OPTIMISING THE REMOVAL BY *B SUBTILIS* AND *B BACTERIUM* OF METALS FOUND AROUND MINING AREAS: EVALUATION OF THE EFFECT OF PHYSICAL AND PHYSIOLOGICAL PARAMETERS

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ABSTRACT

Biological remediation of toxic metal polluting surface water, although a great approach, still required improvement of protocol prior to effective application. *B subtilis'* and *B bacterium's* potentials to uptake silver, chromium and lead from aqueous solution were investigated, and the results revealed maximum sorption (up to 95%) of these metals at neutral pH (7 and 8) as well as at higher temperature (45^{o}C). Cell biomass recovered at the stationary phase was better biosorbents compared to its log phase counterpart. Specificity in metal removal was expressed by the preference of *B subtilis* to chromium while *B bacterium* showed better performance in removing lead. Higher concentration of metal was found to be more toxic to bacteria and slightly influence their ability to uptake metal. Understanding of physiological characteristic of each bacterium involved in bioremediation process as well as its interaction with specific metal could improve the technique to around 20% as found in this study, therefore facilitating its effective application at large scale.

Keywords: Biosorption, indigenous microorganisms, metals, physical and growth parameters, optimization

1. INTRODUCTION

The shortage of water around the world could partly be attributed to global warming, but anthropogenic activities also contribute in degrading the quality of water sources already in shortage. Included in the latter are mining and metallurgical exploitations, which often promote the dispersion of toxic metals in water system (Bueno et al., 2008; Volesky, 2003; Dursun et al., 2003), reducing the amount of usable water especially in underdeveloped areas (Fosso-Kankeu et al., 2008). Various techniques have been developed to address the problem of surface water contamination with metals, but more efforts are now devoted in improving the biological approach, which provides economic and environmental advantages. Extensive studies done in the last decades on the remediation of metals in surface waters showed that living and dead biomass can remove considerable amount of toxic metals from aqueous solution (Tsezos *et al*., 1995; Volesky and May-Phillips, 1995; Kim *et al*., 2007). Despite these significant advances in bioremediation processes, the challenge is to apply these processes at industrial scale, achievable through proper understanding of the interaction between microbial cells and metal ions. Previous works have shown that metal removal by microorganisms could occur through two mechanisms namely biosorption and bioaccumulation , which depend on the microbial membrane surface and composition and metabolic activities respectively (Brandl and Faramarzi, 2006; Gupta et al., 2000). Metal removal by living biomass is therefore growth dependent, and could be affected by environmental conditions that influence the development of microorganisms. Microorganisms generally have specific range of pH or temperature suitable for their growth and performance of vital functions. Out of that range, microorganisms are either killed or inhibited (Prescott et al., 2002), during such arch conditions, microorganisms adapt differently and how the microorganisms adapt in such circumstances may affect in a certain way their abilities to remove metals from solution.

The determination of suitable physiological growth state and physical conditions where microorganisms can effectively uptake metals and the identification of appropriate method for the recovery of absorbed metals with possible regeneration of microorganisms are crucial. Although the biological approach provides an unequivocal alternative with cost effective viability as well as environmental friendliness (Allury et al., 2007), there is still an important need to optimize the process and also improve the experimental protocol.

Investigations around diverse mines were systematically carried out to determine residual metals such as chromium, lead and silver in surrounding surface waters. Using indigenous microorganisms isolated during previous studies

(Mulaba et al., 2009), the above metals were effectively removed from aqueous solution while determining the optimal physical and physiological parameters.

2. METHODOLOGY

Culturing Microorganisms

Stock cultures of strains of *B subtilis* and *B bacterium* isolated during previous study (Mulaba et al., 2009), were plated in nutrient agar medium ("Lab-Lemco" powder 1.0; yeast extract 2.0; peptone 5.0; sodium chloride 5.0; Agar 15.0; pH 7.4 ± 0.2 at 25 °C; Merck Chemicals, SA) and incubated at 37°C overnight. Growing colonies were selected and inoculated in nutrient broth ("Lab-Lemco" powder 1.0; yeast extract 2.0; peptone 5.0; sodium chloride 5.0; pH 7.4 ± 0.2 at 25 °C; Merck Chemicals, SA) then incubated overnight at 37°C in a shaking incubator (150 rpm).

Preparation of Metal Solution

Salts of Silver nitrate, Chrome and Lead nitrate were dissolved in sterile distilled water to form a stock solution of 1000 ppm. Adequate volumes were taken from the stock solution to prepare working solutions with various metal concentrations (30, 50, 100 and 200 ppm).

Metal Removal

To assess the ability of *B subtilis* and *B bacterium* to remove silver, chrome or lead from solution, these bacteria were cultured in nutrient broth for twenty hours under the above conditions then aliquots of the culture were transferred into sterile centrifugation tubes, and centrifuged at 8000 rpm for 15 minutes. The supernatant was discarded and the pellet suspended with sterile distilled water and added to metal solution in 250 ml sterile Erlenmeyer to form a biomass concentration of 150 mg of wet cells per 100 ml. The mixture was incubated at 37 °C in a shaking incubator (150 rpm). Five milliliter of the mixture were collected into a centrifugation tube after twenty minutes, one hour, eight hours, twenty hours and twenty four hours and centrifuged at 13000 rpm for five minutes. The supernatant was collected and the concentration of remaining metal measured using Inductively Coupled Plasma – Optical Emission Spectrometer (ICP – OES).

As in previous work (Fosso-Kankeu et al., 2009), all experiments were done in triplicate with an abiotic control that assisted in accounting for metal loss due to precipitation; discrepancy between replicates was less than 10%. The averages of the triplicate values were used when drawing the graphs.

Impact of PH on Metal Removal

B subtilis and *B bacterium* were cultured, centrifuged and recovered as above, then added (1.5g/l) to solutions of sterile distilled water at various pH (3, 5, 7, 8 and 10) with similar concentration (50 ppm) of metal. The mixture was incubated at 37 °C in the shaking incubator (150 rpm). Samples were collected after twenty hours and bacterial sorption activity determined by measuring the concentration of remaining metal in the supernatant.

Effect of Temperature on Metal Removal

In solutions containing metal at concentration of 50 ppm, *B subtilis* and *B bacterium* were added to a concentration of 1.5 g/l of wet cells. The mixture was incubated at various temperatures (28, 37 and 45 °C) in the incubator with shaker (150 rpm). The procedure in determining the amount of metal removed was performed as above.

Removal of Metal At Various Growth Stages

Both strains of *B subtilis* and *B bacterium* were grown in the nutrient broth under the above conditions for various length times (ten hours, twenty hours and twenty-four hours). Cells were recovered by centrifugation and added to metal solution (50 ppm) up to a biomass concentration of 1.5 g/l (wet cells). The mixture was incubated under the above conditions and the samples collected after twenty hours to determine the remaining metal concentration in supernatant.

Microorganisms Survival during Removal of Metal

When performing metal removal experiment at various concentrations, pH and temperatures, one milliliter of the solution was also collected after twenty minutes, one hour, eight hours and twenty hours, then serially diluted (10^{-1} to 10^{-10}) in sterile phosphate buffer, and plated in nutrient agar medium overnight. Colonies were counted and expressed as CFU/ml.

3. RESULTS AND DISCUSSIONS

Sorption Capability of *B Subtilis* and *B Bacterium*

The sorption of three metallic species (silver, chromium and lead) by *B subtilis* and *B bacterium* was examined as a function of contact time and initial metal concentration. The results presented in figures 1 and 2 show a relatively good competency of bacteria to remove metals (up to 95%) from solution. Both microorganisms exhibited almost similar patterns in removing metals at various times and initial metal concentration. A higher removal rate was recorded with 30 and 50 ppm initial concentration of metal. The maximum removal rate for all the elements (Ag-77%; Cr-91%; Pb-91%) and (Ag-87%; Cr-93%; Pb-95%) for *B subtilis* and *B bacterium* respectively, occurring generally between eight and twenty-four hours contact time. It was observed that both microorganisms have little affinity for silver as compared to chromium and lead. Expression of results as ratio of the amount of metal absorbed versus biomass revealed the highest removal in the range of 292 to 500 µg metal/g of cell biomass, occurring with 100 and 200 ppm initial concentration of metal; this implies that metal uptake was also function of cell surface availability, it has been suggested (Congeevaram et al., 2006; Rani and Harapriya, 2003) that at low metal concentration cell biomass it not fully utilized. The passive or physical adsorption occurring within the first 40 minutes of contact (Fosso-Kankeu et al., 2009; Goyal et al., 2003; Kefala et al., 1999), it was observed in this study that more than eighty percent of metal uptake occurred at 20 minutes of contact time, meaning that metal uptake was mainly the results of physicochemical interaction between bacterial cell wall and metal ions, than energy dependant absorption (bioaccumulation). However lead uptake by *B bacterium* was significantly improved as a result of bioaccumulation, more than thirty percent increase of metal removal rate was recorded from eight hours to twenty-four hours.

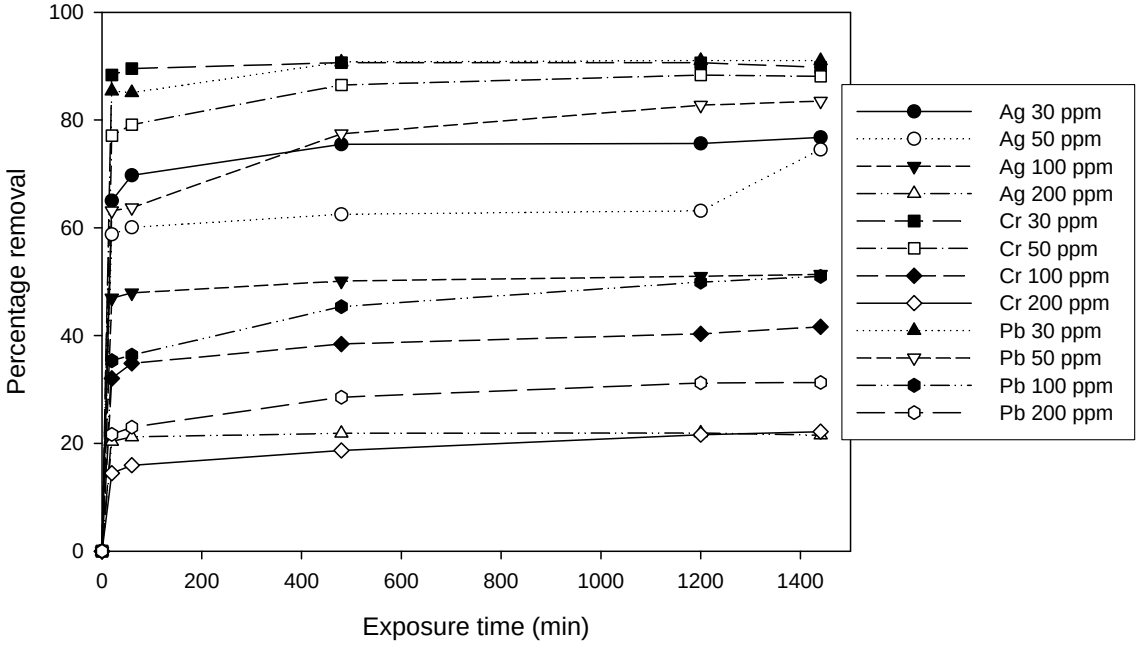

Figure 1. Removal of Ag, Cr (III) and Pb by B subtilis

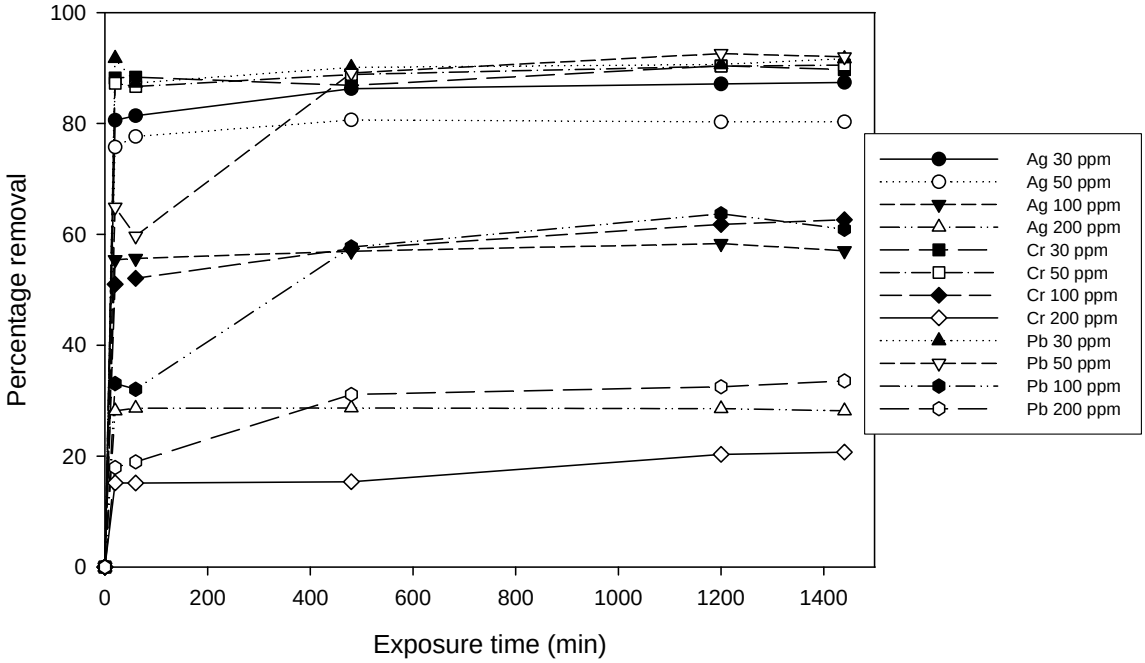

Figure 2. Removal of Ag, Cr (III) and Pb by B bacterium

Effect of PH on Metal Removal

The pH value of the solution is reported to influence the composition of the binding site on the surface of biomass as well as the chemical state of metal in solution (Exposito et al., 2002; Kiran et al., 2005). The optimization of metal uptake by *B subtilis* and *B bacterium* will therefore required the identification of suitable pH condition. The removal of metal as a function of pH keeping constant the initial concentration (50 ppm) of metal showed (Figures 3 and 4) significant variation. Removal rate increase from pH 3 to pH 5, and remain almost constant in the pH range 7 to 10, except in the case of lead where a drastic decrease of the removal rate was observed at pH 10. However maximum removal rate ("Ag-80.7% at pH 7 and Pb-94.65 at pH 5 by *B bacterium*; Cr-88.76% at pH 8 by *B subtilis*) was achieved between pH 5, 7 and 8. In principle at very low (acidic) pH value the presence of proton promote competition with metal cations for the essentially negatively charged binding sites (carboxyl, phosphate etc) on cell wall surface, while at higher (basic) pH, hydroxyl group dominate in solution and complex metal cations, preventing attachment to ligands on cell wall surface (Wang and Chen, 2006). The results found in this study corroborate with previous works, which also reported low metal cations removal rate at very low pH and maximum removal rate at neutral pH (Sar et al., 1999).

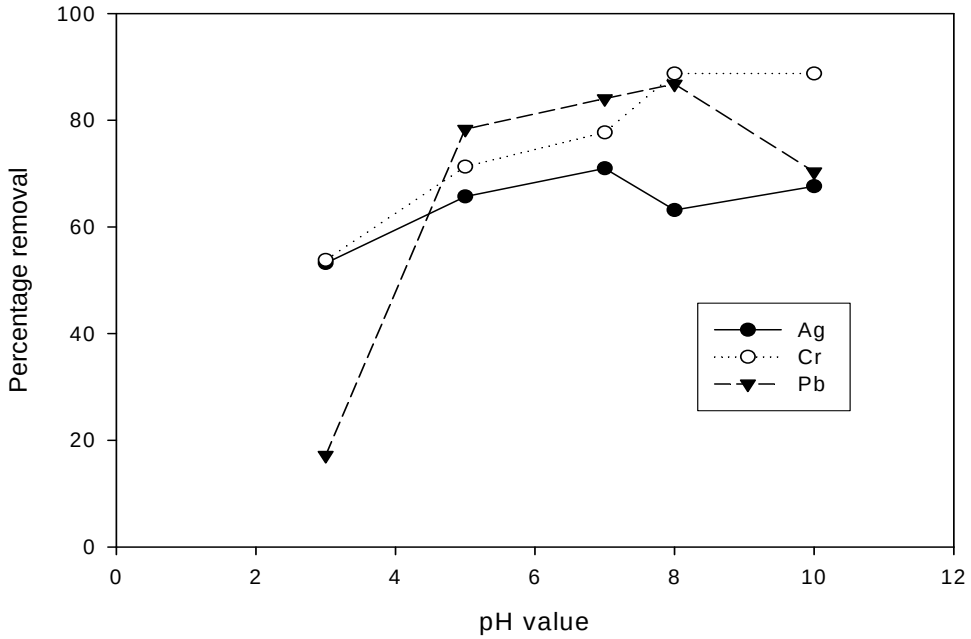

Figure 3. Metal sorption by B subtilis as a function of pH

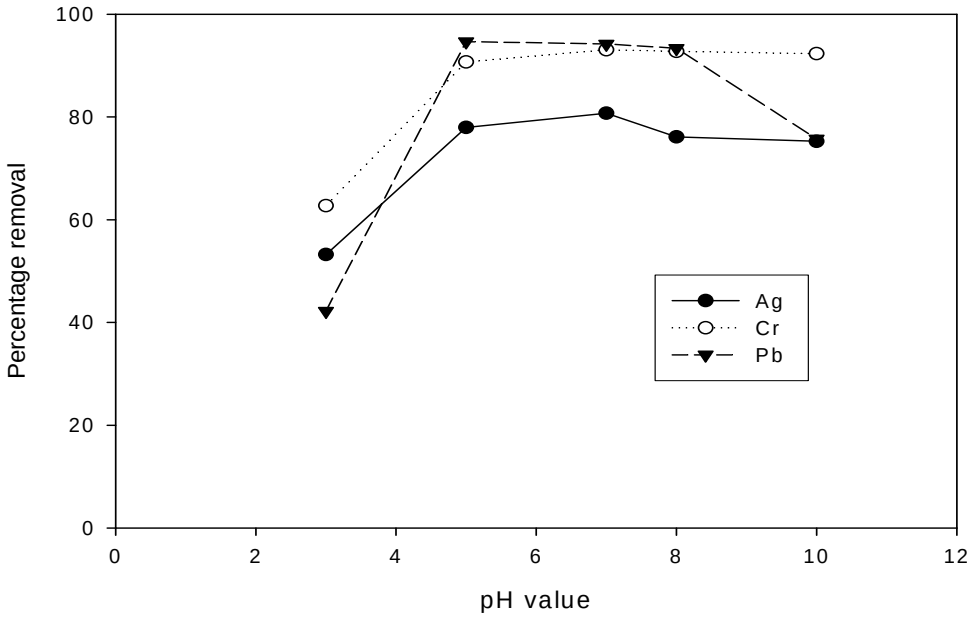

Figure 4. Metal sorption by B bacterium as a function of pH

Effect of Temperature on Metal Removal

The effect of temperature was determined in this study by comparing the removal efficiency of *B subtilis* and *B bacterium* at 37°C and 45°C keeping constant metal concentration (50 ppm) and contact time (twenty hours). It is reported that temperature could influence both the function as well as cell structure of microorganisms (Prescott et al., 2002). Figures 5 and 6 show that irrespective of the microorganism, better removal of all the metals was performed a higher temperature (45°C). The trend of this result is similar to funding by Goyal et al. (2003) for the biosorption of Cr(VI) by *S cerevisiae*. Although we recorded a positive correlation between temperature increase and metal sorption, it also appeared that the degree of variation of metal uptake differed between metals, silver removal increase the most at 45°C especially when using *B subtilis*, 20% removal improvement was achieved. These observations could be explained by the fact that high temperatures to certain extend leads to the increase of metabolic activity (Prescott et al., 2002) and energy of the system, which could promote the active uptake or attachment of metal to cell surface respectively (Goyal et al., 2003; Kefala et al., 1999).

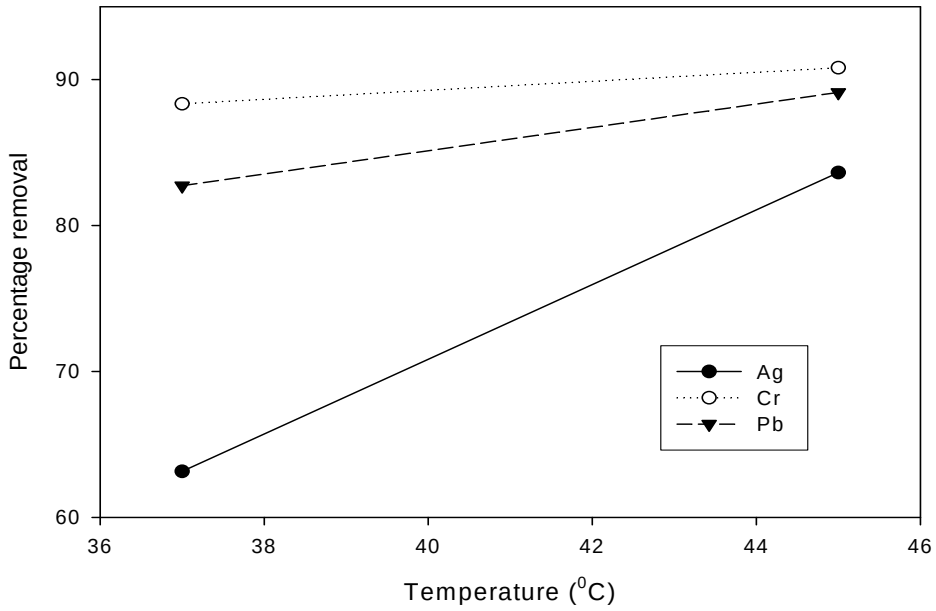

Figure 5. Comparative sorption of metal by B subtilis at 37°C and 45 oC

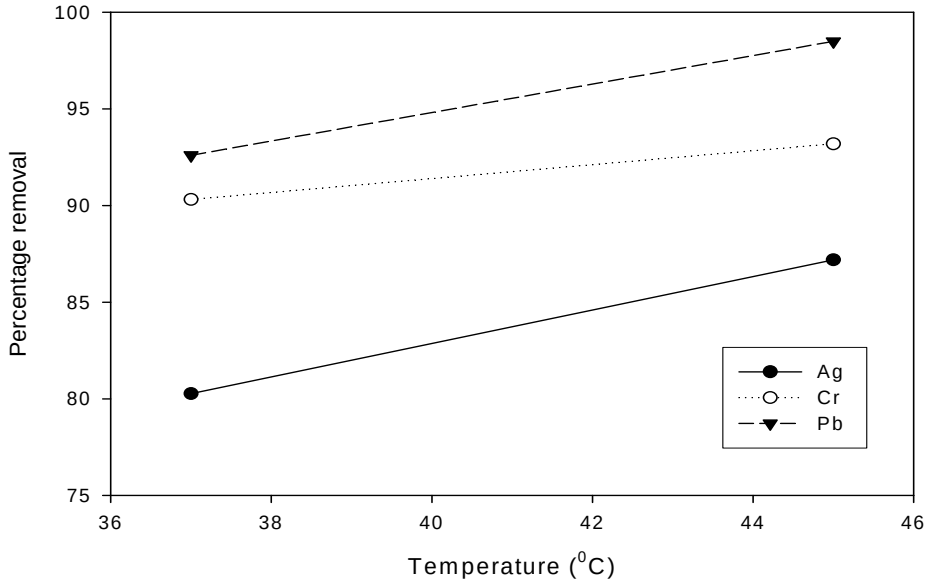

Figure 6. Comparative sorption of metal by B bacterium at 37°C and 45 oC

Sorption Efficiency at Different Bacterial Growth Stage

To determine the physiological impact on the biosorption efficiency, cultures of *B subtilis* and *B bacterium* grown to different stages (middle log phase, early and late stationary phase-ten hours, twenty hours and twenty-four hours respectively according to Alcamo, 2001) were separately used to remove silver, chromium and lead from solution. Results (Figures 7 and 8) show different pattern from *B subtilis* and *B bacterium* as maximum removal rate was reached at different growth stage, twenty and twenty-four hours respectively. It therefore ensue from the above that metal removal in the context of this study was favorable with cells collected at the stationary phase of growth as compared to those of the log phase, a trend also observed by Friis and Keith (1985). However certain authors (Sar et al., 2004) using Pseudomonas for the remediation of uranium did not find any difference of metal removal rate between cells from log and stationary phases while others working with fungi reported better removal rate with cells from the log phase (Kapoor and Viraraghavan, 1997).

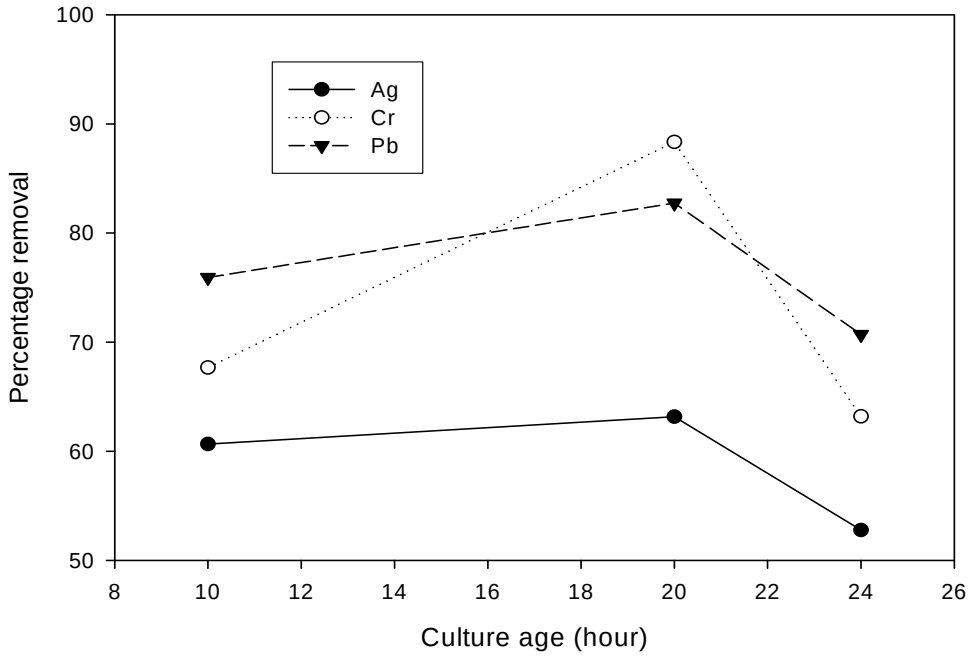

Figure 7. Metal sorption by B subtilis as a function of culture age

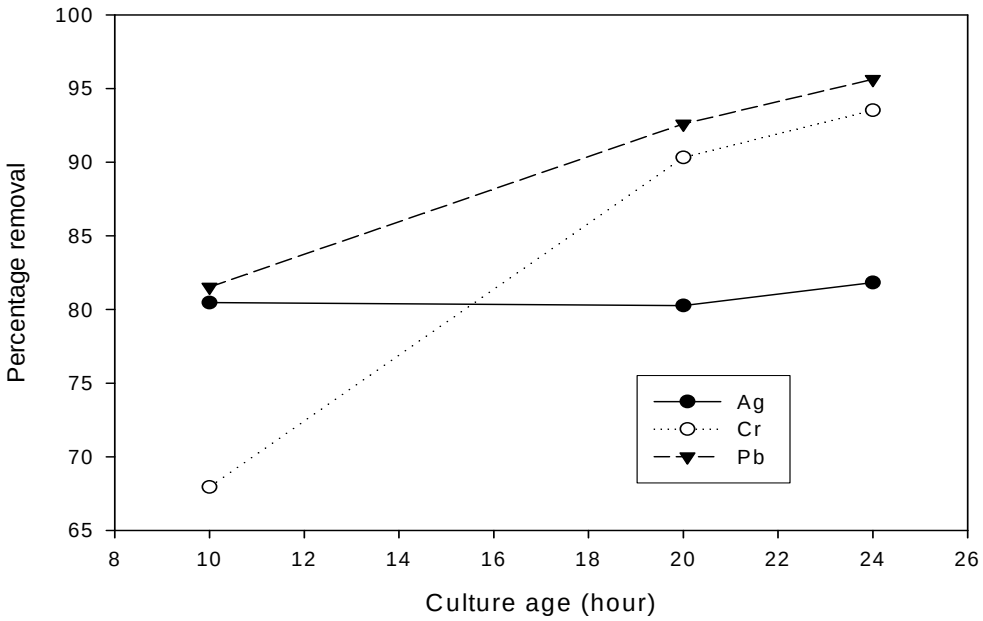

Figure 8. Metal sorption by B bacterium as a function of culture age

Metal Tolerance by Microorganisms

Heavy metals are generally toxic to microorganisms and can therefore inhibit their growth through alteration of cell membrane or blockage of metabolic activities (Nies, 1999), so doing could considerably affect the uptake of metal by microorganisms. Examination of the resistance of B subtlis and *B bacterium* to silver, chromium and lead during sorption experiment showed (Table 1) that both microorganisms' survival in aqueous solution is affected by the presence of metal, this can be interpreted in the table below as a decrease of cell numbers inversely proportional to the concentration of metal and exposure time. It was observed that silver contributed the most to the decrease of microbial cell number. *B bacterium* was most resistant to metals than *B subtilis*, and was not significantly affected by lead probably the reason why we could observe an effective metabolic removal of lead by *B bacterium* (Figure 2).

Table 1. Bacterial colonies count (CFU/ml) after exposure to metal

Microorganism	Metal concentration	Time (min)			
		20	60	480	1200
B subtilis	Ag-50 ppm	650	270	50	nd
	Ag-100 ppm	340	230	10	nd
	Ag-200 ppm	440	180	10	nd
	Cr-50 ppm	1780	1500	230	170
	Cr-100 ppm	1670	1020	190	140
	Cr-200 ppm	1540	790	100	30
	Pb-50 ppm	200 $x10^3$	31 $x10^3$	10 $x10^3$	7 $x10^3$
	Pb-100 ppm	143 $x10^3$	34 $x10^3$	4 $x10^3$	4 $x10^3$
	Pb-200 ppm	112 $x10^3$	17 $x10^3$	4 $x10^3$	4 $x10^3$
B bacterium	Ag-50 ppm	16$x10^3$	20 $x10^3$	1 $x10^3$	nd
	Ag-100 ppm	11 $x10^3$	6 $x10^3$	nd	nd
	Ag-200 ppm	4 $x10^3$	3 $x10^3$	nd	nd
	Cr-50 ppm	116 $x10^3$	107 $x10^3$	10 $x10^3$	11 $x10^3$
	Cr-100 ppm	114 $x10^3$	104 $x10^3$	43 $x10^3$	22 $x10^3$
	Cr-200 ppm	109 $x10^3$	79 $x10^3$	36 $x10^3$	26 $x10^3$
	Pb-50 ppm	247 $x10^5$	309 $x10^5$	34 $x10^5$	nd
	Pb-100 ppm	199 $x10^5$	4 $x10^5$	1 $x10^5$	nd
	Pb-200 ppm	4 $x10^5$	2 $x10^5$	1 $x10^5$	nd

nd: not detected

4. CONCLUSION

The effect of physical as well as physiological factors on metal sorption by *B subtilis* and *B bacterium* was examined in this study and results showed that pH value, temperature and culture age have considerable influence on the uptake of silver, chromium and lead by the above bacteria. Neutral pH (7 and 8) as well as higher temperature (45^{o}C) were observed to be optimal physico-chemical conditions for metal removal while the cell cultures at stationary phase were better biosorbents than cell recovered at log phase. It was observed that metal uptake occurred mainly as a result of passive mechanism rather than energetic sequestration. Specificity in bacterial-metal interaction was also noticed, as *B subtilis* preferably removed chromium while *B bacterium* was most effective in removing lead from aqueous solution. Higher concentration of metal in solution was found to inhibit bacteria over exposure time and particularly affecting metabolic uptake of metal by bacteria. The findings in this study are of major importance for effective use of the sorption capacity of *B subtilis* and *B bacterium* through optimized metal removal protocol applicable at industrial level for big scale treatment of wastewater before discharge in the environment.

5. ACKNOWLEDGEMENTS

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