# Isolation and examination of copper removing bacteria from activated sludge culture

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**Abstract** Experiments were carried out to isolate metal removing bacteria from activated sludge culture and test the metal removal capability of isolated bacteria. Two concentrations of copper solution ($CuCl_2$) used to select the copper-tolerance level of each isolate were 0.05 mM and 2.5 mM. Experimental data showed that isolates could be obtained by using non-selective medium (NSM) in the presence of copper. The isolates were displayed on agar plates with diverse colonial morphological characteristics. A maximum uptake of 81 mg/g dry cell mass was observed at an initial copper concentration of 50 mg/l. The minimum uptake was 9 mg/g dry cell mass. Copper uptake efficiency by isolates from NSM with 0.5 mM $CuCl_2$ was significantly different with those from NSM with 2.5 mM $CuCl_2$.

**Keywords** Activated sludge; copper; copper-removing bacteria; non-selective media

## Introduction

Heavy metals discharged into the environment from various industries constitute one of the major causes of water and soil pollution. Contamination of the environment by these heavy metals is of growing concern because of their known accumulation in the food chain and their persistence in nature so as to cause health risks to human and animals.

Common processes to remove heavy metals from the waste stream are chemical precipitation, solvent extraction, dialysis or electrolysis, electrolytic extraction, membrane separation, reverse osmosis, evaporation, ion exchange and carbon adsorption. These conventional treatment methods may be ineffective or uneconomical when the heavy metal concentration in the polluted environment is in the range of 10 to 100 mg/L and the permissible concentration is less than 1 mg/L.

The need for economical, effective and safe methods for removing heavy metals from wastewaters has resulted in the research for unconventional materials that may be useful in reducing the levels or accumulation of heavy metals in the environment. The newly discovered metal sequestering properties of certain types of microbial biomass of fungi, bacteria and algae show considerable promise (Volesky, 1990). The ability of metal uptake by these microorganisms (known as biosorption and bioaccumulation) has been paid much attention for the remediation of heavy-metal-polluted water. Pure and mixed cultures of bacteria, algae, yeast and other microorganisms have been known to remove metal ions from solution in significant quantities (Gadd, 1988; Volesky and Holan, 1995).

Studies on the genetic information of extracellular polymers secreted by metal tolerant species are essential for the removal of heavy metals. With the results obtained from such studies, it is possible to alter the genes of various microorganisms to enhance the metal removal capability in addition to maintaining their normal functions in wastewater treatment processes. This study was undertaken to isolate copper-removing bacteria from activated sludge in order to examine species and their heavy metal removal capability.

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## Materials and methods

### Sludge characteristics

This research was carried out to investigate the metal removal capability of isolated bacteria from activated sludge. Activated sludge was obtained from Siphraya Municipal Wastewater treatment plant located in Bangkok, Thailand. The original sludge had the average characteristics of pH 7.0 and mixed liquor volatile suspended solid (MVLSS) concentration of 3000 mg/L.

### Culture media

The media used throughout was a modification of that of *Standard Methods* (APHA, 1995). The non-selective medium used consisted of glucose (1.0 g/l), tryptone (5.0 g/l) and yeast extract (2.5 g/l). To achieve the comparison between copper-resistant strains, the culture media was divided into three groups: (1) control culture, the culture grown on NSM in the absence of copper; (2) test culture 1, the culture grown in the presence of 0.5 mM copper concentration in addition to NSM; (3) test culture 2, the culture grown in the presence of 2.5 mM copper concentration in addition to NSM. A certain portion of filtered sewage (around 10% of total volume) was added into each medium to supplement various salts concentration and to enhance the adaptation of the bacteria to the new environment. No precipitation was found by naked eye in culture media. It is known that metal ions are difficult to precipitate in a system that contains soluble organic materials even at high pH.

### Inducing metal

In this research, copper was selected as inducing metal. It has a high toxicity for living organisms and is also known to have capability to trigger the genetic information of bacteria to synthesize metal-complexing biopolymers (Fukushi, 1996).

### Solutions for metal uptake experiment

The solution used in this study to wash and resuspend bacterial cells was phosphate buffer saline (PBS). PBS was selected as it does not complex with heavy metals and promotes cell viability (Bonthrone *et al.*, 1999). The pH of the solution for washing bacterial cells was buffered to around 2.0 by concentrated HCl in order to recover the metal ions from the cells.

### Test metal stock solution

To test the copper removal capacity of isolated bacteria, 50 mg/l of $CuCl_2$ solution was prepared. The pH of the solution was adjusted to 7.0 by one or two drops of PBS. No obvious precipitation was observed by naked eye.

### Growth of bacterial cells

One millilitre of activated sludge was sampled and transferred into 1,000 ml of culture media. It was then incubated in a constant temperature chamber (30°C) for 48 h. To maintain aerobic conditions, the air was pumped (80 ml/min) into the medium by a diffuser. Dissolved oxygen (DO) in the culture was maintained at 2.0 mg/l. To ensure stable composition of the bacterial fauna, cells were sub-cultured for several times in the fresh medium grown in one-litre bottles, generally, for 48 h.

### Selection and isolation of pure colonies

One millilitre of sample was taken from the above culture and serial dilutions were made. It was found that, $10^5$ to $10^8$ dilutions were suitable for displaying various copper-tolerant colonies. Isolation and purification of copper-tolerant bacteria was done by the streak plate method.

#### Methods for analyses

Metal concentration and total suspended solid (TSS) determination was done following *Standard Methods* (APHA, 1995). Metal species passing through 0.45-μm membrane filter were defined as "soluble metal".

Measurements were performed in triplicate for each sample to determine metal and TSS concentrations. The data presented in this paper are the average values of these replicate analyses.



## Results and discussion

The characteristics of activated sludge collected from Siphraya Wastewater Treatment Plant, Bangkok, Thailand are presented in Table 1.

After inoculation of activated sludge sample in the non-selective medium with copper (spread plate), each viable organism formed a colony. Various copper-tolerant species were screened on agar plates. Their growth indicated the ability of the bacteria to survive on agar plates containing low (0.5-mM) or high (2.5-mM) concentration of copper.

#### Isolation of copper-resistant bacteria

Pure colonies were isolated by streak plate method using non-selective medium solidified with agar. Isolates were segregated with respect to their colonial morphology such as size, shape, color, consistency and texture, etc. The isolates were selected for copper tolerance under the following conditions:

(i) isolates were taken only from the activated sludge sample collected from wastewater treatment plant;
(ii) all isolated were grown on non-selective medium with copper under the same environmental conditions such as pH, oxygen, etc;
(iii) they were isolated by using agar plates; and
(iv) all isolates could grow in the presence of copper chloride (low or high concentration).

#### Characteristics of isolated bacteria

Tables 2 and 3 summarize characteristics of isolated bacteria. There are five species (No. 1 to No. 5) isolated from medium with 0.5 mM of copper and four species (No. 6 to No. 9) from medium with 2.5 mM of copper.

According to the characteristics of colonies observed and their culture conditions, preliminary identification was made by reference to *Bergey's Manual of Determinative Bacteriology*. Although characteristics and morphology of isolates are defined in Tables 2 and 3, these isolates were difficult to identify precisely in this study due to the limitation of time, equipment and resources. These isolates were suspected to be species of *Pseudomonas*. They could be discriminated by their ability to accumulate copper. Identification of isolates is suggested for future work.

**Table 1** Characteristics of activated sludge from Siphraya Wastewater Treatment Plant

| Parameter | Amount |
|---|---|
| COD (mg/L) | 7,600 |
| TS (mg/L) | 9,400 |
| TKN | 2.80% TS |
| TVS | 60% TS |
| Total carbon | 33% TS |
| pH | 6.88 |

**Table 2** Characteristics and morphology of colonies isolated from non-selective medium with 0.5 mM of copper

| Isolates | Colonial morphology | | | | |
|---|---|---|---|---|---|
| | Color | Surface | Colony size | Ease of removal | Gram-stain results |
| No. 1 | White | Mucous | Larger size | Difficult | Gram-negative; Spherical shape |
| No. 2 | Light brown | Mucous | Larger size | Difficult | Gram-negative; Rod shape |
| No. 3 | Light yellow | Dry, smooth | Middle size | Easy | Gram-negative; Rod shape |
| No. 4 | Dark yellow | Dry, shining | Middle size | Moderate | Gram-negative; Rod shape |
| No. 5 | Light green | Dry | Smaller size | Easy | Gram-negative; Short rod shape |

**Table 3** Characteristics and morphology of colonies isolated from non-selective medium with 2.5 mM of copper

| Isolates | Colonial morphology | | | | |
|---|---|---|---|---|---|
| | Color | Surface | Colony size | Ease of removal | Gram-stain results |
| No. 6 | White | Mucous | Larger size | Difficult | Gram-negative; Spherical shape |
| No. 7 | Light green | Dry | Smaller size | Easy | Gram-negative; Rod shape |
| No. 8 | Bright yellow | Dry, smooth | Middle size | Easy | Gram-negative; Rod shape |
| No. 9 | Dark green | Dry | Smaller size | Moderate | Gram-negative; Short rod shape |



#### Determination of copper removal capacities of isolated bacteria

Capability of different isolates to accumulate copper were observed and compared to that of control culture (absence of copper). Certain species were found to actively respond to potentially toxic copper concentrations by complexing more of the metal and thus reducing the free ion concentration efficiently. As stated by previous researchers, many environmental conditions influence heavy-metal uptake by the biomass. pH of the solution has significant effect on the free metal ion forms and the cellular wall charge (Artola *et al.*, 1996; Chang *et al.*, 1996). In order to achieve efficient biosorption at similar conditions, the pH of the acidic copper stock solution was adjusted to 7.0 for each isolate. Biomass was taken from stationary growth phase which was measured by stable absorbance. Copper uptake was based on 24 h at 37°C.

Copper removal efficiency was observed ranging from 9 to 81 mg/g dry cell mass. The most significant level was found in species isolated from medium with 0.5 mM copper. In general, species from medium with 2.5 mM copper has lower copper removal capability than that from medium with 0.5 mM copper.

#### Comparison of copper uptake by various isolates

It was found that, in general, copper removal efficiency by isolates from medium with 0.5 mM $CuCl_2$ was much higher when compared with isolates from medium with 2.5 mM $CuCl_2$. This may be partly due to the differences of copper concentration in the initial cultural medium where the isolates were grown. Environmental conditions to which the bacteria were exposed must have an impact on the biosorptive capacity. Different cultural media or growth conditions may produce different microbial culture growth characteristics and different metal uptake capabilities. According to previous research, of the metals of toxicological concern in aquatic environments, copper exhibits the greatest tendency to associate with organic matter (Chang *et al.*, 1996). Microbial exopolymers could therefore chelate copper. Synthesis of metal-binding proteins may lead to immobilization, accumulation and detoxification of metal ions and thus help in reducing toxic ion resistance. Fukushi (1996) demonstrated that microbial culture in the presence of a trace amount of heavy metal

**Table 4** Copper uptakes by isolates from medium with 0.5 mM copper

| Isolate's number | Copper uptake efficiency (mg/g dry cell mass) | Compared with copper uptake by control culture** (%) |
|---|---|---|
| No. 1 | 65.76 (0.407)* | +52 |
| No. 2 | 80.94 (0.202) | +87 |
| No. 3 | 33.16 (0.313) | –30 |
| No. 4 | 21.81 (0.215) | –49 |
| No. 5 | 18.63 (0.531) | –56 |

**Table 5** Copper uptakes by isolates from medium with 2.5 mM copper

| Isolate's number | Copper uptake efficiency (mg/g dry cell mass) | Compared with copper uptake by control culture** (%) |
|---|---|---|
| No. 6 | 59.06 (0.099)* | +37 |
| No. 7 | 17.01 (0.184) | –60 |
| No. 8 | 35.45 (0.363) | –18 |
| No. 9 | 9.08 (0.288) | –79 |

*Number in parentheses is standard deviation
**Copper uptake by control culture: 43.15 mg/g dry cell mass
(+) Increment of copper uptake over the control culture (%)
(–) Decrease of copper uptake over the control culture (%)

induced the formation of extracellular biopolymers to protect biomass from hazardous outer environment. These biopolymers were known to complex heavy metals in a significant amount. From the present investigation, it could be inferred that copper uptake is inducible. The induction of $Cu^{2+}$ leads to enhanced accumulation of the metal, triggering an internal detoxification mechanism that results into the extrusion of the excess copper from the wastewater.

However, copper in low concentration acts as micronutrients that are essential for the bacteria as constituents of special enzyme. Much higher concentration of heavy metal may cause serious upset in the biological system due to higher toxic effects toward the microorganisms.

Results indicated that some copper-tolerant species of bacteria may grow well in the medium with copper, but their accumulation of copper was not significant. These species could tolerate copper to protect themselves from heavy metal toxicity, but they may not have high ability to remove the heavy metal. Some other species not only protect themselves from metal toxicity but also remove metal ions from aqueous solutions efficiently.

## Conclusions

Results obtained from this experimental work suggest that copper-tolerant bacteria could be isolated from activated sludge from municipal wastewater treatment plant, by using non-selective medium with copper. Spread plate and streak plate methods proved to be successful for isolation of copper-tolerant bacteria. In total five species were isolated from medium with 0.5 mM copper and four species from 2.5 mM copper. These species were differentiated by morphological characteristics such as colour, size, shape, and consistency. However, precise identification should be carried out in the future work.

Copper uptake experiments showed that the isolated bacteria exhibited wide range of copper removing efficiency from 9 to 81-mg/g dry cell mass. The isolates from medium with 0.5 mM copper exhibited copper removal efficiency between 19–81 mg/g dry cell mass and those from medium with 2.5 mM copper, between 9–59 mg/g dry cell mass. The

genetic ability of the isolated bacteria, which was thought to be responsible for the biosorption, could be stimulated by the copper present in the culture medium. Some of the isolates exhibited copper removal capabilities of more than 60 mg/g dry cell mass making them a prospective biomaterial for copper.



## References

APHA (1995). *Standard Methods for the Examination of Water and Wastewater Treatment*. American Public Health Association.

Artola, A., Balaguer, M.D. and Rigola, M. (1996). Heavy metal binding to anaerobic sludge. *Wat. Res.*, **31**(5), 997–1004.

Bonthrone, K.M., Quarmby, J., Hewitt, C.J., Allan, V.J.M., Paterson-Beedle, M., Kennedy, J.F. and Macaskie, L.E. (1999). The effect of the growth medium on the composition and metal binding behaviour of the extracellular polymeric material of a metal-accumulating *Citrobacter sp. Environ. Tech.*, **21**, 123–134.

Chang, J.-S., Law, R. and Chang, C.-C. (1996). Bisorption of lead, copper and cadmium by biomass of *Pseudomonas aeruginosa* PU21. *Wat. Res.*, **31**(7), 1651–1658.

Fukushi, K. (1996). Heavy metal removal by microbial biopolymers. Ph.D. dissertation. University of Utah, Salt Lake City, U.S.A.

Gadd, G.M. (1988). Accumulation of metals by microorganisms and algae. *Biotechnology*, **6**, 401–430.

Volesky, B. (1990). Bisorption and biosorbents. In *Biosorption of Heavy Metal* (edited by Volesky, B.) pp. 3–6. CRC Press, Boca Raton, FL.

Volesky, B. and Holan, Z.R. (1995). Biosorption of heavy metals. *Biotechnol. Prog.*, **11**, 235–250.