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Full Length Research Paper

Influence of chemical speciation and divalent cations on cadmium toxicity during microbial biodegradation of crude oil

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Microbial metal toxicity had been attributed to metal concentration and bioavailability, the later factor being dependent on speciation; the distribution of metals into various physicochemical forms. This research was aimed at establishing a correlation between the nature of predominating ligands in natural environments which can associate with metals and influence toxicities to biodegrading microbes. Cadmium, a known inducer of oxidative stress, was chosen because of the nature of the oil-degrading microorganisms used. The organisms were chosen because of their robust accumulation of biomass in crude oil/mineral salts medium under aerobic cultivation conditions. The forms of cadmium used included acetate, chloride and sulfate at 0, 1, 10, 100, and 1000 mg cadmium/L concentrations. Duration of study was 24 h with agitation. At the end of the study, cadmium toxicity was found to positively correlate (r^2 = 0.94, P < 0.01) with chemical species. Acetate was the most toxic of the ligands tested, but not necessarily at the highest cadmium concentration. Attempts at reducing cadmium toxicity with divalent cations like zinc, magnesium and barium; cations found in the study area, revealed that only zinc and magnesium could mitigate cadmium toxicity (P < 0.05) to the bacterium. These findings underscore the unpredictability of metal toxicity in natural environments, and call for better understanding of the physiology and ecology of bioremediating microbes.

Key words: Chemical species, divalent cations, crude oil, bioremediation.

INTRODUCTION

One of the major drawbacks of bioremediation as a treatment option in oil-polluted environments is the relative slow rate at which the clean-up is achieved. Among the contributory factors to this slow rate is the concomitant presence of heavy metals in the oil-impacted areas at concentrations above trace amounts, the effect of which includes extension of acclimation periods of bioremediating organisms, reduction of the rate and extent of biodegradation and prevention of the degradation of target compounds (Said and Lewis, 1991; Kuo and Genthner, 1996). Metals are naturally occurring substances differing in their degree of availability to organisms in both aquatic and terrestrial settings. These differences in bioavailability have often not been properly addressed in the methodologies currently used, but it has become increasingly evident that it is the actual metal species that determines mobility, bioavailability and toxicity of a metal, and that metal speciation depends on the site-specific seasonal and spatial variations existing in a particular water, sediment or soil system (Sandrin and Maier, 2003; Landner and Reuther, 2004). Chemical compounds that differ in isotopic composition, conformation, oxidation or electronic state, or in the nature of their complexed or covalently bound substituents, are referred to as chemical species (Templeton et al., 2000).

Sodium chloride and sodium trioxocarbonate (IV) for instance, are both salts of the same metal sodium but do not play similar roles in biological systems. Sodium carbonate can be used to isolate intracellular membranes from cells, the mechanism which involves conversion of closed vessicles to open membrane sheets with the consequent release of peripheral membrane proteins.

Sodium chloride on the other hand maintains

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electrolyte balance amidst sundry nutritional and preservative functions (Kennelly and Rodwell, 2006). It is also an important determinant of cellular membrane potentials (Nwafor and Coakley, 2003). Worthy of note is the involvement in the determination of acidity and alkalinity of sodium chloride and sodium carbonate respectively (Atlas, 1995), which is another pointer to the role the anionic components of particular metals can play. If the common metal between these two compounds is sodium, then it follows that the relevance of one salt and the non-relevance of the other for a particular role should be a function of the anionic group attached to the metal.

In view of the above exposition, it became pertinent therefore for these authors to investigate the influence of specific ligands which were found abundantly in the study area on the toxicity of cadmium during biodegradation of crude oil by species of *Bacillus* and *Rhodotorula*. Cadmium was chosen because it is a known inducer of oxidative stress especially in aerobic organisms (Sandrin and Maier, 2003). The metal has no known essential biological function (Irwin et al., 1997). It causes behaviour, growth and physiological problems in aquatic life, microorganisms unexcepted (WHO, 1992; Irwin, 1997). Its pristine concentration is low (0.03 mg/kg) in estuaries but naturally bioaccumulates in plants, earthworms and aquatic life to concentrations that impair biological functions (Landner and Reuther, 2004; WHO, 1992). Typical polluted-sediment concentration is < 0.6mg/kg (Irwin et al., 1997). Its mean concentration in Nigerian light crude oil is 0.1 ppm (Osuji and Onojake, 2004). The organisms were chosen based on their ability to accumulate biomass efficiently in crude oil/mineral salts medium making toxicity assessment by spectrophotometric method feasible. Besides, they are well documented hydrocarbon degrading organisms (Atlas, 1981; Atlas and Cerniglia, 1995; Itah and Essien, 2005; Ekpenyong et al., 2007). Apart from these, the organisms are aerobic and biodegradation process is mostly, if not entirely, aerobic. The mono- and dioxygenase enzymes involved in hydrocarbon biodegradation are the main target sites for toxic metals particularly cadmium (Sandrin and Maier, 2003). These authors decided on a short-term labouratory study, the results of which are expected to guide the selection of very efficient bioremediating microorganisms for use in the clean-up of oil polluted areas of Qua Iboe estuary, Nigeria.

MATERIALS AND METHODS

The Nigerian light crude oil used for the study was obtained from Mobil Producing Nigeria Unlimited, Ibeno, Akwa Ibom State. The oil was filter-sterilized using Whatman 0.45 µM membrane filter, and preserved at room temperature until required.

The bacterial and yeast cultures used in the study were isolated from sediment samples obtained from Qua Iboe Estuary; a well-known oil-impacted area in the Niger Delta region of Nigeria. The organisms were isolated on mineral salts agar medium (MSM) of Zajic and Supplison (1972), supplemented with crude oil in vapour form from the lid of the plate to provide carbon and energy source (Thijsee and van der Linden, 1961). The mineral salts medium contained in g/L; K_2HPO_4, 1.8; KH_2PO_4, 1.2; NH_4Cl, 4.0; $MgSO_4.7H_2O$, 0.2; NaCl. 0.1; $FeSO_4.7H_2O$, 0.01; Agar, 15; adjusted to pH 7.2. The bacterial isolate was purified on nutrient agar (NA) plates, and the yeast on Sabouraud Dextrose Agar (SDA) plates.

Oil utilization tests were conducted using the method of Okpokwasili and Okorie (1988) on mineral salts medium of Zajic and Supplison (1972), adjusted to pH 7.0, and supplemented with 1% Nigerian light crude oil as sole source of carbon and energy. Triplicate tubes were prepared and incubated stationary at 30°C for 16 days. Good turbidity development in bacterial and yeast screen tubes after 16 days incubation selected for oil utilizers used for the study.

Efficient oil utilizing bacterium assessed spectrophotometrically was maintained on nutrient agar (NA) slants, while the yeast was maintained on Sabouraud dextrose agar (SDA) slants. The slant cultures were preserved at 4°C in a refrigerator until required for further studies.

Characterization involving cultural morphology included color, size and pigment production. Morphological characteristics included cellular morphology, cell size, Gram and spore reactions. Physiological characteristics included oxygen tolerance, pH and temperature optima and ranges. Biochemical characteristics included carbon utilization, carbohydrate oxidation or fermentation and enzyme patterns. Identification of the bacterium followed the scheme of MacFaddin (1980) and that of Barnett and Pankhurst (1974) for the yeasts.

The concentrated stock solutions of cadmium sulphate, zinc sulphate, magnesium sulphate and barium sulphate were prepared by dissolving one gram (1g) of each metal salt in 5 ml aliquot of concentrated acid mixture (3:1 v/v; H_2SO_4 and HNO_3) and then diluted to one litre with sterile deionized distilled water. The stock solutions were analyzed using atomic absorption spectrophotometer (Model UNICAM 939/959) (Asuquo et al., 2004). Cadmium concentrations of 1, 10, 100 and 1000 mg/L were then prepared by serial dilutions from concentrated stock solutions. Ten milligrams of each of the divalent cations namely zinc, magnesium and barium were similarly prepared by serial dilutions of their concentrated stock solutions.

Crude oil biodegradation studies

All crude oil biodegradation studies were conducted using 25 ml of mineral salts medium (MSM) in 100 ml Erlenmeyer flasks. The stock cultures maintained at 4°C were re-grown on fresh plates containing media used for their purification. The bacterial plates were incubated at 30°C for 24 h, while the yeast plates were incubated at 30°C for 72 h. Growth of isolates on plates were transferred onto mineral salts agar medium supplemented with Nigerian light crude oil supplied in vapour form from the lid of the plate to provide vapour phase transfer of carbon source. Bacterial plates were incubated at 30°C for 48 h while the yeast plates were incubated at 30°C for 96 h. Microbial inocula used for the biodegradation studies were prepared by transferring appreciable amount of inoculum from the MSM/Oil agar plates to MSM/Oil broth tubes, containing 0.1 ml Nigerian light crude oil in 9.9 ml MSM. The cells were allowed to grow overnight to an optical density of 0.8 (bacterium), 0.7 (yeast) at 540 nm (Spectro 22RS; Digital Spectrophotometer Lab Med Inc. England).

A 0.1 ml portion of each inoculum was used to inoculate each of the degradation study flasks, which contained different quantities of crude oil ranging from 0.1 to 0.5 ml. A control flask for each of the microbial isolates contained 9.9 ml MSM but 0.1 ml of sterile distilled water in place of crude oil. The flasks were incubated at 30°C for 24 h with an 8 hourly mechanical gentle swirling to provide

some level of aeration, increase the surface area of contact between test cultures and the oil, and to prevent possible build-up of toxic metabolites at the oil-water interface. Growth was monitored turbidimetrically at 540 nm.

Cadmium toxicity studies

The influence of cadmium on growth of bacterium and yeast on crude oil/mineral salts medium was investigated using different chemical forms of cadmium: acetate, chloride and sulphate salts, each at concentrations of 0, 1, 10, 100 and 1000 mg/l. Precisely, 0.1ml of each dilution of each cadmium salt was added to each of the degradation study flasks containing each test culture and 0.3 ml of crude oil. This concentration of crude oil has previously been found by these investigators to support optimal growth of the test isolates. Control cultures were grown under the same conditions but without any of the cadmium salts. The mineral salts medium (MSM) contained all the ingredients as listed for isolation experiments except that ammonium chloride and sodium chloride were replaced with their nitrates. The test and control cultures were prepared in triplicates. Conditions of incubation were similar to that of the crude oil degradation studies. Growth was expressed as a percentage of the final growth in the control cultures (without cadmium).

Toxicity mitigation by divalent cations

Since we observed the highest possible growth inhibition with cadmium acetate, the influence of divalent cations namely; zinc (Zn^{2+}), magnesium (Mg^{2+}) and barium (Ba^{2+}) on cadmium toxicity to the bacterial and yeast isolates during biodegradation of crude oil at 30°C for 24 h, was investigated on cadmium acetate. Zero-point-five milliliter (0.5 ml) of 10 mg/l concentration of each of the divalent cations was incorporated into each of the study flasks containing 0, 1, 10, 100 and 1000 mg cadmium/l. Conditions of incubation were as described above.

Statistical analysis

Data obtained from these studies was subjected to analysis of variance (ANOVA) and correlation analysis to determine the differences if any, in responses to the factors tested.

RESULTS

The screen test for crude oil utilization was used to select a bacterium and yeast that showed best ability as expressed by highest turbidity and optical density reading at 540 nm. Some test cultures showed very little turbidity but increased shredding of overlaid oil suggesting oil viscosity reduction by presumably biosurfactant production (Ekpenyong and Antai, 2007).

Cultural, biochemical and physiological characteristics identified the isolates as *Bacillus* sp.BSB-02 and *Rhodotorula* sp.ESY-03.

Figure 1 shows the effect of various concentrations of Nigerian light crude oil on the growth of *Bacillus* sp.BSB-02 and *Rhodotorula* sp.ESY-03 at the end of 24 h. No growth was observed in the control flasks, but there was a linear relationship between growth in test flasks and the crude oil concentration for both organisms.

The results of the effect of different chemical species of cadmium on microbial growth in liquid mineral salts medium containing 0.3 ml Nigerian light crude oil as sole carbon and energy source are shown in Figures 2a and 2b. The figures compare the responses of the test organisms to different concentrations of the different chemical species of cadmium over a 24-h period. The figures reveal clearly the many cadmium toxicity response patterns that may exist within and between the test organisms depending on the particular metal ligand and the operable concentration.

In Figures 3a and 3b, the possible interactions that exist between divalent cations (at the fixed concentration that was used) and cadmium toxicity at different concentrations are shown. The figure shows that metal toxicity reduction might involve a reordering of the entire toxicity pattern to an organism.

Correlation analysis and analysis of variance (ANOVA) data show significant ($P < 0.01$) in toxicity response of *Bacillus* sp.BSB-02 in the presence of Zinc and Magnesium. No significant ($P > 0.05$; 0.01) difference in toxicity among the three divalent cations tested was observed for *Rhodotorula* sp.ESY-03.

DISCUSSION

Figure 1 shows a linear response of *Bacillus* sp.BSB-02 and *Rhodotorula* sp.ESY-03 to increasing concentrations (0.1, 0.2, 0.3, 0.4, 0.5 ml) of Nigerian light crude oil. This result suggests that as microbial growth increases with crude oil concentration, growth measured turbidimetrically can therefore be used as an indication of biodegradation, which could also be extended to quantify heavy metal toxicity associated with biodegradation (Malakul et al., 1998). The no growth situation in the controls suggests that sterile distilled water alone could not serve as a carbon and energy source for *Bacillus* sp.BSB-02 and *Rhodotorula* sp.ESY-03 which were the test organisms in this study.

The toxicity of cadmium to the bacterial and yeast test cultures was highly dependent on the concentration of the toxic metal and on the prevailing chemical species at any particular concentration (Figures 2a and 2b). Least toxicity effect (LTE) for instance, was observed for *Bacillus* sp. at different concentrations of cadmium for the three different chemical forms: Acetate (56.8%) at 100 mg/L, chloride (70.4%) at 1000mg/L and sulphate (54.6%) at 10mg/L. This suggests that the different metal ligands have specific concentrations at which they can interact with biological membranes maximally, optimally and minimally to mediate toxicity. For instance, acetate exerted its most profound effect on the bacterium and yeast at 1000 mg cadmium/L. It is possible that acetate at 1000 mg/L 'opened the gate' (Nies, 1999), making the organisms take up cadmium ions by the fast, unspecific system.

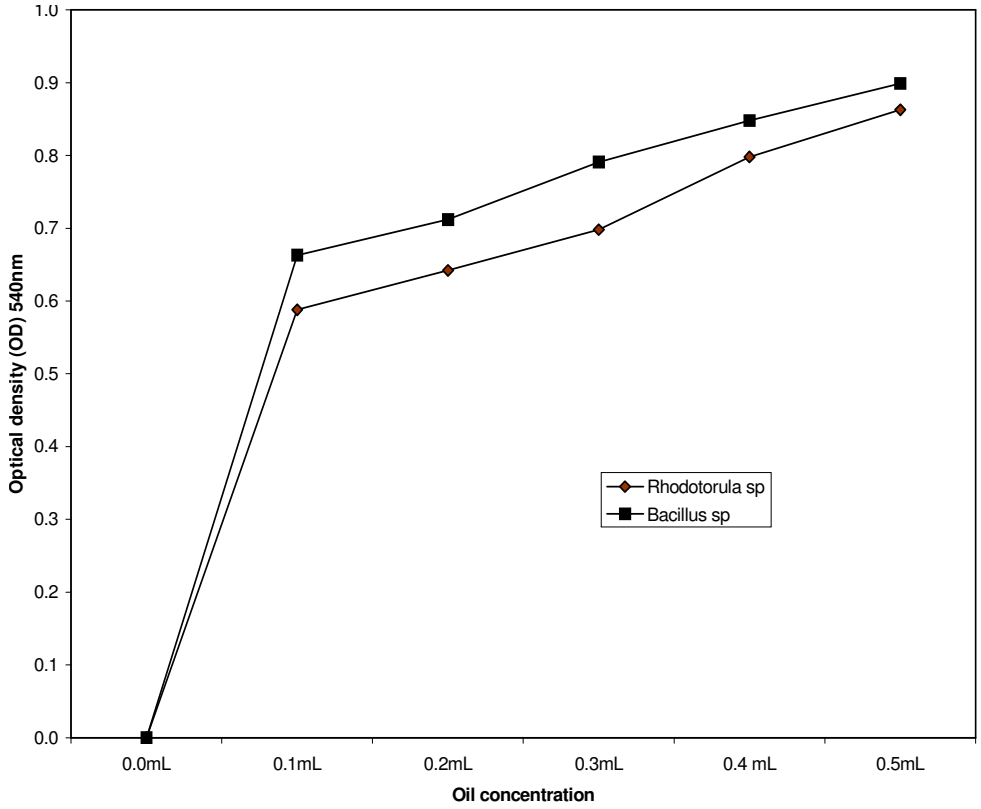

Figure 1. A 24-h growth of *Bacillus* sp. BSB-02 and *Rhodotorula* sp. ESY-03 on different concentrations of Nigerian light crude oil.

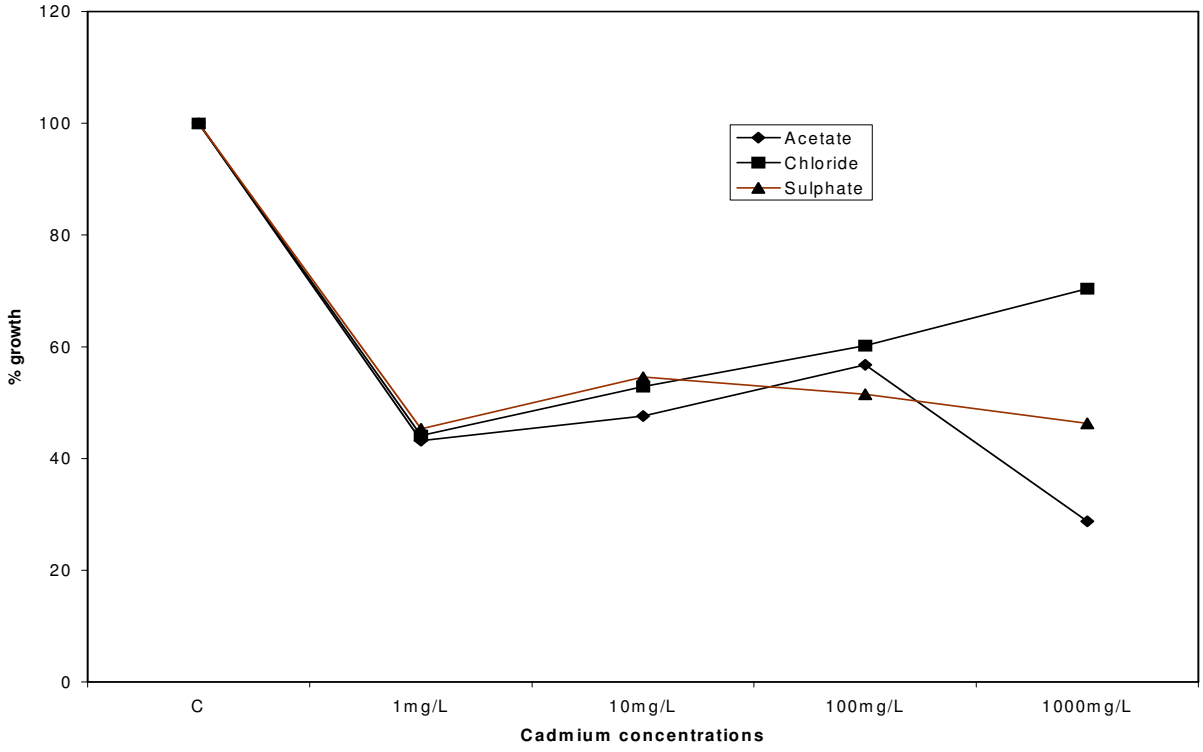

Figure 2a. A 24-h effect of concentrations of different cadmium salts on growth of *Bacillus* sp. BSB-02 (mean effects of triplicate determinations).

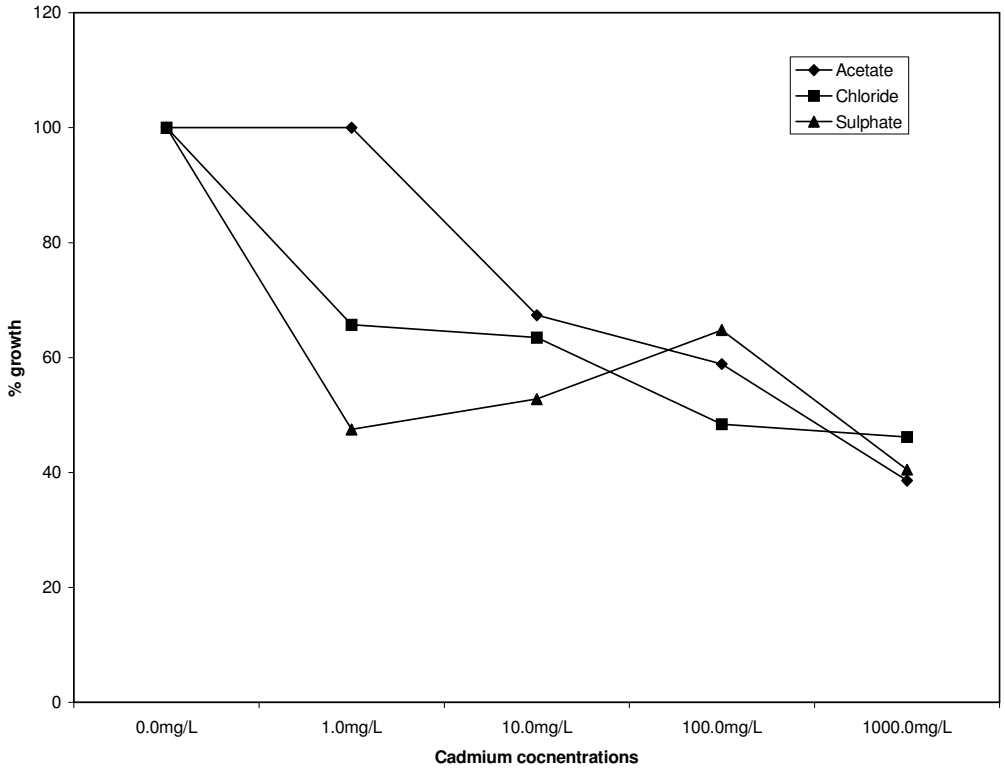

Figure 2b. A 24-h effect of concentrations of different cadmium salts on growth of *Rhodotorula* sp. ESY-03 (mean effects of triplicate determinations).

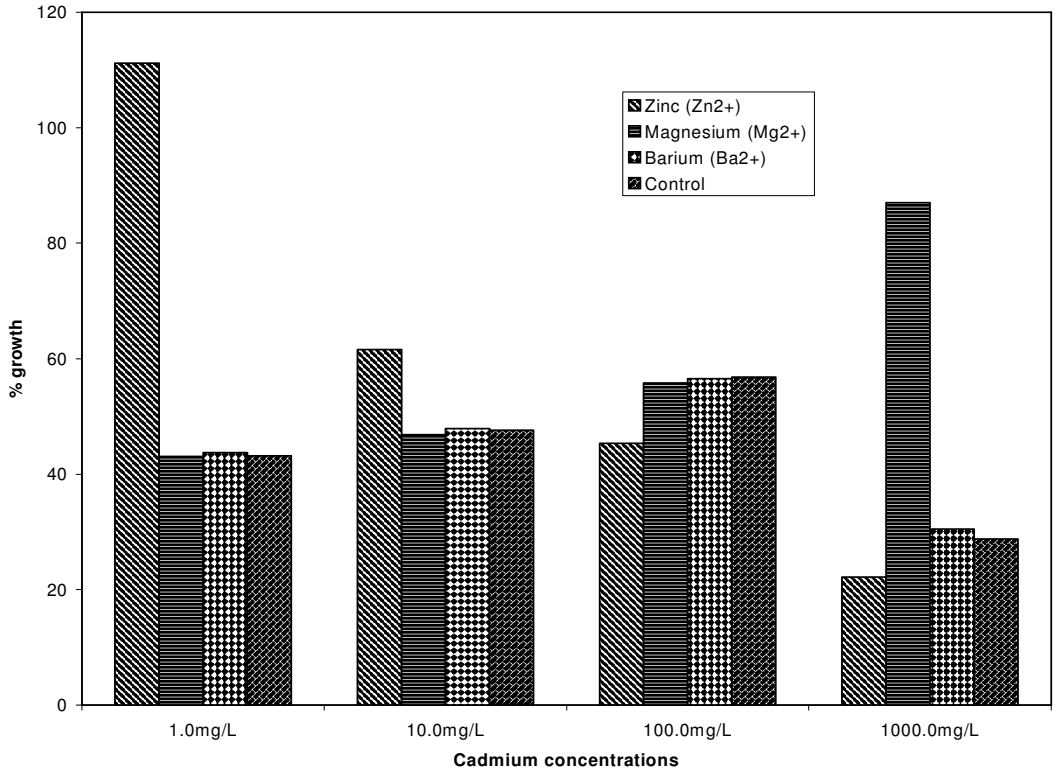

Figure 3a. A 24-h effects of divalent cations on cadmium toxicity to *Bacillus* sp. BSB-02 (mean effects of triplicate determinations).

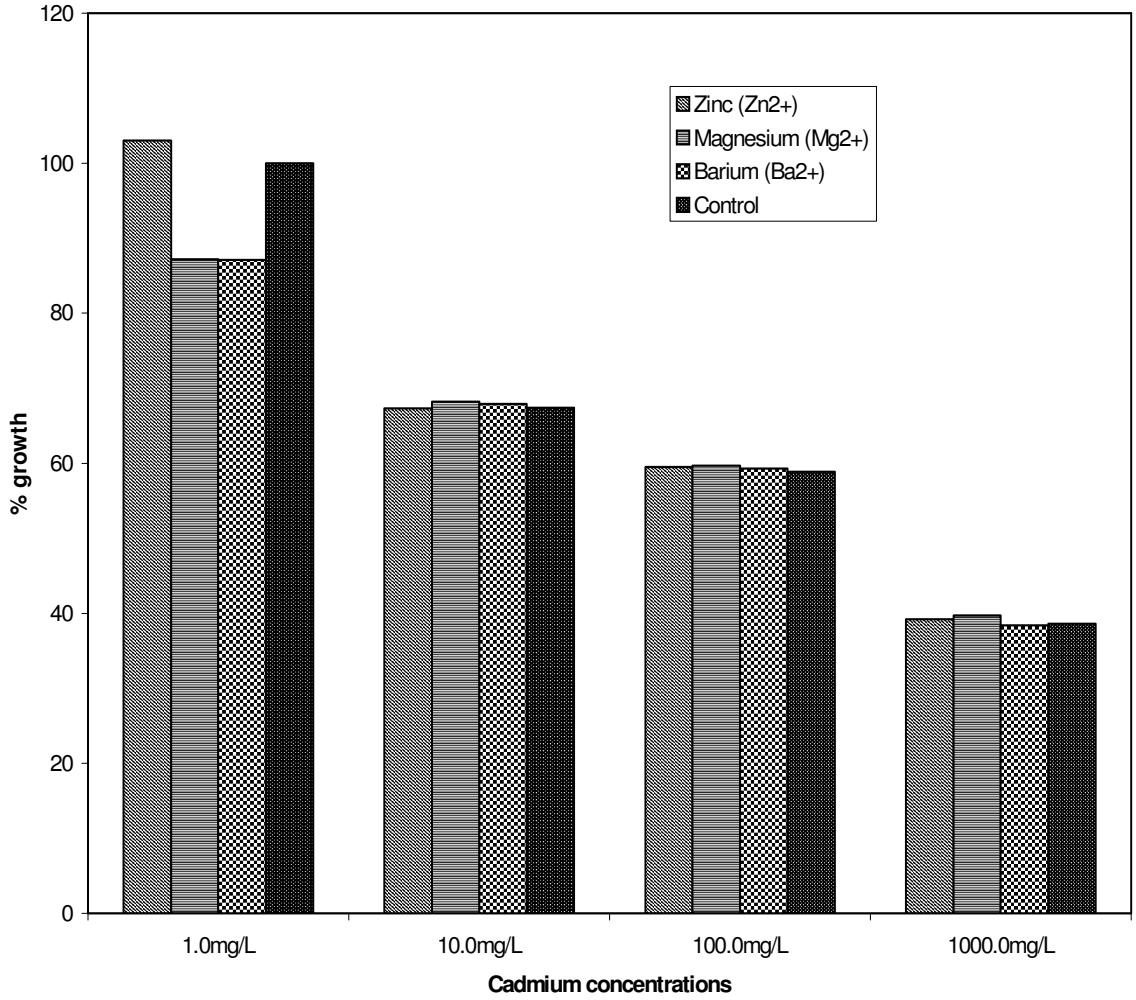

Figure 3b. A 24-h effect of divalent cations on cadmium toxicity to *Rhodotorula* sp. ESY-03 (mean effects of triplicate determinations).

The toxicity of cadmium chloride to *Bacillus* sp.BSB-02 decreased with increasing cadmium concentrations. This response will agree with the study of Laddaga and Silver (1985), who reported that the initial rate of cadmium uptake by *E. coli* K-12 was lower at a higher concentration (5.0 µM) than at a lower one (2.5 µM). Sandrin and Maier (2003) proposed that it is possible that high metal concentrations more rapidly induce a metal-resistance mechanism important in cadmium detoxification (e.g. an efflux pump) than low concentrations. Overall, significant ($P< 0.05$) differential toxicities of the cadmium species to the bacterium were observed only at 1000 mg/L cadmium concentration suggesting chemical species-specific toxicity mediation.

The lack of inhibition by cadmium acetate observed for *Rhodotorula* sp.ESY-03 at 1 mg/L is supported by Malakul et al. (1998) who observed no inhibition for Pseudomonas putida at cadmium concentrations less than 10 ppm. Still, the inhibition observed for the same organism at 1 mg/L for the chloride and sulphate forms of cadmium is supported by Laddaga and Silver (1985), who reported that growth inhibition was first noticed at cadmium concentrations between 0.1 and 10 ppm. These differences therefore strongly point to the differences in the specific metal ligands that mediate the toxicity, and of course, the particular organism in the test system. Overall significant ($P< 0.05$) difference in cadmium toxicity was observed at 1 mg/L cadmium concentration.

It is obvious from Figure 3a that zinc at the concentration tested (10 mg/L) stimulated the growth of *Bacillus* sp. BSB-02 in a medium containing 1 mg/L cadmium. This is a strange occurrence, but very possible, in that zinc is accumulated by the fast, unspecific, uptake system but cadmium is not (Nies, 1999). Therefore, it is possible that this uptake system became expressed in the presence of a very low concentration of cadmium, and zinc, a physiologically important metal ion was only taken up in preference to a metabolically toxic one. The tested divalent cations did not significantly ($P> 0.05$) influence cadmium toxicity to *Rhodotorula* sp.ESY-03 at

any of the tested concentrations. There was no significant ($P > 0.05$) difference in cadmium toxicity to *Bacillus* sp.BSB-02 at cadmium concentrations ≤ 100 mg/L in the presence or absence of magnesium ions. A significant ($P < 0.05$) difference in toxicity response was observed at cadmium concentration 1000 mg/L in the presence of 10 mg/L Mg^{2+}. Abelson and Aldous (1950) reported magnesium interference in metal toxicity to yeasts but the mechanism is still not clear. Sandrin (2000) investigated possible differences in microbial heavy metal resistance at varying concentrations of the bioavailable metal. He investigated the ability of various divalent cations to induce the activity of a highly effective metal resistance mechanism which normally is inactive at low but toxic concentrations. Formation of metallothionein complexes by organisms to bind toxic metals which threaten the organism's existence is a major mechanism by which microorganisms tolerate the presence of toxic metal concentrations. It is not just enough to consider the background concentration of a toxic metal, but to also take into consideration different controlling factors like calcium and magnesium concentrations, pH and the buffering capacity of the system (Fairbrother and McLaughlin, 2002).

Probably 1000 mg/L cadmium concentration was just the amount required by magnesium to trigger the yeast's signalling molecules leading to the proliferation of sulfur-containing amino acids that typify metallothionein compounds which are physiologically responsible for metal-binding. The net effect of all this is reduction in the overall cadmium concentration that is biologically available to the test organism for interaction with intracellular receptor sites (Irwin et al., 1997). The implications are that 100 mg/L cadmium can be more toxic to an organism than 1000 or even 10,000 mg/L, since a definite exposure concentration of the toxin might be required to trigger definite survival cadmium tolerance mechanisms. Secondly, the organisms used in this study do not and will not have this highly effective cadmium tolerance mechanism since the concentration of cadmium in Qua Iboe estuary (0.057 mg/kg) at the time of sampling was not up to the test concentration that enabled magnesium to induce the tolerance mechanism.

Conclusion

Heavy metals alter the electrochemical properties of biological systems, but the degree of such alterations depends strongly on the concentration of the chemical form of the metal available in solution to regulate binding to the biological membrane and enhancing penetration of metal into the cell (Zhang and Crow, 2001). Differences in ligand operable concentrations (LOC); concentrations at which the specific ligand exerts a definite effect; toxic or otherwise result in seeming reordering of metal toxicity pattern whereby a metal ion at an expected toxic concentration becomes metabolically useful to the bioremediating organism. A more detailed understanding of the physiology and ecology of bio-remediating microorganisms is therefore strongly recommended.

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