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ORIGINAL ARTICLE

Resistance and bioaccumulation of Cd^{2+}, Cu^{2+}, Co^{2+} and Mn^{2+} by thermophilic bacteria, *Geobacillus thermantarcticus* and *Anoxybacillus amylolyticus*

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Abstract In this study, bioaccumulation and heavy metal resistance of Cd^{2+}, Cu^{2+}, Co^{2+} and Mn^{2+} ions by thermophilic *Geobacillus thermantarcticus* and *Anoxybacillus amylolyticus* was investigated. The bacteria, in an order with respect to metal resistance from the most resistant to the most sensitive, was found to be $Mn^{2+} > Co^{2+} > Cu^{2+} > Cd^{2+}$ for both *G. thermantarcticus* and *A. amylolyticus*. It was determined that the highest metal bioaccumulation was performed by *A. amylolyticus* in Mn^{2+} (28,566 μg/g dry weight), and the lowest metal bioaccumulation was performed by *A. amylolyticus* in Co^{2+} (327.3 μg/g dry weight). The highest Cd^{2+} capacities of dried cell membrane was found to be 36.07 and 39.55 mg/g membrane for *G. thermantarticus* and *A. amylolyticus*, respectively, and the highest Cd^{2+} capacities of wet cell membrane was found to be 14.36 and 12.39 mg/g membrane for *G. thermantarcticus* and *A. amylolyticus*, respectively.

Keywords Bioaccumulation · Heavy metals · Resistance · Thermophilic bacteria

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Introduction

The increase of industrial activities has intensified environmental pollution and the deterioration of ecosystems, especially aquatic, with the accumulation of pollutants, such as heavy metals, synthetic compounds, nuclear wastes, etc. (Papageorgiou et al. 2008). Heavy metals are ubiquitous and persistent environmental pollutants that are introduced into the environment through anthropogenic activities, such as mining and smelting, as well as through other sources of industrial waste. Heavy metals contaminate drinking water reservoirs and freshwater habitats and can alter macro- and microbiological communities (Teitzel and Parsek 2003). According to the water standards used in most countries, levels of heavy metal ions in wastewater must be controlled and reduced to permissible limits (Dursun et al. 2003). Several methods are available for removing heavy metals from waste streams. Among these conventional processes for removal of metals from industrial wastewaters are chemical precipitation, oxidation-reduction, filtration, electrochemical techniques, and other sophisticated separation procedures using membranes (Green-Ruiz et al. 2008). These processes are expensive when metals are found in relatively moderate concentrations, such as 1–100 mg/L. Biological methods such as biosorption or bioaccumulation strategies for the removal of metals ions may provide an attractive alternative to existing technologies (Wuyep et al. 2007)

The microbial processes for bioremediation of toxic metals and radionuclides from waste streams employ living cells, nonliving biomass, or biopolymers as biosorbents (Volesky and Holan 1995). Microbial interaction with metallic elements is a frequent event that often leads to intracellular accumulation of these cations from their environment. Although very low

levels of several metals are essential, micro-organisms show cation uptake often at concentrations high enough to be detrimental to them (Sar et al. 2001). The application of biological processes is an appropriate concept for the solution of the environment metal contamination. Biological processes for the bioremediation are dependent on the nature of the site and the chemical environment (Gadd 2000). It has been found that both living and dead microbial cells adsorb metal ions. Microbial biomass can bind heavy metals either actively or passively or by a combination of both processes (Madrid and Camara 1997; Ansari and Malik 2007; Ozdemir et al. 2009). The active process of metal removal by living cells is referred to as bioaccumulation, and the passive sorption of the dead cell walls is called biosorption. In general, microorganisms take up toxic metal ions by two distinct mechanisms. Biosorption, a rapid phase of metal binding to the surface cell wall fraction, is followed by a slower phase of metal ion bioaccumulation into the intracellular region. Both the above principles are considered as attractive alternatives to conventional metal removal techniques (Gupta et al. 2000). Metals can be bioaccumulated by living organisms through complexation, coordination, ion exchange, chelation, and adsorption (Gupta and Keegan 1997). Heavy metal bioaccumulated biomass can be reused for heavy metal biosorption or bioaccumulation studies after desorption.

Many organisms have developed chromosomally- or extrachromosomally-controlled detoxification mechanisms to overcome the detrimental effects of heavy metals (El-Helow et al. 2000). A first-resistance mechanism involves extracellular binding whereby cells synthesize and release organic materials that chelate metals to reduce their bioavailability or the metal ions may be bound to the outer cell surface. These complex forms are generally more difficult to transport into the cell. Secondly, cells can increase the rate of metal ion excretion using energy-driven efflux pumps. A third method of resistance is through internal metal sequestration. This is one of the most important mechanisms by which bacteria combat heavy metal exposure and subsequent accumulation (Ybarra and Webb 1999)

The main objectives of the present study were to investigate, evaluate, and optimize the bioaccumulation capacity that thermophilic bacteria that has compared with the chemical functionality of ordinary bacteria (Ozdemir et al. 2009, 2012). Thermophilic microorganisms are able to grow at a wide range of temperatures (45–80 °C). Several adaptations are required for biological membranes for optimal functioning at high temperatures. In general, the phospholipid composition of bacteria changes with the growth temperature. Thus, they may possess different metal adsorption mechanisms compared to mesophilic species. In the literature, there have been insufficient studies on heavy metal resistance and bioaccumulation by the thermophilic bacteria *Geobacillus thermantarcticus* and *Anoxybacillus amylolyticus*. Metal adsorption reactions onto thermophilic microorganisms may differ quantitatively and qualitatively from the mesophilic species that have been studied to date. A wide range of geological and anthropogenic thermal environments exhibit high concentrations of dissolved metals. In response to these conditions, microorganisms isolated from these habitats may have unique cell wall structures (Hetzer et al. 2006). Thus, studies of thermophilic microorganisms can supplement our present knowledge of metal biosorption and accumulation, which is completely based on mesophilic organisms. In this study, Cd^{2+}, Cu^{2+}, Co^{2+}, and Mn^{2+} were selected in order to investigate their bioaccumulation on thermophilic bacteria due to their increasing levels in the environment as a result of anthropogenic mining activities. These elements were selected by considering their toxicity to living organisms. Special interest was focused on Cu^{2+} of which there is important pollution due to anthropogenic mining activities. Other elements are the heavy metals which are also the result of mining activity. Concentrations of elements in waste are at the level of ppm. By considering the long-term exposure of heavy metals to the environment, we selected these elements for bioremedation.

Materials and methods

Microorganisms growth conditions and preparation of the powdered dried dead cells

Geobacillus thermantarcticus and *Anoxybacillus amylolyticus* were obtained from the Istituto di Chimica Biomolecolare, CNR, Napoli/Italy. *G. thermantarcticus* was grown in 250-mL Erlenmayer flasks in a medium containing 0.6 % yeast extract and 0.3 % NaCl (Culture medium A) at pH6.0, shaking at 60 °C for 24 h, as described by Nicolaus et al. 1996. *A. amylolyticus* was cultivated in 250-mL Erlenmayer flasks containing 0.6 % yeast extract, 0.6 % NaCl, and 0.2 % starch (Culture medium B) at pH5.6, shaking at 60 °C for 24 h (Poli et al. 2006). The pH of the medium was adjusted with 0.1 M H_2SO_4.

Preparation of metal solution

The heavy metal solutions were prepared from their chloride and sulfate salts: $CdCl_2$, $CuCl_2{\cdot}2H_2O$, $CoCl_2{\cdot}6H_2O$, and $MnCl_2{\cdot}4H_2O$. Stock solutions were prepared in distilled water, slightly acidified with HNO_3 (2–3 drops of concentrated HNO_3), and were sterilized at 121 °C for 15 min. These solutions, in various concentrations according to the metal tested, were kept at 25 °C. The glassware used was leached in 3 N HNO_3 and rinsed several times with distilled water before use to avoid metal contamination.

Table 1 Operating conditions of the ICP-OES

Parameter	Value
RF power (W)	1,450
Plasma gas flow rate (L/min)	15
Auxiliary gas flow rate (L/min)	0.2
Nebulizer gas flow rate (L/min)	0.8
Sample flow rate (L/min)	1.5
View mode	Axial–Radial
Read	Peak area
Source equilibration time (s)	15
Read delay (s)	60
Replicates	3
Background correction	2-point (manual point correction)
Spray chamber	Scott type spray chamber
Nebulizer	Cross-Flow GemTip Nebulizer (HF resistant)
Detector	CCD
Purge gas	Nitrogen
Shear gas	Air
Gas	Argon
Analytical wavelengths (nm)	Cd 228.802
	Cu 327.393
	Co 228.616
	Mn 57.610

Determination of minimum inhibitory concentrations (MIC) of heavy metals

The metal-tolerance pattern of each bacterial strain was determined by the minimum inhibitory concentration (MIC) approach. Solutions of the metal salts were added to the culture media A and B agar plates in various concentrations, which were then spot inoculated with approximately 3×10^6 for each organism. The plates were incubated at 60 °C for both *G. thermantarcticus* and *A. amylolyticus* for 72 h. The lowest concentration of the metal which inhibited the bacterial growth was considered as the MIC of the metal against the strain tested (Hetzer et al. 2006).

Effect of metal ion concentration on growth

To assess the effect of the metal ion concentration on cell growth, microorganisms were inoculated into 100 mL of the medium containing the metal ions at different concentrations. A culture grown in the absence of the metal served as the control. Suspensions of growing cells were incubated as batch cultures for 24 h. Growth of the bacteria was monitored periodically (0, 4, 8, 12, 16, 20 and 24 h) by measuring the OD at 540 nm. The effect of metal ion concentration on the growth of the bacteria was carried out by inoculating 100 mL of the medium in a 250-mL flask with 2 mL of a day-old culture (Yılmaz 2003).

Effect of metal ion concentration on bioaccumulation

Microorganisms were grown in 100 mL of bioaccumulation medium including various concentration of metals in 250-mL Erlenmayer flasks by shaking at 60 °C for both *G. thermantarcticus* and *A. amylolyticus* for 24 h. Interval samples of cultures (10 mL) were centrifuged at 10 min at 10,000 rpm. Supernatant and pellets were dried overnight at 80 °C and then the pellets were weighed. Supernatant and pellets (after acid digestion by nitric acid:perchloric acid, 5:3) were separetely used to estimate the bioaccumulated metal concentration by using ICP-OES (Optima 2100 DV; Perkin Elmer) (Yılmaz 2003). The operating conditions of the ICP-OES are given in Table 1. Uptake values were calculated as the difference between the initial metal ion concentration and the one in the sample. All the experiments were carried out at least twice.

Determination of the cell membrane's metal biosorption capacity of wet and dried powdered cells of *Geobacillus thermantarcticus* and *Anoxybacillus amylolyticus* which were exposed to different Cd concentrations

The microorganisms were grown in 100 mL of growth medium for 24 h in 250-mL Erlenmeyer flasks by shaking at 60 °C to determine the cell membrane's biosorption capacity of *G. thermantarcticus* and *A. amylolyticus*. Following bacterial growth, the samples were centrifuged (Sigma Christ 2K15) at 10,000 rpm for 10 min, then the pellets were washed twice with 0.9 % NaCl and dried in an oven at 80 °C for 24 h. To obtain a fine powder, dried cells were ground in a porcelain mortar, then were autoclaved at 121 °C for 15 min to assess the complete death of the dried cells. The cell membrane's metal biosorption capacity of wet and dried powdered cells of *G. thermantarcticus* and *A. amylolyticus* were determined accordinng to method of Ozdemir et al. (2012) and Hsieh et al. (2007).

Results and discussion

Minimum inhibitory concentrations (MIC) of heavy metals

The study of minimum inhibitory concentration obtained after 72 h incubation is shown in Table 2. When the minimum inhibition concentration (MIC) during 72 h of incubation was compared to the experiment results for these two bacteria, it was determined that: the most resistant strain was *A. amylolyticus* 0.574 mM; the most sensitive strain was *G. thermantarcticus* 0.41 mM for Cd^{2+}; the most resistant strain was

Table 2 Minimum inhibitory concentrations (MIC) of heavy metals

Metal	*Geobacillus thermantarcticus* MIC (mM)	*Anoxybacillus amylolyticus* MIC (mM)
Cd^{+2}	0.41	0.574
Co^{+2}	4.1	1.435
Cu^{+2}	2.05	0.616
Mn^{+2}	22.02	22.02

G. thermantarcticus 4.1 mM; the most sensitive strain was *A. amylolyticus* 1.435 mM for Co^{2+}; the most resistant strain was *G. thermantarcticus* 2.05 mM; and the most sensitive strain was *A amylolyticus* 0.616 mM for Cu^{2+}. The MIC values of Cd^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+} were found to be 0.41, 4.1, 2.05, and 22.02 mM for *G. thermantarcticus*, and 0.574, 1.435, 0.616, and 22.02 mM for *A. amylolyticus*, respectively. The bacteria in an order with respect to metal resistance from the most resistant to the most sensitive were found to be $Mn^{2+} > Co^{2+} > Cu^{2+} > Cd^{2+}$ for both *G. thermantarcticus* and *A. amylolyticus*. In this study, it was determined that the most toxic metal was Cd^{2+} and the least toxic metal was Mn^{2+} for these thermophilic bacteria. Hassen et al. (1998a), in their study, determined that the MIC values of *Pseudomonas aeruginosa*, *Bacillus thuringiensis*, and *Staphylococcus aureus* were: 1.2, 0.4, 1.5, and 1.5; 0.5, 0.05, 1.2, and 0.5; and 0.2, 0.2, 0.1, and 0.2 mM for Cu^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+}, respectively. In addition to this, it can be seen that the results are compatible if the MIC values obtained in this study are compared with both the MIC values for Cd^{2+} by thermophilic bacteria conducted by Hetzer et al. (2006) and the MIC values by mesophilic bacteria conducted by other researchers.

Effect of metal ion concentration on growth

The effect of different metal concentrations (Cd^{2+}, Cu^{2+}, Co^{2+}, and Mn^{2+}, respectively) on the growth and bioaccumulation capacity of *G. thermantarcticus* is shown in Fig. 1a–d. The growth was not significantly affected at a concentration of 0.732 mg/L Cd^{2+}, and it was observed that microbial growth was inhibited by 46 % in the presence of 4.575 mg/L Cd^{2+} at 12 h (Fig. 1a). As presented in Fig. 2b, the growth were partially affected in the presence of 6.784 and 16.96 mg/L Cu^{2+} in the first 16 h when compared with the control. At a concentration of 42.4 mg/L Cu^{2+}, the growth of *G. thermantarcticus* also showed a longer lag phase than the control. As seen from Fig. 1c, the growth was not significantly affected in the presence of 9.512 mg/L Co^{2+}, but in the presence of 59.45 mg/L Co^{2+}, growth were inhibited by 47 % at 8 h and there was almost no effect at 16–24 h. As presented in Fig. 2d, the growth was not affected in the presence of 9.89 and 19.78 mg/L Mn^{2+}, and was partially increased compared with the control between 16 and 24 h.

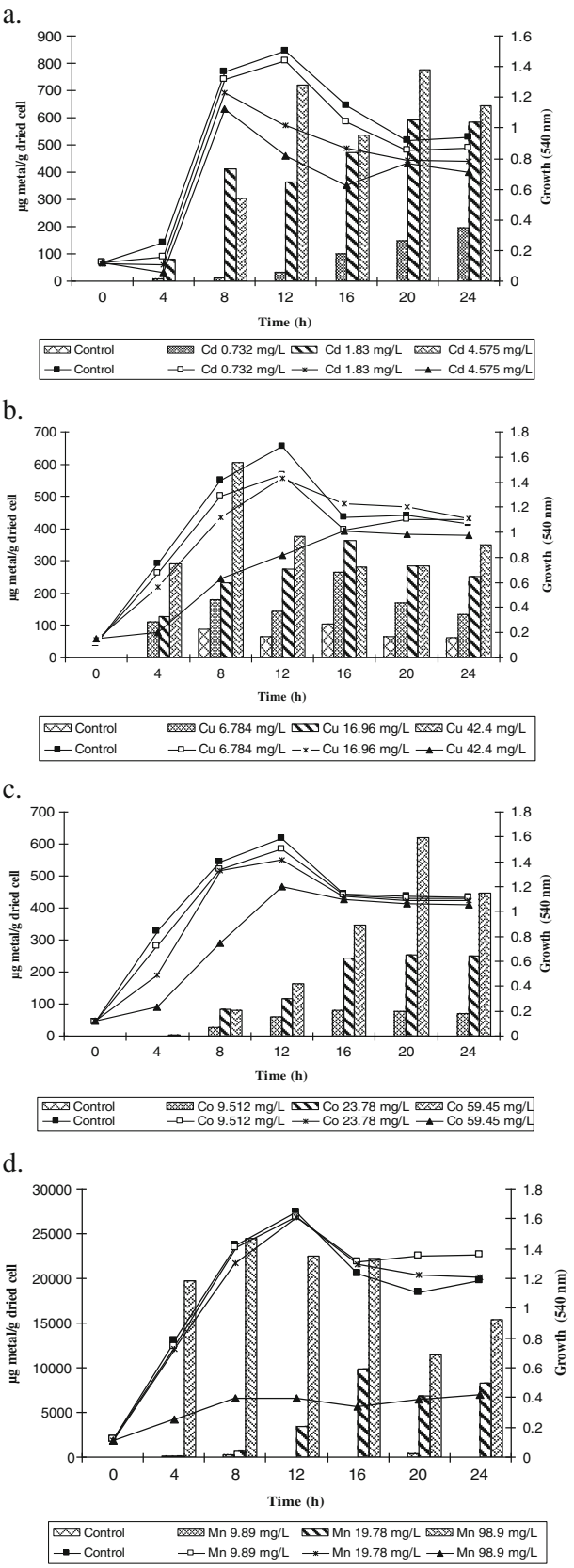

Fig. 1 Effect of metal ion concentration on growth and bioaccumulation by *Geobacillus thermantarcticus*: Cd (**a**), Cu (**b**), Co (**c**), and Mn (**d**). *Lines* bacterial growth, and *bars* metal bioaccumulation

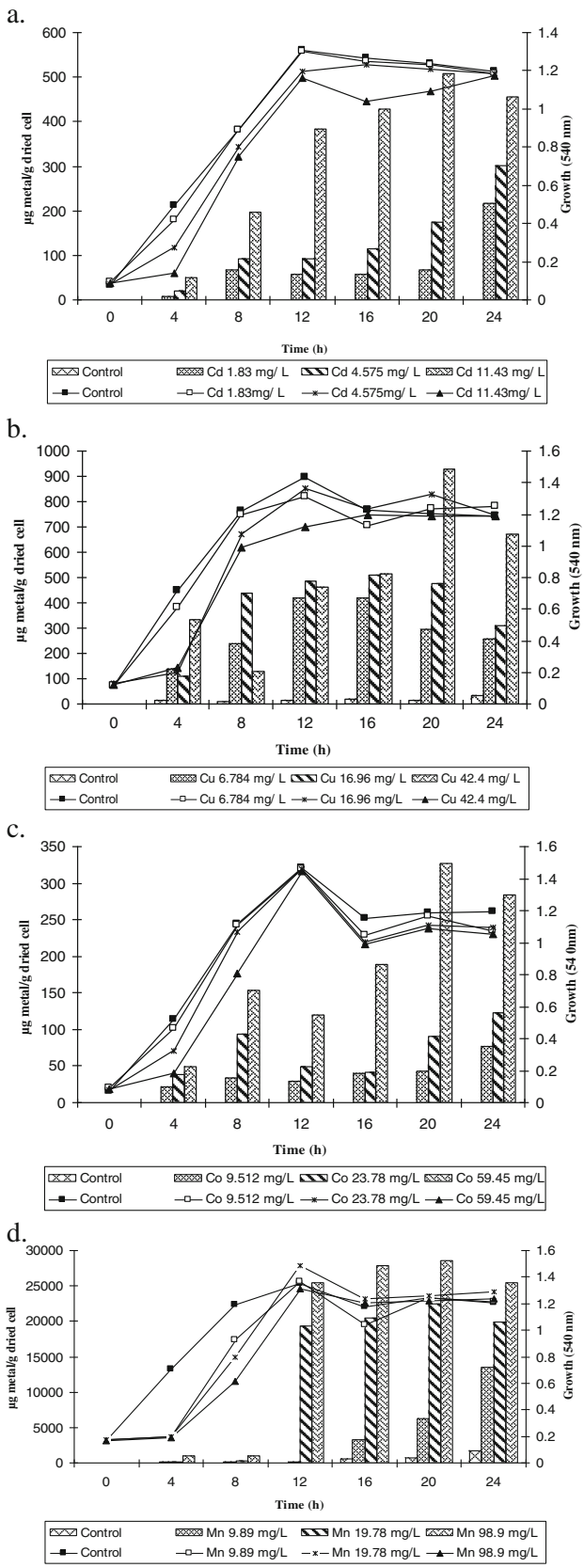

Fig. 2 Effect of metal ion concentration on growth and bioaccumulation by *Anoxybacillus amylolyticus*: Cd (**a**), Cu (**b**), Co (**c**), and Mn (**d**). *Lines* bacterial growth, *bars* metal bioaccumulation

The effect of different metal concentrations (Cd^{2+}, Cu^{2+}, Co^{2+}, and Mn^{2+}, respectively) on the growth and bioaccumulation capacity of *Anoxybacillus amylolyticus* is shown in Fig. 2a–d. Bacterial growth was not affected in the presence of 1.83 mg/L Cd^{2+} and the growth was partially affected in the presence of 4.575 mg/L Cd^{2+}. In the presence of 11.43 mg/L Cd^{2+}, the growth was inhibited by 16 % at 16 h (Fig. 2a). As seen in Fig. 2b, the bacterial growth was not significantly affected in the presence of 6.784 mg/L Cu^{2+}. The growth showed a much longer lag phase when compared with the control in the presence of 16.96 mg/L Cu^{2+}, however, and it was determined that the growth in this metal concentration between 12 and 24 h had a value near that of the control. In the presence of 42.4 mg/L Cu^{2+}, the growth of *A. amylolyticus* was greatly inhibited in the first 12 h when compared with the control. It was determined that reproduction between 16 and 24 h had a value near that of the control. The growth of *A. amylolyticus* was partially affected in the presence of 9.512 and 23.78 mg/L Co^{2+}. At a concentration of 59.45 mg/L Co^{2+}, bacterial growth was inhibited by 27 % at 8 h (Fig. 2c). At concentrations of 9.89, 19.78, and 98.9 mg/L Mn^{2+}, the growth medium caused an increase in the lag period. When compared with the control in the presence of 19.78 mg/L Mn^{2+} between 12 and 24 h, microbial growth was slightly increased (Fig. 2d).

When the effect of different metal concentrations on bacterial growth after 24 h of incubation in the liquid medium was compared with the solid medium MIC values, it was found that bacteria in liquid medium was more sensitive. These results are also in agreement with the previous reports of Hassen et al. (1998b) and Yılmaz (2003). This situation gives rise to the thought that complexation by diffusion of the metals is different from solid media (Yılmaz 2003), and the solubility of the metals in liquid media is higher, and the bacteria have more interactions with the metals.

Effect of metal ion concentration on bioaccumulation

As presented in Fig. 1 (a–d), the highest bioaccumulation capacity performed during 24 h incubation by *G. thermantarcticus* for Cd^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+} was found to be 774.8 (20 h), 620.1 (20 h), 604.05 (8 h), and 24,503.07 (8 h) µg/g dry weight, respectively. According to these results, it was determined that the highest metal capacity which was bioaccumulated by *G. thermantarcticus* was Mn, while the lowest was Cu^{2+}. It was determined that the highest bioaccumulation capacity performed during 24 h incubation by *A. amylolyticus* for Cd^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+} was 507.39 (20 h), 327.3 (20 h), 929.68 (20 h), and 28,566 (20 h) µg/g dry weight, respectively (Fig. 2–d). As a result, it was determined that the highest bioaccumulation capacity was

by Mn^{2+} and lowest bioaccumulation capacity was by Co^{2+} during 24 h incubation by *A. amylolyticus*.

Hassen et al. (1998a), in their study, determined that the bioaccumulation capacity for Cd^{2+} and Cu^{2+} was 6 and 1.8 μg/mg dry weight, respectively, after 36 h incubation by *Pseudomonas aeruginosa*. Dönmez and Aksu (1999), investigated the possible use of growing yeast for bioaccumulating of Cu^{2+} ions and found the maximum binding capacity values as 1.27 mg/g for *Schizosaccharomyces pombe*. In another study, Cu^{2+} had the leading capacity (1.91 mg/g) reported by using *Saccharomyces cerevisiae* (Huang et al. 1990). Kapoor et al. (1999) determined the bioaccumulation capacity for Cd^{2+} using *Aspergillus niger*. They found the maximum binding capacity values to be 1.31 mg/g for live *A. niger*. It is obvious that the maximum uptake values of Cd^{2+} and Cu^{2+} found in this work are comparable to these values found in the literature.

It is understood from the experimental studies that the metal biosorption mechanism is determined through the cell type and the main compounds of the microorganisms that were applied. What was determined from this study is that there was variation in the different periods of the growth phases of metal bioaccumulation capacity, which was shown by micobial cells. Accordingly, the bioaccumulation capacity of Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} by *Bacillus circulans* strain EB1 also showed variation in different periods of growth phases (Yılmaz 2003). Furthermore, generally, it was at the end of the stationary phase (20 h) that the maximum capacity occurred. In the 24-h growth process, by the variation of metal bioaccumulation by bacteria, we are supposed to think that an active mechanism controls the absorption, and, as the cells have a live and active metabolism, there is a role of resistance together with absorption in the metal absorption. In addition, when these studied bacteria cells interact with metals, their cells may be made less permeable against metal ions by leading some conformational alterations in the cytoplasmic membrane, or through recogniton mechanisms developed by them. By seperating those harmful divalent cations from the ones that are necessary for the cell (Mn^{+2}, Ca^{+2}, Mg^{+2}), they may prevent the entrance of harmful metals into the cell by penetration through these canals (Kondo et al. 1974). Within the study of bioaccumulation, we discovered that Mn^{2+} had the highest bioaccumulation level among Mn^{2+}, Cd^{2+}, Co^{2+}, and Cu^{2+}. Moreover, the low bioaccumulation of Cd^{2+}, Co^{2+}, and Cu^{2+} brings possible efflux systems to mind.

Determination of the cell membrane's metal biosorption capacity of wet and dried powdered cells of *Geobacillus thermantarcticus* and *Anoxybacillus amylolyticus*

Cd^{2+} capacities of wet and dried powdered cells of *G. thermantarcticus* and *A. amylolyticus* can be seen in Table 3. It was observed that, when the metal concentration was increased, the mg metal accumulated in both cell membranes increased. In addition to these, for *G. thermantarcticus* and *A. amylolyticus*, dried cell membrane uptake capacities were higher than for wet cell membranes. The maximum metal uptake capacities of dried cell membranes was found to be 36.07 and 39.55 mg/g membrane for *G. thermantarcticus* and *A. amylolyticus*, respectively, and the maximum metal uptake capacities of wet cell membrane was found to be 14.36 and 12.39 mg/g membrane for *G. thermantarcticus* and *A. amylolyticus*, respectively. It was observed that dried cell membrane biosorption capacity was much more than for wet cell membranes for *G. thermantarcticus* and *A. amylolyticus*. This maybe because the dead cells work as an ion exchange resin composed of a network of cell membranes (Sag et al. 2003), and it is well known that living cells are sensitive to high toxicant concentrations, when uptake is usually low. In addition to these, the resistance and active metabolism play a role together against the harmful effects of heavy metals on bacteria cells (Vijayaraghavan and Yun 2008).

Conclusions

In this study, the effects of Cd^{2+}, Cu^{2+}, Co^{2+}, and Mn^{2+} on the growth and bioaccumulation of thermophilic bacteria *Geobacillus thermantarcticus* and *Anoxybacillus amylolyticus* were studied. Maximum metal ion uptake capacities were obtained for both microorganisms at the end of the exponential growth phase. It was determined that the highest bioaccumulation capacity performed during 24 h incubation by *G.*

Table 3 Cd^{+2} biosorption capacity of wet and dried cell membranes in *Geobacillus thermantarcticus* and *Anoxybacillus amylolyticus*

Membran Type	*Anoxybacillus amylolyticus*			*Geobacillus thermantarcticus*		
	Cd^{+2} Concentration (mg/L)			Cd^{+2} Concentration (mg/L)		
	0.732	1.83	4.575	0.732	1.83	4.575
Wet Cell Membran (mg/g membran)	1.91	3.25	12.39	2.35	5.75	14.36
Dried Cell Membran (mg/g membran)	2.49	4.02	36.07	3.47	7.46	39.56

thermantarcticus and *A. amylolyticus* for Mn was 24,503.07 (8 h) and 28,566 (20 h) μg/g dry bacteria, respectively. It was concluded that *G. thermantarcticus* and *A. amylolyticus* could be used for the removal of Mn^{2+} ions. The present study is the second report on the effects on the metal bioaccumulation capacity of thermophilic bacteria. Further studies are needed on the metal bioaccumulation in thermophilic bacteria to evaluate the use of these organisms in metal removal.

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