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Factors affecting cobalt uptake by cobalt-trained *Mucor rouxii* NRRL 1894 biomass

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Abstract
Studies on the mechanism of cobalt biosorption by cobalt-trained *Mucor rouxii* NRRL 1894 biomass were conducted to determine the environmental conditions that would speed up this process. The rate of CO^{+2} removal from solution proceeded rapidly and it appeared to be virtually complete during the initial 5 min., and very slowly increased by time until reached to the maximum after 3h. of incubation of the biomass with cobalt solution. This pattern of accumulation was identical in biomass treated with metabolic inhibitors (NaN_3 or DNP) or heat-killed biomass. Cobalt removal from solution by the biomass was not affected by the change in temperature (25ºC to 50ºC) and was only affected by reduced temperature (5ºC). The effect of pH was very significant in terms of the potential applicability of recovery since cobalt removal from solutions increased linearly with the increase in pH within the range of pH 1.5 to 6.0. The amount of CO^{+2} removed increased as the initial concentration increased, and the removal was efficient (80.9%) in solution containing up to 147.8 ppm CO^{+2}, while the uptake capacity increased at 197 ppm CO^{+2}, suggesting a saturation kinetics with respect to CO^{+2} concentration. The efficiency of sorption of cobalt and zinc dust mixture was that of sorption of cobalt from its pure solution or zinc dust from its pure suspension, on the other hand, the *M. rouxii* biomass removed 93.36-96.80% of zinc dust. The uptake of cobalt from $42x10^{-1}$m eq/l of cobalt solution was not affected by the presence of either Ca^{+2}, Mg^{+2} or Na^{+2} (light metal ions) or from a solution containing all these light meal cations.

Keywords: Cobalt-trained *Mucor rouxii*, Biomass Factors affecting, CO^{+2}, Biosorption, Zinc dust, Light metal ions.

1. Introduction
Biological methods of metal removal, defined as biosorption, have been recommended as cheaper and more effective techniques to solve the water pollution problem[1-3]. In biosorption, either live or dead microorganisms or their derivatives are used, which complex metal ions through the action of ligands or functional groups located on the outer surface of the cell[4]. Biosorption regarded as physicochemical interactions of metal ions with the cellular compounds of biological species[5]. The mechanism of uptake can be due to ion exchange, chelation, chemical complexation with microbial cell surface groups, adsorption, and diffusion through cell walls and membranes[6-8], which differ depending on the species used, the origin and processing of the biomass and solution chemistry. The use of fungi as biosorbents have been proven more efficient and economical for removal of toxic metals from dilute aqueous solutions and treating effluents charged with toxic metallic ions by biosorption because of its filamentous morphology and high percentage of cell walls[12-13]. Moreover, fungi can also be easily grown in substantial amounts using inexpensive growth media to obtain large quantity of biomass[14]. Some workers[9-11] suggested that the binding of heavy metals by the cell may be one of the mechanisms involved in the development of tolerance in fungi to these metals. The major factors that affect the biosorption processes are (a) initial metal ion concentration, (b) temperature, (c) pH, and (d) biomass concentration in solution. Aksu *et al.*[15] reported that temperature does not influence the biosorption processes in the range of 20ºC - 35ºC. However, pH seems to be the most important parameter in the biosorption processes. It affects the solution chemistry of the metals, the activity of the functional groups in the biomass and the competition of the metalic ions[16-17]. Biomass concentration in the solution seems to influence between the binding sites. Fourest and Roux [18] invalidated this hypothesis attributing the responsibility of the specific uptake decrease due to metal concentration shortage in solution. Hence, this factor needs to be taken into consideration in any application of microbial biomass as biosorbent. The heavy metal cobalt (Co) is extensively used for industrial purposes like production and refining of alloys, jet engines, gas turbines and electrochemical materials [19]. Cobalt is an essential metal and is needed in trace amount by the organisms; it is used as a cofactor of vitamin B12 and other enzymes in yeast, animals, bacteria, archaea and plants [20]. However, at higher concentrations, cobalt becomes toxic for

living systems but the exact mechanism of this toxicity is still poorly understood [21].This paper reports on factors affecting the removal of Co^{+2} by cobalt-trained *Mucor rouxii* NRRL 1894, namely the effect of time, temperature, pH, Co^{+2} concentrations, pretreatment of biomass, light metal ions, and zinc dust.

2. Materials and Methods

2.1 Microorganism and culture conditions

Mucor rouxii NRRL 1894 was obtained from ARS culture collection (NRRL), Peoria, Illinios, USA. The fungus was maintained on PDA medium and subcultured every two weeks.

2.2 Media

Potato dextrose medium: contained (g/l) potato, 300, glucose monohydrate, 20 and agar, 20.

Peptone glucose medium (Its comparatively low metal binding affinity)[23]: contained (g/l), peptone, 10 and glucose monohydrate, 20 with or without agar, 20.

2.3 Growth conditions

Fungal biomass was obtained from batch static cultures grown on peptone glucose liquid medium at 30ºC. The biomass was harvested by filtration, washed several times with distilled water and dried between two filter papers.

2.4 Cobalt-trained fungus [24]

The fungus was trained on peptone, glucose solid medium amended with 50 ppm of Co^{+2} (as $CoCl_2$. 6 H_2O) to tolerate high concentration of cobalt by serial transfer on slants (five transfers) for 25 days. The cobalt-trained strain was maintained on peptone, glucose solid medium containing 5ppm Co^{+2}.

3. Kinetics and mechanism of cobalt uptake by cobalt trained *M. rouxii* NRRL 1894

Factors that influence cobalt uptake by fungal biomass were investigated as follows:

3.1 Equilibrium experiments

In order to determine the minimum time of equilibration for the maximum sorption of cobalt by the cells, several equilibrium experiments were conducted at different times ranging from 5 min to 3h.

3.2 Effect of temperature

The effect of different temperatures (5, 25, 30, 40 and 50 ˚C) on the uptake of cobalt during 10 min period was investigated.

3.3 Effect of pH

The effect of pH was studied within the range of pH 1.5 to 8.0 by adjusting the metal solution to the desired pH with either 0.1N NaOH or 0.1N HCl before adding the fungal biomass.

3.4 Effect of cobalt concentration

The dependence of cobalt uptake on initial metal concentration 12.3-246.3ppm Co^{+2} was studied at short times (10 min.) of incubation at 30˚C.

3.5 Effect of light metals

The effect of light metals associated with cobalt solution was studied. The light metals such as Na, Ca, Mg. cobalt-trained *M. rouxii* biomass (5.0g wet weight= 0.39g dry weight) was added to 100ml cobalt solution amended with a light metal or light metals.

3.6 Effect of zinc dust

The Effect of zinc dust (as an insoluble inorganic compound) on the cobalt uptake by cobalt-trained *M. rouxii* was studied in three sets of experiments: **I -** To 100 ml cobalt solution (16.8 m eq/l) in 250 ml flask, 0.5g of zinc dust and 5.0g (wet weight) of the biomass were added. The flasks were incubated shaken (150rpm) at 30 ºC for 5 min. up to 180 min. **II -** To the cobalt solution (100ml) in 250ml flask 5.0g (wet weight) of biomass was added and the flasks were incubated shaken (150rpm) for 10min. thereafter, 0.5g of zinc dust was added and reincubated for different periods of time up to 180 min. **III-** Biomass (5.0g: wet weight) was added to distilled water in 250 ml flask amended with 0.5g of zinc dust and incubated shaken at 30ºC for 30 min. Thereafter, an appropriate volume of cobalt solution was added to the suspension to bring cobalt concentration to 16.8 $x10^{-1}$ m eq/l. Then the flasks were incubated shaken at 30ºC for an additional 2.5h. In all the 3 sets of experiments cited above and by the end of the period of the treatment, two replicate flasks were selected. Then, the contents of the flasks were filtered through pre-weighed filter paper (Whatman No.1) to remove the biomass and zinc dust. The filtrate was kept a side for cobalt determination. The biomass was removed from the filter paper and the non adhering zinc dust was removed by washing the biomass with distilled water onto the filter paper, which was then dried at 45ºC to a constant weight (usually 24h.). The amount of zinc dust adsorbed by biomass was calculated by subtracting the weight of zinc dust on the filter paper from the weight of zinc dust added. Appropriate controls were prepared and treated simultaneously as the experiment. These controls were as follows: **(a)** Zinc dust in distilled water without biomass. **(b)** Zinc dust in cobalt solution. **(c)** Biomass (5.0g, wet weight) in 100 ml of cobalt solution. (**d)** Biomass (5.0g, wet weight) in 100ml of distilled water amended with 0.5g zinc dust

3.7 Effect of pretreatment of biomass on cobalt uptake

Fungal biomass (each 5g, wet weight= 0.39g dry weight) was treated prior to contact with cobalt solution (83.6 X 10^{-1} m eq/l pH 6.0) as follows:

1. Boiling with distilled water for 15 min..
2. Soaking in 5% KOH solution for 10 min., then separated by filtration and washed with 1N HCl then thoroughly by washed with distilled water.
3. Soaking in 1.85 X 10^{-5} mM sodium azide solution for 30 min, separated by filtration and washed with distilled water.
4. Sodium azide 1.85 X 10^{-5} mM or 2, 4 dinitrophenol (DNP) (5X10-^{5}M) were added to the cobalt uptake reaction mixture.

Appropriate controls for the treatment were prepared and treated simultaneously as the experiments. Each of the treated biomass was added to cobalt solution. By the end of incubation period (1h), the biomass was separated by centrifugation and the supernatant was analyzed for the determination of residual cobalt.

4. Analytical methods

Cobalt concentration was determined by atomic absorption spectrophotometer SpectrAA 220.

Metal uptake by the biomass was determined as difference between the initial and final cobalt concentration, and the uptake capacity was calculated.

Uptake capacity = µg Co^{+2} /100mg biomass dry weight.

5. Results and Discussion

5.1. Time course of cobalt uptake

In own a previous work[24], cobalt- trained *Mucor rouxii* showed a promising biosorbent for the removal of cobalt from solution. Thus, studies on the mechanism of cobalt biosorption by cobalt-trained *Mucor rouxii* biomass were conducted to determine the environmental conditions that would speed up this process. The rate of cobalt removal from solution proceeded rapidly and it appeared to be virtually complete during the initial 5min. and very slowly increased by time until reached to the maximum after 3h. of incubation of the biomass with cobalt solution (Fig.,1). It is likely that this period represented surface binding by adsorption to binding sites on the fungal cell wall or exterior of the membrane. Some workers[6-8]reported that, the mechanism of uptake can be due to ion exchange, chelation, chemical complexation with microbial cell surface groups, adsorption, and diffusion through cell walls and membranes, which differ depending on the species used, the origin and processing of the biomass and solution chemistry.

5.2. Effect of temperature

The effect of different temperature (5-50˚C) on the uptake of cobalt was studied. As shown in Fig., (2) the amounts of cobalt taken up by fungal biomass were not affected by the increase of temperature in the range of 25-50˚C, and was only affected by reduced temperature (5ºC). Aksu *et al*.[16] reported that, temperature does not influence the biosorption processes in the range of 20-35ºC. Volesky[27] reported that, the physiological state of the organism, the age of the cells, the availability of micronutrients during their growth and the environmental conditions during the biosorption process (such as pH, temperature, and the presence of certain co-ions)are important parameters that affect the performance of a living biosorbent.

5.3. Effect of pH on the cobalt uptake

Results of the effect of pH on cobalt uptake by cobalt-trained *M. rouxii* biomass (Fig., 3) show that cobalt uptake increased linearly with pH from 1.5 to 6.0 and then a slight increase in uptake was observed within the range of pH 6.0 to 8.0. This may be related to a competition effect for binding sites between H^+ and CO^{+2} [25]. Therefore, it is recommended that pH control would be necessary to maintain the optimum pH conditions for metal uptake. Also, Galli *et al.*[26] reported that the pH value of the metal solutions affects the surface charge of the biosorbents and the degree of ionization.

5.4. Effect of cobalt concentration

The amount of CO^{+2} removed by cobalt trained *M. rouxii* biomass, increased as the initial concentration increased (Fig.,4) and the removal was efficient (80.9%) in solution containing up to 147.8 ppm CO^{+2} , then a decrease in the percentage of cobalt removal was observed, but the uptake capacity increased at 197 ppm CO^{+2}, suggesting a saturation kinetics with respect to CO^{+2} concentration. Volesky[27] reported that, the efficiency of metal concentration on the biosorbent is influenced by metal solution chemical features.

5.5. Effect of light metal ions

The possible interferences of relatively high concentration of Ca^{+2}, Mg^{+2} and Na^+ (light metal cations) on the sorption of CO^{+2} by the biomass of cobalt-trained *M. rouxii* was studied. The uptake of cobalt from $42x10^{-1}$ m eq/l of cobalt solution was not affected by the presence of either Ca^{+2}, Mg^{+2} or Na^+ in a concentration of $36x\ 10^{-1}$, $39\ x10^{-1}$ or $117.4\ x\ 10^{-1}$ meq/l, respectively or from a solution containing all these light metal cations. However, with relatively diluted cobalt solution (16.8 m eq/l), the uptake of CO^{+2} was slightly affected. The results indicated that these cations either separately or in combination scarcely affected the uptake of cobalt by cobalt-trained *M. rouxii* biomass. Thus, it may be possible that these light metal ions are only minimally sorbed by biomass and in turn they did not seriously interfere in the removal of the heavy ions from solution. Similar findings were reported by Nakajima *et al*.[28] they founded that, Na^+, K^+ and Ca^{+2} did not affected the uptake of uranium by *Chlorella regularis* cells.

5.6. The effect of zinc dust on cobalt uptake

In view of the possible interference of inorganic insoluble particulates on the uptake of heavy metal ions, this experiment was designed to test the effect of the presence of zinc dust on cobalt uptake by fungal biomass.

Data in Table (1) indicate that equilibration of the biomass with cobalt solution for 10 min., didn't affect adsorption of added zinc dust as compared with the control (biomass with zinc dust suspension only).

Equilibration of the biomass with zinc dust suspension for 30 min., didn't affect the uptake of added cobalt. The uptake of cobalt was of the same order as the nontreated biomass.

The presence of cobalt and zinc dust in biomass suspension didn't affect the adsorption of either cobalt or zinc dust. These facts were emphasized by the finding that the efficiency of sorption of cobalt and zinc dust mixture was as that of sorption of cobalt from its pure solution or zinc dust from its pure suspension, on the other hand, the *M. rouxii* biomass removed 93.36-96.80% of zinc dust. These results indicated that biosorption of heavy metal ions or particulates is a relatively non specific process with each metal binding site being able to be used by any number of metal species depending on their concentrations, chemical properties and the nature of the ligand and external physicochemical factors[29].

5.7. The effect of metabolic inhibitors on the uptake

To examine whether the uptake of cobalt by nongrowing cobalt-trained *M. rouxii* biomass depended on the biological activity. The uptake of cobalt by killed biomass (boiled biomass) or by preincubated biomass in NaN_3 or DNP were tested. The experiments were performed to test effect of inhibitors on the short term uptake (10-60 min.). Results in Table (2) indicate that cobalt uptake was very rapid during the initial 10 min., of incubation followed by very low uptake over the subsequent 50 min., this pattern of accumulation was indicated in biomass treated by boiling H_2O or KOH or that treated with metabolic inhibitors, indicating that uptake of cobalt by the fungal biomass was independent of cellular metabolism. Tobin *et al*.,[30] suggested that, a wide variety of ligands may be involved in biosorption of metals and these include carboxyl, hydroxyl, sulf- hydryl, amine and phosphate groups, although the relative importance of each is difficult to determine. Asku *et al*.,[15] reported that extracellular accumulation/ precipitation may be facilitated by using viable microorganisms, cell-surface sorption or complexation can occur with alive or dead microorganisms, while intracellular accumulation requires microbial activity.

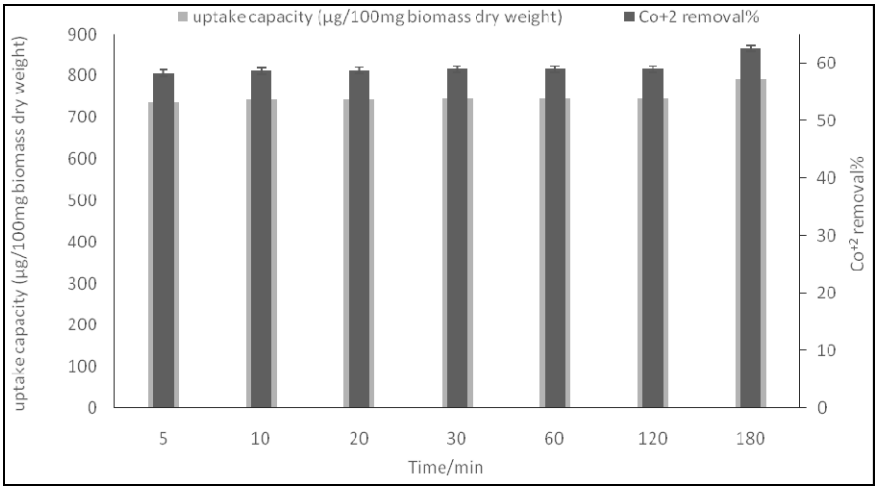

Fig. (1): Time course of cobalt uptake by cobalt-trained *M. rouxii* biomass.

Time course of cobalt uptake by cobalt-trained *M.rouxii* biomass (5g wet weight = 0.39g dry weight were suspended in 100 ml cobalt solution containing 49.3 ppm, pH 6.0, incubated shaken (150 rpm) at 30˚C.

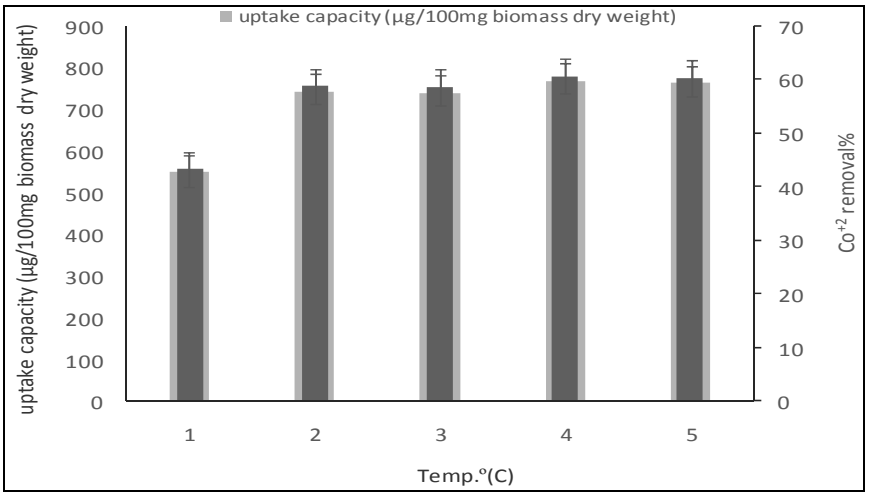

Fig. (2): Effect of temperature on the cobalt uptake by cobalt-trained *M. rouxii* biomass.

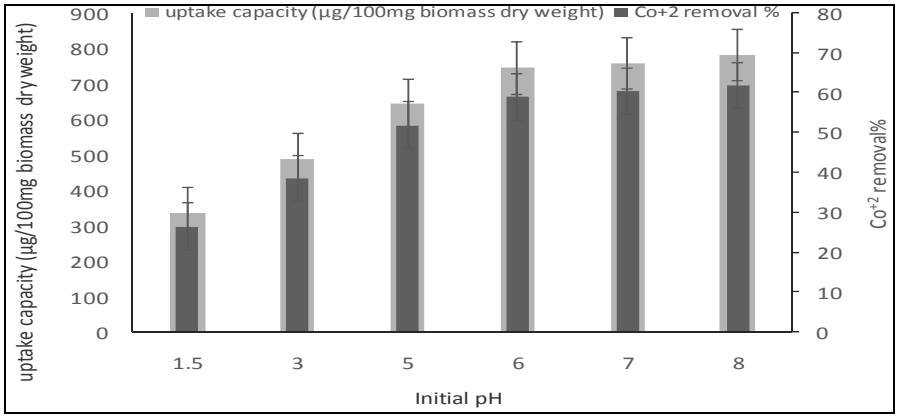

Fig., (3): Effect of pH on the cobalt uptake by cobalt-trained *M. rouxii* biomass.

The experimental conditions were the same as described in Fig., (1) except that the pH was adjusted with 1N HCl or 1N NaOH as indicated for each treatment.

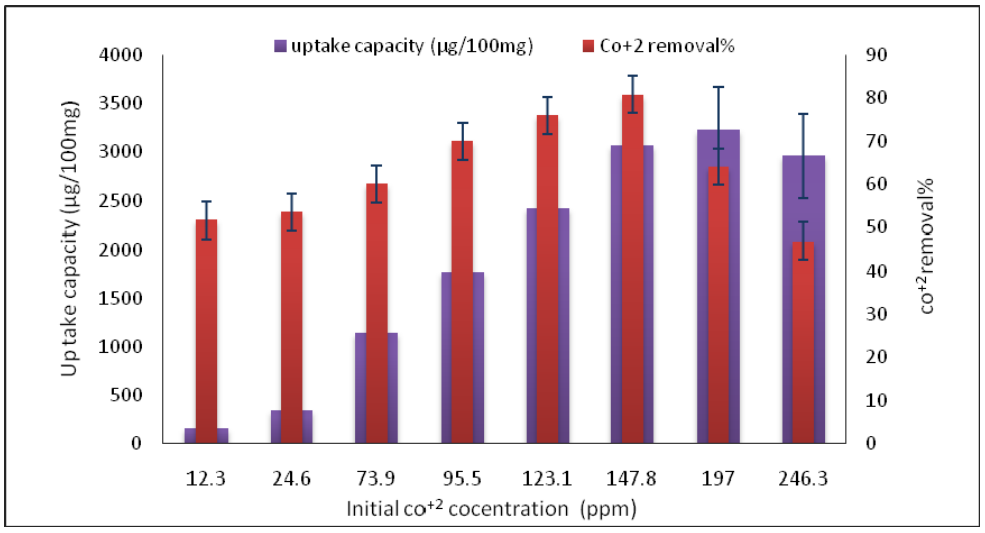

Fig., (4): Effect of Co^{+2} concentrations on the cobalt uptake by cobalt- trained *M. rouxii* biomass.

Cobalt-trained *M. rouxii* biomass (5g wet weight = 0.39g dry weight) from cobalt solutions (100 ml, containing different amounts of Co^{+2}, pH 6) incubated shaken (150 rpm), at 30˚C for 10 min.

Table 1: Effect of zinc dust on uptake by cobalt trained *Mucor rouxii* biomass.

Cobalt removal %				
Time min.	**CO^{+2}solution "control"**	**Zinc dust added at t_0 to CO^{+2} solution**	**Zinc dust added at t_{10} to CO^{+2} solution**	**CO^{+2} added at t_{30} to zinc dust suspension**
5	58.20	57.10	57.67	
10	58.65	57.70	59.40	
30	58.92	58.09	60.45	
40				57.74
60	58.92	58.60	60.60	58.89
90				60.06
120	58.92	59.32	61.45	
150				60.10
180	62.53	59.75	62.09	
210				61.25
adsorbed zinc dust %	Control 95.36	96.56	96.80	93.36

The experimental conditions were described in 3.5.

Table 2: Effect of metabolic inhibitors on the cobalt uptake by trained *M. rouxii* biomass.

	Cobalt removal% during incubation period (min.)		
Pretreatment*	10	30	60
None (control)	46.58	47.18	47.55
Boiled in H_2O for 15 min.	46.44	46.26	46.81
Potassium hydroxide (5%) 10 min.	47.10	47.20	48.85
Sodium azide (1.85 x 10^{-5}Mm) 30 min.	47.65	47.63	48.43
Sodium azide (1.85 x 10^{-5}Mm) in CO^{+2} solution	47.37	47.36	47.70
2,4 dinitrophenol (5x10^{-5}M)in Co^{+2} solution	46.81	47.37	48.71

* Fungal biomass (each 5g wet weight= 0.39g dry weight was treated prior to contact with cobalt solution (83.96x10^{-1} m eq/l CO^{+2}) as indicated.

6. Conclusion:

Tolerant *Mucor rouxii* NRRL 1894 to CO^{+2} and resistance developed by repeated subculturing the original strain with relatively high levels of cobalt (50ppm) enabled the fungus to adapt to and, thus, to tolerate higher levels of CO^{+2} than that original one, therefore, studies on the mechanism of cobalt biosorption by cobalt-trained *M. rouxii* biomass were conducted to determine the environmental conditions that would speed up this process. The major factors that affect the biosorption processes are (a) Initial metal ion concentration, (b) Temperature, (c) Time and (d) Biomass source. The results indicated that the rate of cobalt removal from solution proceeded rapidly and it appeared to be virtually complete during the initial 5 min. of incubation of the biomass with cobalt solution. This pattern of accumulation was identical in biomass treated with metabolic inhibitor (NaN_3 or DNP) or heat killed biomass. Cobalt removal from solution by biomass was not affected by the change in temperature (25-50 ºC) and was only affected by reduced temperature (5ºC), also, cobalt removal increased linearly with the increase in pH within the range of pH 1.5 to 6.0. The amount of CO^{+2} removed increased as the initial concentration increased. Cobalt uptake was not affected by the presence of either light metals or zinc dust.

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