Biosorption of Cu^{+2} ions from wastewater by chemically-immobilized *Rhizobium* BJVr 12 cells and exopolysaccharides

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ABSTRACT

The biosorption efficiency of biotraps consisting of chemically-immobilized *Rhizobium* BJVr 12 exopolysaccharides in adsorbing Cu^{+2} ions was studied. Preliminary screening using the solubility test revealed that the most suitable microbial carrier is the ratio $2Al(OH)_3$: 1 SiO_2 of 20% aluminum hydroxide and silicon dioxide. The effect of flow rate, time of collection, and kind of biotrap (w/ or w/o EPS) in the percent removal of Cu^{+2} ions in wastewater was determined. Atomic Absorption Spectroscopy was used to determine the residual Cu^{+2} ion concentration of the collected 10 ml samples. Three-factor Factorial in CRD to analyze simultaneously the effect of the three factors (flow rate, time of collection and kind of biotrap) and the Duncan's Multiple Range Test to determine the optimum condition for each factor were used to analyze the data. Results showed that flow rate and time of collection significantly affect the percent removal of Cu^{+2} ions in the wastewater. Maximum percent removal (%) of Cu^{+2} ions (98.25%) occurred at the 6-min (0.10 hr) time interval. A slower flow rate (1 ml/min) showed greater adsorption efficiency. Both biotraps (w/ and w/o EPS) exhibited high percentage removals of Cu^{+2} ions. Although the percent removal of Cu^{+2} ions by the biotraps without EPS is significantly greater than the biotraps with EPS for the 2.5 ml/min and 5 ml/min flow rates, no significant difference between the two was noted for the 1 ml/min flow rate.

INTRODUCTION

A. Background of the Study

Toxic metals create the greatest environmental and health hazards. Wastewater streams from metal processing, mining and mining operations carry tons of metals into the environment every year.

Copper is the most widely used metal in our industry today, with products ranging from pipes used in homes to conductors used in industries. It is one of the essential micronutrients living organisms need but high concentrations of this metal lead to toxicity and poisoning.

The role of biological processes in metal transformations and concentration has a recent resurgence because of the concern over the accumulation of toxic metals (copper, cadmium, chromium, lead, mercury, zinc, gold, manganese, arsenic, selenium) in the environment. The use of microorganisms in the bioremediation of wastewater is at present being studied extensively as alternative to costly treatment methods in removing toxic metals in wastewater effluents. Furthermore, biotechnological approaches in solving these constrains are preferred over pure physical or chemical means due to higher efficiency, and greater sustainability (Cheng *et al.*, 1995).

Biosorption is one such process that allows the use of biological materials to remove metals in solutions. The Environmental Biotechnology Program of the **National**

Institute of Biotechnology and Applied Microbiology (NIBAM or BIOTECH) at the University of the Philippines, Los Baños covers a project on biosorption using biotraps. They particularly focus on the use of *Rhizobium* BJVr12, a nitrogen-fixing bacterium, as an agent in the removal of metals from effluents. It was shown that the *Rhizobium* synthesize prodigious amounts of mucilaginous polysaccharide which can adsorb metal ions in solution due to its numerous metal binding sites. Different modes of immobilization or carriers have been studied and used to facilitate the stability of the exopolysaccharide in sorption studies.

B. Statement of the Problem

The main problem of the study is to determine if chemically-immobilized *Rhizobium BJVr 12* cells and exopolysaccharide can efficiently reduce the concentration of Cu^{+2} ions in wastewater.

C. Objectives of the Study

1. To determine which chemical [$Al(OH)_3$, SiO_2, or a mixture of both] is the most suitable carrier to immobilize *Rhizobium BJVr 12* cells and create a biotrap for the adsorption of metal ions in solution.

2. To determine the capacity of the chemically-immobilized *Rhizobium BJVr 12* to adsorb Cu^{+2} ions in the wastewater effluent.
3. To determine the optimum flow rate for the biosorption of Cu^{+2} ions.
4. To determine the fraction of time where maximum adsorption of the Cu^{+2} ions occurred.
5. To determine which kind of biotrap (chemicals with EPS or chemicals alone) is more efficient in the biosorption of Cu^{+2}.

D. Hypotheses

1. A combination of $Al(OH)_3$ and SiO_2, owing to its insolubility in water, is the most suitable carrier for the exopolysaccharide in creating a biotrap that will absorb Cu^{+2}.
2. The chemically-immobilized *hizobium BJVr 12* cells significantly reduce the amount of Cu^{+2} ions in the mining wastewater.
3. The slowest flow rate, 1.0 ml/min, is the most effective in adsorbing the Cu^{+2} ions in the solution.
4. Maximum adsorption of the *Rhizobium BJVr 12* cells occurs within 30 minutes.
5. The biotrap with EPS is more efficient in removing Cu^{+2} from the wastewater.

E. Significance of the Study

Rhizobium BJVr 12, has been known to efficiently remove several metal ions (Cr^{+3}, Ag^{+}, Au^{+3}, Cd^{+2}, Cu^{+2}, Hg^{+}, and Pb^{+2}) from solution (Mamaril *et al.*, 1997). In order to achieve its maximum adsorption capacity however, immobilization of the cells is necessary. Several means of immobilization have already been employed wherein all involved the entrapment of cells in a matrix. This study investigated the effectiveness of chemicals ($Al(OH)_3$ and SiO_2), as a method of cell immobilization because of their ability to complex with polysaccharides and their insolubility in water; and thus creating a relatively inexpensive but effective biosorption gadget, or biotrap in removing metal ions in solution. Aside from effectively removing metals, recovery and reuse of these metals will be possible with the use of biotraps. Since metal ions are also used as raw materials in industries as part of the production process, recycling of these raw materials will be cost-effective and energy-conserving for companies. Environmentally speaking, biotraps when fully developed, is a promising device in the reduction of metal ions in wastewater from mining sites, car battery-manufacturing plants as well as laboratories in research and educational institutions.

F. Scope and Limitations

1. Only one kind of carrier was used for the column run based on a preliminary test determining the most effective biotrap according to the degree of solubility.
2. Only three flowrates 1.0, 2.5, and 5.0 ml/min were used for the column run.
3. The physical (odor, color, etc.) and chemical (dissolved O_2, hardness, etc) characteristics of the mining wastewater effluent were not determined. Only the concentration of Cu^{+2} ions in the wastewater is measured.
4. Due to non-detectable amounts of Cu^{+2} ions in the wastewater effluent (as determined by Atomic Absorption Spectroscopy), the Cu^{+2} content was adjusted to 30 ppm using laboratory grade anhydrous $CuSO_4$ crystals.

REVIEW OF RELATED LITERATURE

A. Copper in the industry and the environment

Toxic metals, alternatively called trace metals, is a general collective term usually applied to elements such as Cd, Cr, Cu, Hg, Ni, Pb, and Zn. Since metals occur naturally in rocks and ore minerals, traces of these elements can be found in soils, sediments, waters and living organisms. Some of these elements constitute enzymes and other important proteins essential to the normal functioning of organisms. However, anomalously high concentrations of these metals relative to the normal background levels are toxic and harmful to living organisms (Mido *et al.*, 1995).

Copper, a reddish metal, is one of the most abundant trace metals. It is widely used in its metallic state, either in the pure form or in alloys. Copper exhibits oxidation states of +2, which is the most common, and +1 which is stable only in aqueous solution if part of a stable complex ion. For almost all organisms it is an essential micronutrient. For plants and animals, it usually occurs as part of the oxidizing enzymes (high molecular weight proteins containing 0.05%-0.35% of Cu^{+2}) bound to O, S, or N ligand sites which play an important role in oxidation and reduction reactions (Grolier, 1993).

Copper is commonly used today as material for electric conductors, generators, car radiators, and pipes. Alloys of this element are also used in tableware, electroplating jewelry, and in brass-making. In agriculture, copper is used as fungicides or algicides.

Copper sulfate is used widely as algicide in ornamental ponds and even in water supply reservoirs which are affected by blooms of toxic blue-green algae (Mido *et al.*, 1995).

Although copper is of great use in industry, excessive amounts of this metal also give adverse effects to the environment and living organisms including man. Excessive concentrations, which may be as low as 0.5 ppm for algae can cause toxic effects due to immediate exposure to the element (Mido *et al*, 1995). In higher animals, copper when taken excessively can induce lethal convulsions leading to brain damage due to its inhibition of the membrane transport of ions (Peters, 1965). Moreover, a high concentration of copper in the blood was found out to be a risk factor for coronary heart disease along with high blood cholesterol. Copper seems to work in combination with cholesterol to promote atherosclerosis, the thickening of arteries which leads to heart attacks (Webb, 1991).

B. Mining and wastewater treatments

Metalliferous mining is one significant source of metals to the environment. Metals used widely in electronics and machines are obtained from the mining of ore bodies in rocks of the earth's crust. Various methods of extraction of the metal ore concentrates generate mine tailings (finely milled fragments of rock and some ore particles) which need to be disposed of in an environmentally appropriate manner. Modern mineral separation methods involve the use of large volumes of water with much normally recycled within the process, although smaller volumes of effluents containing

metals, frothing agents and other chemicals (including cyanide in gold extraction) do need disposal eventually (Mido *et al.*, 1995).

Conventional physico-chemical treatment methods employed in removing toxic metals from dilute waste-waters include precipitation-filtration, ion exchange, reverse osmosis, oxidation-reduction, electrochemical recovery, membrane separation and other techniques. However, they are often ineffective or uneconomical when the heavy metal concentrations are in the range of 10-100 mg/L. This is why current research has been focused on the metal removal capacities of various biological materials such as bacteria, yeasts, filamentous fungi, algae, and plant cells (Wilkins and Yang, 1996).

C. Biosorption

Biosorption is a process of removing metals, and related elements or compounds from solutions using biological materials with biosorptive capabilities. The application of biosorption in industries has been directed towards the use of microorganisms such as bacteria, algae, and fungi. These microorganisms can accumulate heavy metals, radionuclides, organometallic compounds, metalloids and metal particulates from their external environment with high efficiency. Living and dead cells as well as derived or excreted products such cell walls, pigments and polysaccharides are all capable of metal removal from solution (Gadd, 1992).

Microorganisms can accumulate metals by precipitating or binding the metals onto cell walls and cell membranes because of the presence of carboxyl, hydroxyl, phosphoryl, and other negatively charged sites in anionic walls. Some microorganisms synthesize extracellular polysaccharide (EPS), polymers extending from the outer membrane which also serve as sites of metal accumulation (El Aziz *et al.*, 1991). Exposed OH^- or COO^- group on the EPS may act as ligands for metal binding (Mamaril *et al*, 1989). Given ample nutrients, bacteria will abundantly produce complex polysaccharides with a highly regular repeating sequence (Isaac, 1985). Other microorganisms adsorb metals metabolically. They actively take in metals and compartmentalize them into specific organelles such as vacuoles or render them non-toxic by binding them to proteins or precipitation (Wilkins and Yang, 1996).

Biosorption may occur even when the cell is metabolically inactive, such as when it has been killed by chemical or physical means (Wilkins and Yang, 1996). Inactive biomass has the advantage of being independent of a supply of nutrients for cell growth and maintenance, and it does not involve any time loss due to culture propagation or contamination (Brady *et al.*, 1994). Moreover, the concentration of metals in non-viable biomass often exceeds that of viable cells due to the inactivation of the resistance mechanisms which prevent metal uptake in viable cells. Non-viable cells may also be stored or used for extended periods of time without decay (Stoll and Duncan, 1996).
Certain methods such as heat-killing, chemical modification, and several methods of immobilization improve the biosorptive properties of cells (Brady *et al.*, 1994; Nakajima

and Sakaguchi, 1986). Immobilization of biomass is necessary because freely dispersed biomass may block flow lines, clog filters and separation of biomass and effluent can be difficult and expensive (Tsezos, 1986). The microbial biomass is most ideally immobilized as a particulate form that preserves its biosorptive properties and is easy to recover (Stoll and Duncan, 1996).

D. *Rhizobium* and exopolysaccharides

Rhizobium is a genus of gram-negative rod-shaped soil bacteria often found living in small nodules on the roots of peas, soybeans, alfalfa, string beans and other legumes. Within the nodules, nitrogen is converted by the bacterium into usable forms. This process called nitrogen fixation, uses carbohydrates produced by plants (Cotoras *et al.*, 1992).

Rhizobial exopolysaccharides (EPS) have been studied for their role in plant-host specificity but only recently have their metal sorption capacity been investigated. Studies done on some *Rhizobium* isolates revealed that the bacteria were able to reduce radionuclide concentration and that they were able to tolerate and grow in an environment containing a relatively high concentration of lead. (Cotoras *et al.*, 1992; Mamaril *et al.*, 1989).

Mamaril and colleagues (1989,1991) have concluded in their studies that *Rhizobium BJVr 12* (BIOTECH-Jaica *Vigna radiata* strain # 12) found in the nodules of

mungbean (*Vigna radiata)* produce large amounts of mucilaginous polysaccharides that can sequester and reduce the concentration of heavy metals in dilute aqueous solutions to a high degree. Several studies have been done using this strain and results show that it can adsorb metals including Cr^{+3}, Ag^{+}, Au^{+3}, Cd^{+2}, Hg^{+}, and Pb^{+2}. (Mamaril *et al.*, 1997; Aguilar, 1996; Galan, 1996, Padolina, 1994; Paner *et al.*, 1999).

In a study conducted by BIOTECH to determine the chemical characteristics of the exopolysaccharides (EPS) of the *Rhizobium* BJVr 12 strain, it was found out that based on the average CHO composition, the molecular formula of the EPS is $C_6H_{12}O_7$. Glucose is the predominant sugar with mannose and galactose in lesser quantities. Spectroscopic analysis revealed that the functional groups of the EPS is OH (hydroxyl), CHO (aldehyde), and C-O (alcohol) (BIOTECH, unpub. data 1999)

Several methods have been employed in immobilizing *Rhizobium* BJVr 12 exopolysaccharide (EPS). Some of them include styrofoam, scotch brite, ceramic beads, and aquacel and the cells were able to significantly reduce the amount of metal ions present in solution. However, the search for other means of immobilization is encouraged. According to a study done by Padolina in 1994, while experimentation, some of the biomass was released from the ceramic beads. The supernatant, after centrifugation, still appeared to be viscous evident that the EPS is soluble in water. In this study, Si O_2 and $Al(OH)_3$ are used as alternative immobilizing agents because of their low solubility in water.

E. Silicon dioxide (SiO_2) and aluminum hydroxide ($Al(OH)_3$)

Silicon is an ubiquitous element present in significant quantities in nearly all living organisms. Silicon is taken in by animals with food, in soluble forms with water or inhaled as with dust particles. Man has a daily intake of about 0.5g silicon and about 0.001% of his body weight is taken by silicon. In fact, certain tissues and organs including connective tissues, skin, lungs, glands, bones, dental enamel teeth and hair were found to have high silicon contents (Corey *et al.*, 1988).

Living organisms that inhabit the ocean such as the foraminiferans, radiolarians and siliceous sponges take up silica from the external environment and use it for the formation of their shells and frameworks. In plants, they have been found in enzymes, that facilitate the conversion of inorganic compounds of silicon to organosilicon derivatives (Corey *et al*, 1988).

Silica were also used as immobilizing agents of immunoglobulins, *Escherichia coli* with penicillin amidase and glucose dehydrogenase of *Bacillus megaterium* to facilitate their stability, reproducibility and reutilization (Joensson *et al.*, 1985; Babu and Panda, 1991 ; Baron *et al.*, 1997). Furthermore, silica-immobilized Zoogleoea and Zooglan were found to have high adsorption capacity for Cu and Cd ions (Ahn *et al.*, 1998).

Aluminum hydroxide is one of the most important metal hydroxides used commercially. A large portion of it is used in the production of aluminum metal while a significant portion of it is directed to uses other than metal production. $Al(OH)_3$ is used

in the manufacture of paint and fire retardants for polymers such as plastic and glass (for building panels, machine housings, automotive parts, etc.). It is also used as an abrasive filler for toothpastes. (Downs, 1993)

Among the characteristics of $Al(OH)_3$ which makes it widely applicable include: (1) its effectiveness as a smoke suppressant for many polymer systems, (2) non-evolvement of any corrosive or toxic product on decomposition, (3) safety; it presents no health hazard when handled, (4) insolubility in water; and thus will not leach out of a filled polymer, (5) its electrical properties which makes it an ideal filler for insulators, (6) non-volatility; it will not exude out of the polymer on aging, and (7) its relatively low cost (Downs, 1993).

Owing to its properties, $Al(OH)_3$ has been used as immobilizing agents for enzymes such as penicillin G cyclase (Bahulekar *et al.*, 1991) and as a component of media used in batch adsorption tests to enhance adsorptive capabilities (Chen and Koopman, 1997).

MATERIALS AND METHODS

A. Materials

1. Microbial strain

Rhizobium BJVr 12 (BIOTECH-Jaica *Vigna radiata* strain #12) was obtained from the Microbial Culture Collection, at the Institute of Molecular Biology and Biotechnology in UP Los Banos. The nitrogen-fixing bacterium was isolated from the root nodules of mungbean (*Vigna radiata w.*) submitted by Dr. S.N. Tilo and Dr. E.S. Paterno of the Department of Soil Science, College of Agriculture, UPLB.

2. Culture media

Rhizobium BJVr 12 was maintained on slants of Yeast Extract Mannitol Agar (YEMA). For cell biomass production, sterile coconut water and brown sugar is used as culture media.

3. Wastewater effluent

Wastewater from a mining site was used in the experiment. Initial copper analysis of the sample by AAS revealed that the Cu^{+2} content is too low to be detected. For this reason, Cu^{+2} content is adjusted to 30 ppm using laboratory grade anhydrous $CuSO_4$ crystals.

4. Microbial carrier

Solutions of $Al(OH)_3$ and SiO_2 and ratios of both was prepared by dissolving the powder in distilled water. Preliminary testing was done to determine which concentration is the most suitable carrier based on the solubility of the mixture (EPS + solution). The most suitable concentration was used for the column run.

B. Procedure

1. Preparation of seed culture and mass production of cell biomass

For the seed culture, 250 mL Erlenmayer flasks containing 150 ml sterile coconut water, was inoculated with a loopful of *Rhizobium* BJVr 12 cells. The cultures were incubated for 3 days at room temperature (30°C) under regularly shaken conditions (120 rpm).

For the mass production of cell biomass, the inoculum (from the seed culture) was transferred to 1.5 L of sterile coconut water containing 5g brown sugar in a 3 L fermentor (L.E. Marubishi MD-300). The cells were incubated for 5 days at 30°C with constant stirring. Afterwards, the cells were refrigerated until use.

2. Screening of a suitable microbial carrier

In 100 mL volumetric flasks, 100 mL of 1%, 5%, 10% and 20% solutions each of $Al(OH)_3$ and SiO_2 were prepared by dissolving 1g, 5g, 10g, and 20g of powder (Sigma

brand), respectively in distilled water. The solutions were shaken occasionally to prevent the powder from settling down at the bottom of the flasks. In 8 petri plates lined with plastic, 20g of *Rhizobium* EPS were mixed with 10 ml each of the prepared solutions. 10 ml of 95% ethanol were added to facilitate in the mixing and to aid in the preservation of the mixture. The fresh weight of the mixtures was recorded and these were air-dried for 15 minutes. Afterwards, these were oven-dried overnight at 50^0C using Yamato Incubator IC63. The dry weight of the samples was recorded.

To test for the solubility of the samples in water, 3 ml of distilled water were placed in eight 10 ml test tubes containing 0.5 g of the samples cut into strips. This set-up was left for 4 hours and the appearance of the water as well as the texture of the samples was noted. For each of the two chemicals, the one with the clearest suspension indicated the least solubility.

The solution with the least solubility for each of the chemicals was mixed in ratios of 1:1, 1:2 and 2:1 with the EPS. 5 ml both of solution A and solution B were mixed with 20g EPS for the 1:1 ratio; 3.3ml of solution A and 6.7 ml of solution B for the 1:2 ratio and 6.7ml solution A plus 3.3 ml of solution B for the 2:1 ratio. These mixtures were oven-dried and tested for solubility as described in the above procedure. The least soluble sample here was compared with those where A or B are used alone.

3. Biosorption of Cu^{+2} by immobilized cells in columns (biotraps)

The biotraps were prepared using a 30 ml plastic syringe with the immobilized cells cut into 1x1 cm strips and supported at the bottom by a piece of filter paper slightly larger than the area of the syringe.

50 ml of the wastewater were passed through the biotraps at flow rates: 1 ml/min, 2.5 ml/min, 5ml/min . The flow rates were controlled by a peristaltic pump (Eyela Microtube Pump MP-3, Tokyo Rikakikai Co. Ltd.) attached by a plastic tube to the syringe. Fractions of 10 ml were collected at intervals of 0 min, 6 min, 15 min, 30 min, 1 hr, 2 hrs and 4hrs and analyzed for their Cu^{+2} content. All experiments were run in triplicates.

4. Acidification of samples

Collected samples of 10 ml each were poured into 25 ml volumetric flasks. To create a solution with 1.16 normality (N), 2.5ml concentrated HCl were pipetted to each flask and the samples were diluted up to the 25 ml mark with triple distilled water. Acidified samples were refrigerated before analysis and kept inside film containers.

5. Determination of Cu^{+2} concentration of samples

The Cu^{+2} concentrations of the samples were determined by Atomic Absorption Spectroscopy (AAS) using a Model Perkin Elmer 5000 Atomic Absorption

Spectrophotometer with a multi-element cathode lamp.Cu^{+2} concentrations of 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 ppm were used to prepare the standard calibration curve.

C. Experimental Design

Completely Randomized Design (CRD) was used to test the hypotheses of the study. Six columns were used for the column run (3 columns containing the biotrap consisting of the EPS + chemical concentration from the preliminary experiment and 3 columns for the control). 3 different flow rates were used (1 ml/min, 2.5 ml/min, and 5 ml/min) and 10 ml fractions were collected at 7 different time intervals for the elemental analysis for Cu^{+2}. A total of 126 samples were analyzed (6 columns x 3 flow rates x 7 time intervals). The factorial experiment was used to analyze the effect of the three variables (flow rate, time, EPS) simultaneously. The Duncan's Multiple Range Test was also used to determine which flow rate, at what fraction of time maximum adsorption occurred.

RESULTS

A. Screening for a suitable microbial carrier

The selection of a suitable chemical microbial carrier is based upon the degree of solubility of the sample (EPS + carrier) in water. The exopolysaccharide is very soluble in water and means of containing the cells are necessary to ensure maximum adsorption efficiency of the biotrap. The absence of a carrier will wash away the cells together with the effluent, decreasing the removal of ions from solution.

Cells immobilized in 20% $Al(OH)_3$ and 20% SiO_2 had relatively clearer water suspensions and are thus less soluble than lower concentrations (1%, 5%, 10%, and 15%) of the chemicals. A comparison of the solubility of the ratios made from the 20% concentration [$1Al(OH)_3$: $1SiO_2$, $1Al(OH)_3$: $2SiO_2$, and $2Al(OH)_3$: $1SiO_2$ of 20% $Al(OH)_3$ and 20% SiO_2] showed that the EPS immobilized in $2Al(OH)_3$: $1SiO_2$ ratio is the least soluble. After 4 hours, the sample remained intact and the water was clear. This ratio was then used to prepare the biotraps for the column run (Table 1).

B. Biosorption of Cu^{+2} ions by chemically-immobilized cells in columns

At the flowrate of 1 ml/min (Table 8) (Figure 1), maximum percent (%) removal of Cu^{+2} ions for the biotraps with EPS is 99.033% which occurred at 6 min (0.10 hr) and is 99.99% for the biotraps without EPS which occurred at 15 min (0.25 hr). Afterwhich, the trend decreases to a lowest removal rate of 91.287% and 91.843% at 4 hrs respectively.

At the flow rate of 2.5 ml/min (Table 9) (Figure 2), maximum % removal of Cu^{+2} ions is 98.887% at 6 min. for the biotraps with EPS and is 99.957% at 15 min for the biotraps without EPS. The graph also decreases to a lowest % removal of 90.367% and 91.610% at 4 hrs respectively.

At the flow rate of 5 ml/min (Table 10)(Figure 3), maximum % removal of Cu^{+2} ions occurred at 6 min, at the rate of 94.067% for the biotraps with EPS and at the rate of 98.310% for the biotraps without EPS. As the preceding flow rates, the graph also decreases to a lowest % removal of 86.853% (with EPS) and 88.300% (without EPS) at 2 hrs.

1. Effect of flow rate on % Cu^{+2} removal

As the flow rate increases, the % removal of Cu^{+2} ions decreases (Tables 8-10 and Figures 1-3). The ANOVA table and Duncan's Multiple Range Test (Appendix C – flow rate vs. % removal) shows that the % removals in the 3 flow rates differ significantly. DMRT further shows that the flow rate which elicited the greatest % removal is 1 ml/min at 83.369% which is followed by 2.5 ml/min at 82.211% and the last being the 5 ml/min flow rate at 77.877% ($\alpha = 0.05$).

2. Effect of time on % Cu^{+2} removal

Duncan's Multiple Range Test (Appendix C – time interval vs. % removal) shows that the time interval for which maximum removal of Cu^{+2} ions occurred is at 6 min (0.10 hr) at 98.25% followed by (in decreasing order): 15 min, 30 min, 1 hr, 2 hr, 4 hr (at $\alpha = 0.05$).

3. Effect of the kind of biotrap (w/ or w/o EPS) on % Cu^{+2} removal

The ANOVA table shows that there is a significant difference between the % Cu^{+2} removals of the biotraps with and without EPS with the latter exhibiting a greater percentage removal. Biotraps w/ EPS adsorbed 80.393% while the biotraps w/o EPS adsorbed 81.912%.

4. Effect of flow rate and kind of biotrap on % Cu^{+2} removal

Both the biotraps (with EPS and without EPS) are effective in removing the Cu^{+2} metal ions from water although the biotrap without EPS works better than the biotrap with EPS at flow rates greater than 1 ml/min.

Duncan's Multiple Range Test (flow rate vs sample) shows that there is no significant difference between the percent removal of the biotraps (with EPS and without EPS) at flow rate 1 (1ml/min) but not for the two other flow rates (2.5 ml/min and 5 ml/min) (at $\alpha = 0.05$) where the adsorption of the biotrap without EPS is higher. Percentage (%) removal of the biotraps w/ EPS for the 1 ml/min flow rate is 83.116% while that of the biotraps w/o EPS is 83.622%. For the 2.5 ml/min flow rate, % removals of the biotraps w/ and w/o EPS are 81.408% and 83.015% respectively. For the 5 ml/min flow rate, % removals of the biotraps w/ EPS is 81.408% while that of the biotraps w/o EPS is 83.015%.

DISCUSSION

At the 3 flow rates, a decreasing trend in the graphs (decreasing % removal of Cu^{+2}) may be due to the increasing saturation of the binding sites present in the EPS, therefore lesser available binding sites for the metal ions as the fraction of time increases.

1. Effect of flowrate on % Cu^{+2} removal

The percent removal of Cu^{+2} ions is greatest at the 1 ml/min flow rate this is because, a slower flow rate allows for a longer residence time to allow the system to equilibrate and maximize adsorption. There is a time lag for metal ions to reach and interact on the cell surface. If the residence time is too long however, desorption may take place (Mamaril *et al.*, 1997).

2. Effect of time on % Cu^{+2} removal

The short time it took (6 min) to attain maximum % removal is indicative of the efficiency of the biotrap in adsorbing the Cu^{+2} ions. At a short time of 6 min, the biotrap was already capable of removing Cu^{+2} ions from solution at a relatively high rate.

After 6 min., the biotrap is still able to adsorb high amounts of Cu^{+2} ions in solution though eliciting a lower rate of removal than the 6-min time interval. As previously discussed, this may be attributable to the increasing saturation of the binding sites present in the EPS.

3. Effect of the kind of biotrap on % Cu^{+2} removal

The high capacity of the biotrap without EPS in removing metal ions from solution may be due to the fact that the chemicals [$Al(OH)_3$ and SiO_2] also have negative binding sites for metals in which the nature of interaction is ionic (Corey *et al.*, 1988 and Downs *et al.*, 1993). The nature of the interaction between the negatively charged sites in the EPS and the metal ions, on the other hand, is covalent (Isaac, 1985). Since ionic bonds are stronger than covalent bonds, this probably explains why the biotraps w/o EPS exhibited a greater % removal than the biotraps w/ EPS. Furthermore, biotraps w/ EPS contain lesser amounts of chemicals and thus lesser binding sites for ionic interaction which probably explains why the expected additive effect of the chemicals to the biosorption of metal ions is not achieved. Varying the amounts of chemicals to amounts greater than what is used in this study may elicit different results. But this, however, remains to be demonstrated.

4. Effect of flow rate and kind of biotrap on % Cu^{+2} removal

Both the biotraps (with EPS and without EPS) are effective in removing the Cu^{+2} metal ions from water although the biotrap without EPS works better than the biotrap with EPS at flow rates greater than 1 ml/min (no significant difference was observed for the 1 ml/min flow rate as shown by DMRT).

5. Probable disadvantages of using chemicals in wastewater treatments

The use of chemicals in sorptive systems has its drawbacks and limitations. First, it is difficult to resorb Cu^{+2} ions from the chemicals which can be reused for production,

while it is very much easier to resorb ions (via acidification) from the exopolysaccharides. (at $\alpha = 0.05$). Second, as a result of the difficulty in resorption of the metal ions, the disposal of chemical sorbents will be a problem whereas disposal of biosorbents will not be, due to the biodegradability of exopolysaccharides. Third, the use of chemicals for sorptive processes will be highly expensive even in small amounts. And lastly, strong binding of ions to chemicals prevents resorption of the adsorbed ions. Therefore, the use of exopolysaccharide in wastewater treatment remains to be more favorable.

CONCLUSION

Biotraps consisting of chemically-immobilized Rhizobium BJVr 12 were developed. A 2:1 ratio of 20% Al $(OH)_3$ and 20% SiO_2 is the most suitable immobilizing agent for Rhizobium BJVr 12. Slower flow rate of 1 ml/min showed higher Cu^{+2} uptake for all biotraps. Maximum % removal of Cu^{+2} (98.25%) was obtained within 6 minutes. Both biotraps (w/ and w/o EPS) exhibited high percentage removals of Cu^{+2} ions. Although the percent removal of Cu^{+2} ions by the control is significantly greater than the experimental for the 2.5 ml/min and 5 ml/min flow rates, no significant difference between the two was noted for the 1 ml/min flow rate. The use of EPS still remains to be favorable because of its biodegradability, reusability, and lower costs. Also, resorption of the adsorbed ions is possible. These resorbed ions can then be recycled and used as raw materials for production.

RECOMMENDATIONS

The study only dealt with the analysis of the water samples. Future studies which includes the analysis of columns is highly recommended which directly measures the amount of ions absorbed by the biotrap. Different concentrations and mixtures of the chemicals is a good area for further studies in order to compare the capacity of the different mixtures for ion uptake as well explorations on comparative studies between immobilizing agents (physical and chemical) .

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TABLES

Table 1.0 Physical characteristics of samples and water after leaving for 2 hours for the solubility test

Samples	Characteristics of water	Characteristics of samples
With SiO2		
1%	Cloudy	Slightly soluble, gel-like consistency of EPS
5%	Turbid	Flakes of sample seen
10%	Turbid	Samples are soft
20%	Less turbid than 10%	Sample intact but soft
With Al (OH)3		
1%	Turbid	Plastic like, slightly soluble
5%	Turbid	Plastic-like samples
10%	Slightly turbid	Sample is powdery
20%	Water comparatively clear	Sample intact
With 20% Al $(OH)_3$ and SiO_2 in ratio		
1A:1S	Less turbid than 1:2 ratio	Samples intact
1A:2S	Slightly turbid	Samples are soft
2A:1S	Clear	Sample intact

FIGURES

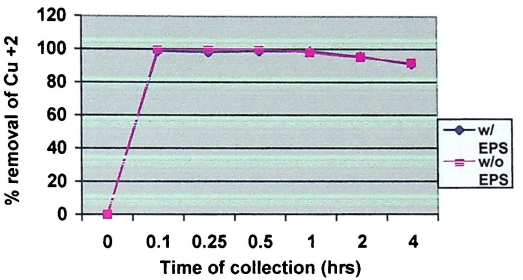

Figure 1. Average % Removal of Cu^{+2} in waste water that passed through a biotrap with 1 ml/min flow rate

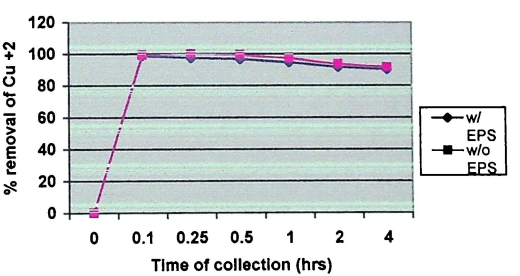

Figure 2. Average % Removal of Cu^{+2} in waste water that passed through a biotrap with 2.5 ml/min flow rate

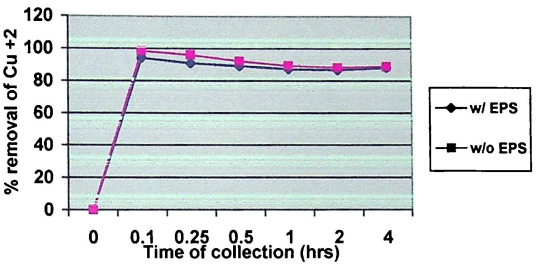

Figure 3. Average % Removal of Cu^{+2} in waste water that passed through a biotrap with 5 ml/min flow rate

Figure 4 (From L-R) 1%,5%,10% and 20% $Al(OH)_3$ with *Rhizobium* BJVr 12 EPS

Figure 5 (From L-R) (From L-R) 1%,5%,10% and 20% SiO_2 with *Rhizobium* BJVr 12 EPS

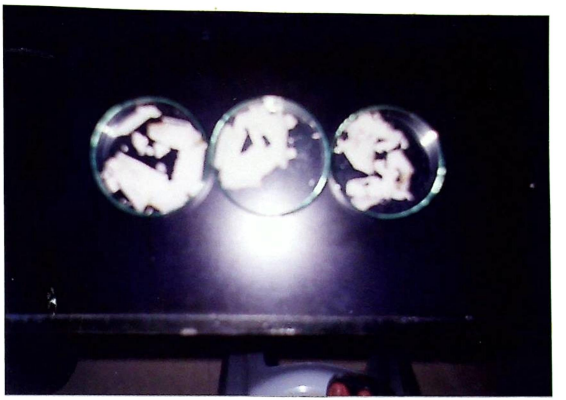

Figure 6 (From L-R) 1A:1S;2S:1A; 1A:2S with *Rhizobium* BJVr 12 EPS (A-20% $Al(OH)_3$; S-20% SiO_2)

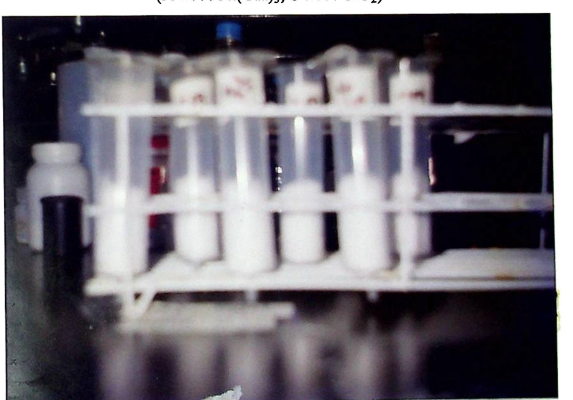

Figure 7 Biotrap Columns

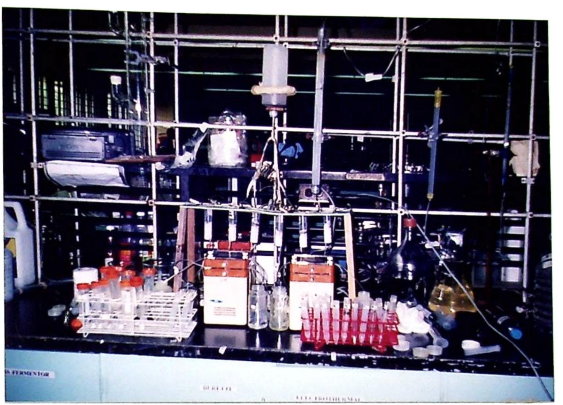

Figure 8.a Biotrap set-up

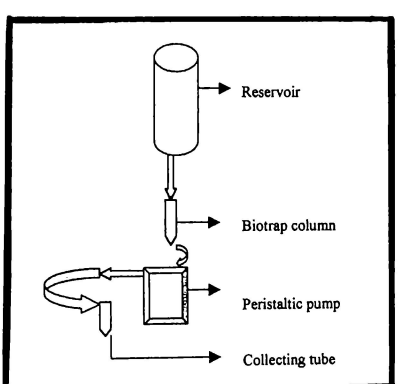

Figure 8.b Diagramatic sketch of biotrap set-up

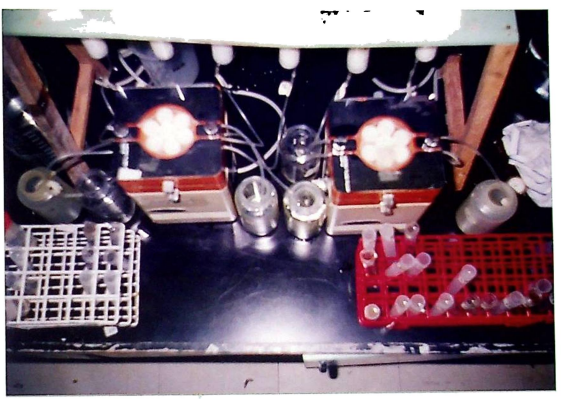

Figure 8.c Biotrap set-up (top view)

APPENDIX A

Table 2.0 Standard values of absorption with equivalent Cu^{+2} concentration 9ppm) for AAS (Atomic Absorption Spectrophotometry

Ppm Cu^{+2}	Absorption
0	0.0
0.5	0.022
1	0.044
2	0.089
3	0.132
4	0.175
5	0.215

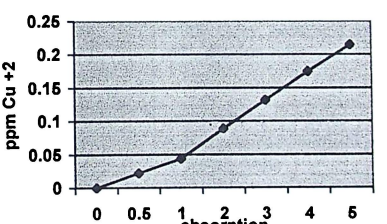

Figure 9.0 Standard Curve of AAS (Atomic Absorption Spectrophotometry) for Cu^{+2}

APPENDIX B

Other Tables

Table3.0 Physical characteristics of the oven-dried samples

Samples	Characteristics
With SiO_2	
1%	EPS forms a network, fine mesh which is plastic-like, amber in color and elastic
5%	EPS mesh no longer visible, elastic, granules of SiO_2 visible with fine texture
10%	Plastic, amber, powdery than that 5% SiO_2
20%	Rubbery, smooth, SiO_2 well-mixed with EPS
With Al $(OH)_3$	
1%	Same as that with SiO_2, forms a mesh but coarser than SiO_2
5%	Elastic, EPS mesh no, longer visible, granules of $AL(OH)_3$ more visible than SiO_2, coarse textured
10%	Rubbery texture, white, with small pores, color is uniform throughout
20%	Brittle, corky, white, less pores than 10%, uniform color
With 10% Si O_2 and Al $(OH)_3$ in ratio	
1:1	Slight yellow, with pores
1:2	Rubbery, smooth, minimal pores, uniformly white
2:1	Plastic-like texture, slightly amber in color, with uniform powdery white, greatest pore number
With 20% Si O_2 and Al $(OH)_3$ in ratio	
1:1	Brittle, rubbery, white
1:2	More brittle, white
2:1	Least brittle , white

Table 4.0 Absorbance and equivalent residual Cu^{+2} (ppm) of water samples passed through biotraps at a flow rate of 1 ml/min

Time (hrs)	Exopolysaccharide + $2Al(OH)_3 : 1SiO_2$		$2Al(OH)_3 : 1SiO_2$	
	Absorbance	Residual Cu^{+2} (ppm)	Absorbance	Residual Cu^{+2} (ppm)
0		30		30
		30		30
		30		30
0.10	0.016	0.34	0.003	0.03
	0.005	0.08	0.001	0.00
	0.021	0.45	0.003	0.03
0.25	0.025	0.54	0.001	0.00
	0.028	0.61	0.000	0.00
	0.021	0.45	0.002	0.01
0.5	0.021	0.45	0.001	0.00
	0.010	0.20	0.001	0.00
	0.012	0.24	0.020	0.01
1	0.033	0.73	0.008	0.15
	0.010	0.20	0.005	0.08
	0.012	0.24	0.007	0.13
2	0.090	2.05	0.042	0.94
	0.029	0.64	0.063	1.43
	0.058	1.31	0.080	1.82
4	0.116	2.66	0.093	2.12
	0.097	2.22	0.111	2.54
	0.129	2.96	0.117	2.68

Table 5.0 Absorbance and equivalent residual Cu^{+2} (ppm) of water samples passed through biotraps at a flow rate of 2.5 ml/min

(hrs)	Exopolysaccharide + $2Al(OH)_3 : 1SiO_2$		$2Al(OH)_3 : 1SiO_2$	
	Absorbance	Residual Cu^{+2} (ppm)	Absorbance	Residual Cu^{+2} (ppm)
0		30		30
		30		30
		30		30
0.10	0.036	0.80	0.002	0.01
	0.008	0.15	0.004	0.06
	0.047	1.05	0.026	0.57
0.25	0.035	0.78	0.001	0.00
	0.025	0.54	0.003	0.03
	0.043	0.96	0.003	0.01
0.5	0.040	0.89	0.009	0.17
	0.042	0.84	0.010	0.20
	0.057	1.29	0.009	0.17
1	0.069	1.57	0.035	0.78
	0.077	1.75	0.034	0.75
	0.058	1.31	0.043	0.96
2	0.107	2.45	0.080	1.82
	0.108	2.47	0.067	1.52
	0.114	2.61	0.090	2.10
4	0.128	2.94	0.114	2.61
	0.127	2.91	0.097	2.22
	0.123	2.82	0.117	2.72

Table 6.0 Absorbance and equivalent residual Cu^{+2} (ppm) of water samples passed through biotraps at a flow rate of 5 ml/min

Time (hrs)	Exopolysaccharide + $2Al(OH)_3 : 1SiO_2$		$2Al(OH)_3 : 1SiO_2$	
	Absorbance	Residual Cu^{+2} (ppm)	Absorbance	Residual Cu^{+2} (ppm)
0		30		30
		30		30
		30		30
0.10	0.059	1.33	0.005	0.08
	0.118	2.70	0.010	0.20
	0.058	1.31	0.055	1.24
0.25	0.122	2.80	0.049	1.10
	0.125	2.87	0.048	1.08
	0.108	2.47	0.054	1.22
0.5	0.135	3.10	0.109	2.49
	0.155	3.56	0.101	2.31
	0.134	3.07	0.094	2.15
1	0.163	3.75	0.142	3.26
	0.171	3.93	0.133	3.05
	0.164	3.77	0.136	3.12
2	0.169	3.89	0.159	3.66
	0.172	3.96	0.152	3.49
	0.173	3.98	0.147	3.38
4	0.153	3.52	0.142	3.21
	0.154	3.54	0.140	3.21
	0.53	3.52	0.149	3.42

Table 7.0 Master table showing average% removal in terms of flowrate, time of collection and kind of biotrap

Time of collection (hrs)	Kind of biotrap	1ml/min	2.5ml/min	5ml/min
0	W/ EPS	0	0	0
	W/o EPS	0	0	0
0.1	W/ EPS	99.023	98.887	94.067
	W/o EPS	99.933	99.290	98.310
0.25	W/ EPS	98.223	97.467	90.957
	W/o EPS	99.990	99.957	96.220
0.5	W/ EPS	99.010	96.643	89.190
	W/o EPS	99.977	99.397	92.277
1	W/ EPS	98.700	94.857	87.277
	W/o EPS	98.267	97.233	89.520
2	W/ EPS	95.557	91.633	86.853
	W/o EPS	95.343	93.620	88.300
4	W/ EPS	91.287	90.367	88.247
	W/o EPS	91.843	91.610	89.067

Table 8.0. Average % Removal of Cu from waste-water that passed through the biotrap with 1ml/min flow rate

Time (hrs)	% Removal of Cu^{+2}	
	Exopolysaccharide + $2Al(OH)_3 : 1SiO_2$	$2Al(OH)_3 : 1SiO_2$
0	0.00	0.00
0.10	99.023	99.933
0.25	98.223	99.990
0.5	99.010	99.977
1	98.700	98.267
2	95.557	95.343
4	91.287	91.843

Table 9.0 Average % Removal of Cu from waste-water that passed through the biotrap with 2.5 ml/min flow rate

Time (hrs)	% Removal of Cu^{+2}	
	Exopolysaccharide + $2Al(OH)_3 : 1SiO_2$	$2Al(OH)_3 : 1SiO_2$
0	0.00	0.00
0.10	98.887	99.290
0.25	97.467	99.957
0.5	96.643	99.397
1	94.857	97.233
2	91.633	93.620
4	90.367	91.610

Table 10.0 Average % Removal of Cu from waste-water that passed through the biotrap with 5 ml/min flow rate

Time (hrs)	% Removal of Cu^{+2}	
	Exopolysaccharide + $2Al(OH)_3 : 1SiO_2$	$2Al(OH)_3 : 1SiO_2$
0	0.00	0.00
0.10	94.067	98.310
0.25	90.957	96.220
0.5	89.190	92.277
1	87.277	89.520
2	86.853	88.300
4	88.247	89.067

APPENDIX C

Statistical Analysis

3-factor Factorial in Completely Randomized Design (CRD)

Function: Factor

Experiment Model Number 3:
Three factor Completely Randomized Design

Data case no. 1 to 126

Factorial ANOVA for the factors:
Replication (Var 4: Replicates) with values from 1 to 3
Factor **A** (Var 1: Flowrate) with values from 1 to 3
1 - 1 ml/min
2 – 2.5 ml/min
3 – 5 ml/min
Factor **B** (Var 2: Time) with values from 1 to 7
1 – 0 hr
2 – 0.10 hr
3 – 0.25 hr
4 – 0.50 hr
5 – 1 hr
6 – 2 hrs
7 – 4 hrs
Factor **C** (Var 3: EPS) with values from 1 to 2
1- with EPS
2- without EPS

Variable 6: % Removal of Cu^{+2}
Grand mean: 81.153 Grand Sum: 10225.220 Total Count=126

Table 11. TABLE OF MEANS

4	1	2	3	6	TOTAL
*	1	*	*	83.369	3501.490
*	2	*	*	82.211	3452.880
*	3	*	*	77.877	3270.850
*	1	*	*	0.000	0.000
*	2	*	*	98.253	1768.560
*	3	*	*	97.136	1748.440
*	4	*	*	96.082	1729.480
*	5	*	*	94.309	1697.560
*	6	*	*	91.884	1653.920
*	7	*	*	90.403	1627.260
*	1	1	*	0.000	0.000
*	1	2	*	99.483	596.900
*	1	3	*	99.107	594.640
*	1	4	*	99.493	596.960
*	1	5	*	99.483	590.900
*	1	6	*	95.450	572.700
*	1	7	*	91.565	549.390
*	2	1	*	0.000	0.000
*	2	2	*	99.088	594.530
*	2	3	*	98.712	592.270
*	2	4	*	98.020	588.120
*	2	5	*	96.045	576.270
*	2	6	*	92.627	555.760
*	2	7	*	90.988	545.930
*	3	1	*	0.000	0.000
*	3	2	*	96.188	577.130
*	3	3	*	93.588	561.530
*	3	4	*	90.733	544.400
*	3	5	*	88.398	530.390
*	3	6	*	87.577	525.460
*	3	7	*	88.657	531.940

*	*	*	1	80.393	5064.760
*	*	*	2	81.912	5160.460
*	1	*	1	83.116	1745.430
*	1	*	2	83.622	1756.060
*	2	*	1	81.408	1709.560
*	2	*	2	83.015	1743.320
*	3	*	1	76.656	1609.080
*	3	*	2	79.099	1660.080
*	1	1	1	0.000	0.000
*	1	1	2	0.000	0.000
*	1	2	1	99.033	297.100
*	1	2	2	99.933	299.800
*	1	3	1	98.223	294.670
*	1	3	2	99.990	299.970
*	1	4	1	99.010	297.030
*	1	4	2	99.977	299.930
*	1	5	1	98.700	296.100
*	1	5	2	98.267	294.800
*	1	6	1	95.557	286.670
*	1	6	2	95.343	286.030
*	1	7	1	91.287	273.860
*	1	7	2	91.843	275.530
*	2	1	1	0.000	0.000
*	2	1	2	0.000	0.000
*	2	2	1	98.887	296.660
*	2	2	2	99.290	297.870
*	2	3	1	97.467	292.400
*	2	3	2	99.957	299.870
*	2	4	1	96.643	289.930
*	2	4	2	99.397	298.190
*	2	5	1	94.857	284.570
*	2	5	2	97.233	291.700
*	2	6	1	91.633	274.900
*	2	6	2	93.620	280.860
*	2	7	1	90.367	271.100
*	2	7	2	91.610	274.830
*	3	1	1	0.000	0.000
*	3	1	2	0.000	0.000
*	3	2	1	94.067	282.200

*	3	2	2	98.310	294.930
*	3	3	1	90.957	272.870
*	3	3	2	96.220	288.660
*	3	4	1	89.190	267.570
*	3	4	2	92.277	276.830
*	3	5	1	87.277	261.830
*	3	5	2	89.520	268.560
*	3	6	1	86.853	260.560
*	3	6	2	88.300	264.900
*	3	7	1	88.247	264.740
*	3	7	2	89.067	267.200

1. Ho: There is no significant difference between the means of the different flow rates (1ml/min, 2.5ml/min and 5ml/min) with regards to % removal of Cu^{+2}
   Ha: There is a significant difference between the means of the three different flow rates
2. Ho: There is no significant difference between the means of the 7 different time interval with regards to % removal of Cu^{+2}
   Ha: There is a significant difference between the means of the different time intervals with regards to % removal of Cu^{+2}
3. Ho: There is no significant difference between the means of the kind of biotrap with regards to % removal of Cu^{+2}
   Ha: There is a significant difference between the means of the kind of biotrap with regards to % removal of Cu^{+2}
4. Ho:There is no interaction between flowrate and time interval with regards to % removal of Cu^{+2}
   Ha: There is an interaction between flow rate and time interval with regards to % removal of Cu^{+2}
5. Ho: There is no interaction between flowrate and the kind of biotrap with regards to % removal of Cu^{+2}
   Ha: There is an interaction between flowrate and the kind of biotrap with regards to % removal of Cu^{+2}
6. Ho: There is no interaction between time interval and kind of biotrap There is no interaction between flowrate and the kind of biotrap with regards to % removal of Cu^{+2}
   Ha: There is an interaction between time interval and kind of biotrap There is no interaction between flowrate and the kind of biotrap with regards to % removal of Cu^{+2}
7. Ho: There is no interaction between the three factors (flowrate, time interval and kind of biotrap) with regards to % removal of Cu^{+2}
   Ha: There is an interaction between the three factors (flowrate, time interval and kind of biotrap) with regards to % removal of Cu^{+2}

Table 12. ANALYSIS OF VARIANCE

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
2	Factor A	2	703.910	351.955	394.6079	0.000
4	Factor B	6	139146.562	23191.194	26001.5878	0.000
6	AB	12	264.287	22.024	24.6929	0.000
8	Factor C	1	72.687	72.687	81.4953	0.000
10	AC	2	19.824	9.912	11.1134	0.001
12	BC	6	28.557	4.760	5.3364	0.001
14	ABC	12	19.223	1.602	1.7961	0.0618
15	Error	84	74.920	0.892		
	TOTAL	125	140329.971			

Coefficient of Variation: 1.16%

S/y for means group 2:	0.1457	Number of observations: 42
S/y for means group 4:	0.2226	Number of observations: 18
S/y for means group 6:	0.3856	Number of observations: 6
S/y for means group 8:	0.1190	Number of observations: 63
S/y for means group 10:	0.2061	Number of observations: 21
S/y for means group 12:	0.3148	Number of observations: 9
S/y for means group 14:	0.5453	Number of observations: 3

* Therefore there is a great significant difference between the three factors. Reject the null hypothesis.

Statistical Analysis
3 factor factorial in CRD

Case Range: 127-129
Variable 6: % Removal
Function: RANGE

Error Mean Square = 0.8920
Error Degrees of Freedom = 84
No. of observations to calculate a mean =42

Duncan's Multiple Range Test
LSD value = 0.4098
s = 0.1457 at alpha (α)= 0.050
x

Flow rate vs % Removal of Cu^{+2} (Factor A)

Original Order	Ranked Order
Mean 1 = 83.37 A	Mean 1 = 83.37 A
Mean 2 = 82.21 B	Mean 2 = 82.21 B
Mean 3 = 77.88 C	Mean 3 = 77.88 C

Note: A, B, C= codes for the significant difference of the data, A with the highest rank

Legend: 1=1ml/min
2=2.5 ml/min
3=5ml/min

Case Range: 132-138
Variable 6: % Removal Cu^{+2}
Function: RANGE

Error Mean Square = 0.8920
Error Degrees of Freedom = 84
No. of observations to calculate a mean = 18

LSD value = 0.6261
s=0.2226 at alpha (α)= 0.050
x

Time of collection vs % removal of Cu^{+2+} (Factor B)

Original Order	Ranked Order
Mean 1 = 0.000	Mean 2 = 98.25 A
Mean 2 = 98.25	Mean 3 = 97.14 B
Mean 3 = 97.14	Mean 4 = 96.08 C
Mean 4 = 96.08	Mean 5 = 94.31 D
Mean 5 = 94.31	Mean 6 = 91.88 E
Mean 6 = 91.88	Mean 7 = 90.40 F
Mean 7 = 90.40	Mean 1 = 0.000 G

Note: A, B, C= codes for the significant difference of the data , A with the highest rank

Legend: 1= 0.0 hr
2=0.10
3=0.25
4=0.5
5=1.0
6=2.0
7=4.0

Case Range: 141-161
Variable 6: % Removal Cu^{+2}
Function: RANGE

Error Mean Square = 0.8920
Error Degrees of Freedom = 84
No. of observations to calculate a mean = 6

LSD value = 1.084
s=0.3856 at alpha (α)= 0.050
x
Flow rate vs. Time of collection (AB)

Original Order					Ranked Order				
Mean	1	=	0.000	H	Mean	4	=	99.49	A
Mean	2	=	99.48	A	Mean	2	=	99.48	A
Mean	3	=	99.11	AB	Mean	3	=	99.11	AB
Mean	4	=	99.49	A	Mean	9	=	99.09	AB
Mean	5	=	98.48	AB	Mean	10	=	98.71	AB
Mean	6	=	95.45	C	Mean	5	=	98.48	AB
Mean	7	=	91.57	EFE	Mean	11	=	98.02	B
Mean	8	=	0.0000	H	Mean	16	=	96.19	C
Mean	9	=	99.09	AB	Mean	12	=	96.04	C
Mean	10	=	98.71	AB	Mean	6	=	95.45	C
Mean	11	=	98.02	B	Mean	17	=	93.59	D
Mean	12	=	96.04	C	Mean	13	=	92.63	DE
Mean	13	=	92.63	DE	Mean	7	=	91.57	EF
Mean	14	=	90.99	F	Mean	14	=	90.99	F
Mean	15	=	0.000	H	Mean	18	=	90.73	F
Mean	16	=	96.19	C	Mean	21	=	88.66	G
Mean	17	=	93.59	D	Mean	19	=	88.40	G
Mean	18	=	90.73	F	Mean	20	=	87.58	G
Mean	19	=	88.40	G	Mean	8	=	0.0000	H
Mean	20	=	87.58	G	Mean	15	=	0.0000	H
Mean	21	=	88.66	G	Mean	1	=	0.0000	H

Note: A, C, B. . . . . H: codes for significant difference, A with the highest rank

Legend: 1-7 (1ml/min with time intervals 0,0.1,0.25,0.5,1,2,4)
8-14 (2.5 ml/min with time intervals 0,0.1,0.25,0.5,1,2,4)
15-21(5 ml/min with time intervals 0,0.1,0.25,0.5,1,2,4)

Case Range: 168-173
Variable 6: % Removal Cu^{+2}
Function: RANGE

Error Mean Square = 0.8920
Error Degrees of Freedom = 84
No. of observations to calculate a mean = 21

LSD value = 0.5796
s=0.02061 at alpha (α)= 0.050
x

Flow rate vs Sample (AC)

Original Order					Ranked Order				
Mean	1	=	83.12	A	Mean	2	=	83.62	A
Mean	2	=	83.62	A	Mean	1	=	83.12	A
Mean	3	=	81.41	B	Mean	4	=	83.02	A
Mean	4	=	83.02	A	Mean	3	=	81.41	B
Mean	5	=	76.66	D	Mean	6	=	79.10	C
Mean	6	=	79.10	C	Mean	5	=	76.66	D

Note: A,B. C,D: codes for significant difference of data, A with the highest rank.
Legend:

1= 1ml/min, with EPS
2=1ml/min without EPS
3=2.5ml/min with EPS
4=2.5ml/min without EPS
5=5ml/min with EPS
6=5ml/min without EPS

* Flow rate 1 (1ml/min)- no significant difference between samples with and without EPS
* Flow rate 2 (2.5 ml/min)- there is a significant difference between samples with and witout EPS; sample without EPS has no significant difference with those of flow rate 1
* Flow rate 3 (5 ml/min)- there is a significant difference between the samples with and without EPS

Case Range: 176-189
Variable 6: % Removal Cu^{+2}
Function: RANGE

Error Mean Square = 0.8920
Error Degrees of Freedom = 84
No. of observations to calculate a mean = 9

LSD value = 0.8854
s=0.3148 at alpha (α)= 0.050
x

Time of collection vs. sample

Original Order					Ranked Order				
Mean	1	=	0.000	H	Mean	4	=	99.18	A
Mean	2	=	0.000	H	Mean	6	=	98.72	A
Mean	3	=	97.33	B	Mean	3	=	97.33	B
Mean	4	=	99.18	A	Mean	8	=	97.22	B
Mean	5	=	95.55	C	Mean	5	=	95.55	C
Mean	6	=	98.72	A	Mean	10	=	95.01	C
Mean	7	=	94.95	C	Mean	7	=	94.95	C
Mean	8	=	97.22	B	Mean	9	=	93.61	D
Mean	9	=	93.61	D	Mean	12	=	92.42	E
Mean	10	=	95.01	C	Mean	11	=	91.35	F
Mean	11	=	91.35	F	Mean	14	=	90.84	FG
Mean	12	=	92.42	E	Mean	13	=	89.97	G
Mean	13	=	89.97	G	Mean	2	=	0.000	H
Mean	14	=	90.84	FG	Mean	1	=	0.000	H

Note: A,B,C. . .H=codes for significant difference between data, A with highest rank
Legend:
Odd nos: with EPS (time interval 0,0.1,0.25,0.5,1,2,4 consecutively)
Even nos: without EPS(time interval 0,0.1,0.25,0.5,1,2,4 consecutively)

Case Range: 192-233
Variable 6: % Removal Cu^{+2}
Function: RANGE

Error Mean Square = 0.8920
Error Degrees of Freedom = 84
No. of observations to calculate a mean = 3

LSD value = 1.534
s=0.5453 at alpha (α)= 0.050
x

Interaction of three factors (ABC: flow rate, time and sample)

Original Order					Ranked Order				
Mean	1	=	0.0000	P	Mean	6	=	99.99	A
Mean	2	=	0.0000	P	Mean	8	=	99.98	A
Mean	3	=	99.03	AB	Mean	20	=	99.96	A
Mean	4	=	99.93	A	Mean	4	=	99.93	A
Mean	5	=	98.22	ABC	Mean	22	=	99.40	A
Mean	6	=	99.99	A	Mean	18	=	99.29	A
Mean	7	=	99.01	AB	Mean	3	=	99.03	AB
Mean	8	=	99.98	A	Mean	7	=	99.01	AB
Mean	9	=	98.70	AB	Mean	17	=	98.89	AB
Mean	10	=	98.27	ABC	Mean	9	=	98.70	AB
Mean	11	=	95.56	EFG	Mean	32	=	98.31	ABC
Mean	12	=	95.34	EFG	Mean	10	=	98.27	ABC
Mean	13	=	91.29	JK	Mean	5	=	98.22	ABC
Mean	14	=	91.84	JK	Mean	19	=	97.47	BCD
Mean	15	=	0.000	P	Mean	24	=	97.23	BCD
Mean	16	=	0.000	P	Mean	21	=	96.64	CDE
Mean	17	=	98.89	AB	Mean	34	=	96.22	DEF
Mean	18	=	99.29	A	Mean	11	=	95.56	EFG
Mean	19	=	97.47	BCD	Mean	12	=	95.34	EFG
Mean	20	=	99.96	A	Mean	23	=	94.86	FGH
Mean	21	=	96.64	CDE	Mean	31	=	94.07	GH
Mean	22	=	99.40	A	Mean	26	=	93.62	HI

Mean	23	=	94.86	FGH	Mean	36	=	92.28	IJ
Mean	24	=	97.23	BCD	Mean	14	=	91.84	JK
Mean	25	=	91.63	JK	Mean	25	=	91.63	JK
Mean	26	=	93.62	HI	Mean	28	=	91.61	JK
Mean	27	=	90.37	KLM	Mean	13	=	91.29	JK
Mean	28	=	91.61	JK	Mean	33	=	90.96	JKL
Mean	29	=	0.000	P	Mean	27	=	90.37	KLM
Mean	30	=	0.000	P	Mean	38	=	89.52	LMN
Mean	31	=	94.07	GH	Mean	35	=	89.19	MN
Mean	32	=	98.31	ABC	Mean	42	=	89.07	MN
Mean	33	=	90.96	JKLK	Mean	40	=	88.30	NO
Mean	34	=	96.22	DEF	Mean	41	=	88.25	NO
Mean	35	=	89.19	MN	Mean	37	=	87.28	O
Mean	36	=	92.28	IJ	Mean	39	=	86.85	O
Mean	37	=	87.28	O	Mean	16	=	0.000	P
Mean	38	=	89.52	LMN	Mean	2	=	0.0000	P
Mean	39	=	86.85	O	Mean	29	=	0.000	P
Mean	40	=	88.30	NO	Mean	30	=	0.000	P
Mean	41	=	88.25	NO	Mean	15	=	0.000	P
Mean	42	=	89.07	MN	Mean	1	=	0.000	P

Note: A,B,C,. . . P: codes for significant difference between data, A with the highest rank

Legend: 1-14 =1ml/min with time intervals 0,0.1,0.25,0.5,1,2,4; odd nos w/EPS, even nos. w/o EPS
15-28 =2.5 ml/min with time intervals 0,0.1,0.25,0.5,1,2,4; odd nos w/EPS, even nos. w/o EPS
29-42=5 ml/min with time intervals 0,0.1,0.25,0.5,1,2,4 odd nos w/EPS, even nos. w/o EPS