration and percentage detoxification (%) | | Condition | Enzymes / Bacteria | References |
|---|---|---|---|---|---|---|---|
| | Cr(VI) | Phenol | Cr(VI) | Phenol | | | |
| *P. ambigua* G-1 | - | - | 150 (39.4) | - | - aerobic<br>- 50°C<br>- NADH as $H_2$ donor<br>- contact time 30 min. | - | Horitsu etal., 1987 |
| Methanogenic | - | - | - | 1430 (50) | - anaerobic<br>- product – $CH_4+CO_2$ | 2 bacteria :<br>(1) Methanothrix<br>*(2) Methanobacterium formicicum* | Wang et. al., 1989 |
| *P. aeruginosa* PAO1 (puM505) | - | - | 208 | - | aerobic | - | Cervantes et.al., 1990 |
| *Streptomyces* sp. 3M | - | - | 1000 (82.7) | - | - aerobic<br>- PYE broth<br>- 28°C<br>- $K_2Cr_2O_7$<br>- NADH | - | Das and Chandra, 1990 |
| *E. clocae* HO1 | - | - | 26 (100) | - | - anaerobic<br>- 30°C<br>- KSC medium<br>-incubation period 6hr.<br>-contact time30min. | 2 enz. :<br>(1) chromate reductase<br>(2) membrane fraction | Wang et.al., 1990 |
| *P. putida* PRS2000 | - | - | - | - | - aerobic<br>- 30°C<br>- VB broth<br>- NADH or NADPH | - | Ishibashi et. al., 1990 |

| Organisms | Max. resistance (μg/ml) of | | Test concentration and percentage detoxification (%) | | Condition | Enzymes / Bacteria | References |
|---|---|---|---|---|---|---|---|
| | Cr(VI) | Phenol | Cr(VI) | Phenol | | | |
| *E. clocae* HO1 | - | - | 104 | - | - anaerobic | Cytochrome c | Wang et.al., 1990; Ohtake et.al., 1990 |
| *P. ambigua* G-1 | - | - | 1040 | - | - aerobic<br>- NADH | Cr(V) as intermediate | Suzuki et.al., 1992 |
| *Agrobacterium radiobacter* EPS-916 | - | - | 26 | - | - anaerobic better than aerobic<br>- 25-30°C, pH 7-7.5<br>- glucose-mineral salts medium<br>- contact time 6 hr. | - | Llover et.al.,1993 |
| *P putida* PaW85 | - | - | - | 235 | - aerobic<br>- LB broth | 2 enz. :<br>(1) catechol1,2 dioxygenase (gene ***pheB***)<br>(2) phenol monooxygenase (***gene pheA***) | Kasak et.al., 1993; Peters et.al., 1997 |
| *E. aoli* ATCC33456 | - | - | 14.5 | - | - anaerobic better than aerobic | Aerobic – soluble protein fraction+NADH as $e^-$ donor (soluble reductase)<br>Anaerobic – membrane-bound reductase | Shen and Wang, 1993 |
| *Desulfovibrio vulgaris* | - | - | 26 | - | - anaerobic | - $H_2$ ($e^-$donor)<br>- $C_3$ cytochrome as Cr(VI) reductase | Lovley abd Phillips, 1994) |

| Organisms | Max. resistance (μg/ml) of | | Test concentration and percentage detoxification (%) | | Condition | Enzymes / Bacteria | References |
|---|---|---|---|---|---|---|---|
| | Cr(VI) | Phenol | Cr(VI) | Phenol | | | |
| *E. coli* ATCC33456 | - | - | 385 (30-40) | - | - aerobic (grow), anaerobic (reduce) - NB broth (glucose, yeast extract as C-sources) - 35°C,pH7 -incubation period 8hr. - contact time 24 hr. | 2 enz. : (1) soluble Cr(VI) reductase (NADH as $e^-$ donor) (2) membrane-bound protein reductase (respiratory chain linked) | Shen and Wang, 1994 (a); 1994(b) |
| *Streptomyces* sp. | - | - | - | 500 (96) | - aerobic - 30°C - contact time 12 hr. | - | Ambujom and Manilal, 1995 |
| *Candida tropicalis* M4 (Yeast) | - | - | - | 1598 | aerobic | - | Chang et.al., 1995 |
| *E.coli* ATCC33456 (Cr(VI) reducer) *P. putida* DMP-1 (phenol degrader) | - | - | 124 | 1402 | - NB broth for *E.coli* - BM medium for *P. putida* -300 μg/ml as carbon and energy sources -incubation period 24 hr.,30°C | - | Shen and Wang, 1995 (a); 1995(b) |

| Organisms | Max. resistance (μg/ml) of | | Test concentration and percentage detoxification (%) | | Condition | Enzymes / Bacteria | References |
|---|---|---|---|---|---|---|---|
| | Cr(VI) | Phenol | Cr(VI) | Phenol | | | |
| *P. pictorum* MU174 | - | - | - | 500 (99.9) | - aerobic - contact time 24 hr.,31°C | - | Chitra et.al., 1996 |
| *Escherichia* sp. CrR-2 | 2400 | 1000 | - | - | Aerobic 37°C, pH 7 NB (grow) BM medium (activity) Contact time 15 min. incubation period 6 hr. 300 μg/ml phenol as C and energy sources | - | This Study |
| *Pseudomonas* sp. CrR-14 | 2400 | 1000 | - | - | | | |
| *Enterobacter* sp. CrR-15 | 2400 | 1000 | - | - | | | |
| *Klebsiella* sp. PhR-26 | 500 | 2000 | - | - | Aerobic 37°C, pH 7 NB (grow) BM medium (activity) Contact time 15 min. incubation period 6 hr. 300 μg/ml phenol as C and energy sources | - | This Study |
| *Pseudomonas* sp. PhR-33 | 500 | 2000 | - | - | | | |
| *Escherichia* sp. PhR-64 | 500 | 2000 | - | - | | | |
| *Pseudomonas* sp. CPR-4 | 1200 | 1200 | 2000 (94.5) | 2000 (35.5) | Aerobic 37°C, pH 7 NB (grow) BM medium (activity) Contact time 15 min. incubation period 6 hr. 300 μg/ml phenol as C and energy sources | - | This Study |
| *Proteus* sp. CPR-16 | 1200 | 1200 | 2000 (94.5) | 2000 (35.3) | | | |
| *Escherichia* sp. CPR-17 | 1200 | 1200 | 2000 (95) | 2000 (37.8) | | | |