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ORIGINAL PAPER

Factors affecting immobilization of heavy metals by purple nonsulfur bacteria isolated from contaminated shrimp ponds

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Abstract In order to remove heavy metals (HMs) from contaminated shrimp pond at the highest concentrations found of; 0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}, two strains of purple nonsulfur bacteria isolated from shrimp ponds (NW16 and KMS24) were investigated for their ability to immobilize HMs in 3% NaCl in both microaerobic-light and aerobic-dark conditions. Based on metabolic inhibition and metabolic-dependent studies, it was concluded that both strains removed HMs using biosorption and also bioaccumulation. The efficiency of removal by both strains with both incubating conditions tested was in the order of lead (Pb) > copper (Cu) > zinc (Zn) > cadmium (Cd). Optimal conditions for removal of HMs by strain NW16 were; cells in the log phase at 4.5 mg DCW/ml, pH 6.0, and 30°C for 30 min. With microaerobic-light conditions, the relative percent removal of HMs was: Pb, 83; Cu, 59; Zn, 39; Cd, 23 and slightly more with the aerobic-dark conditions (Pb, 90; Cu, 69; Zn, 46; Cd, 28). Cells in the log phase at 5.0 mg DCW/ml, pH 5.5, and 35°C for 45 min were optimal conditions for strain KMS24 and there were no significant differences for the removal percentages of HMs with either incubating conditions (averages: Pb, 96; Cu, 75; Zn, 46; Cd, 30). The presence of Ca^{2+} and Mg^{2+} significantly decreased the removal capacity of HMs for both strains.

Keywords Immobilization · Heavy metals · Photosynthetic bacteria · Shrimp ponds

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Introduction

Thailand is recognized as one of top ten aquaculture producers in the world (FAO 2007). Contamination of seafood by heavy metals (HMs) such as lead (Pb), cadmium (Cd), copper (Cu) and zinc (Zn) has become a barrier for export due to the well documented health hazards associated with ingestion of HMs (Petroczi and Naughton 2009). For example, chronic exposure to HMs like Cu, Pb and Zn has been reported to be associated with Parkinson's disease (Gorell et al. 1997) and these are present in shrimp farm cultivation water that is normally derived from the coastal environment (Cheevaporn and Menasveta 2003; Pradit et al. 2009). In order to ensure that cultivated shrimp are safe, it has been recognized for a long time that removal of HMs from shrimp ponds particularly Cd and Pb that have been accumulating within the aquatic food chain is essential (Mdegela et al. 2009; Mokhtar et al. 2009; Yap et al. 2004).

Traditional methods for removal of HMs e.g., chemical precipitation, ion exchange, and reverse osmosis have some significant disadvantages e.g., a high cost of treatment, low efficiency at low concentrations of HMs and the remnants of some toxic substances etc. (Lloyd and Lovley 2001). An alternative method, like bioremediation, that uses microbes has many advantages such as high efficiency, low cost, easy

to operate, and environmentally friendly. Furthermore, perhaps the most important benefit is that the residual soils, sediments, water, or sludge after removal by microbial actions can be reused (Gazso 2001; Lloyd and Lovley 2001). Immobilization of HMs by microbes occurs by precipitation, biosorption and bioaccumulation (Gazso 2001). These processes have been considered to remove HMs from water as part of the microbial biomass as not only living cells but also dead cells can bind HMs either actively, or passively or by a combination of both processes (Al-Momani et al. 2007; Bai et al. 2008; Gavrilescu 2004). Biosorption of metal ions on the cell surface is based on non-enzymatic processes such as adsorption while bioaccumulation involves the intracellular uptake of metal ions and requires an energy dependent transport system (Gazso 2001; Pardo et al. 2003). The capacity of any biosorbent is mainly influenced by the biomass characteristics, physico-chemical properties of the target metals, and the micro-environmental factors of the contact solution including pH, temperature, and interaction with other ions (Chan and Wang 2007).

Microorganisms living in a contaminated environment are often well adapted themselves to survive in the presence of existing contaminants by evolving mechanisms to tolerate the presence of HMs such as by using an efflux of metal ions, or by accumulating and complexing metal ions inside the cell (Gazso 2001; Nies 1999). Thus, bacteria that can stay alive in the environment of HMs contamination should be isolated and used as inoculants for bioremediation. Many research workers have studied removal of HMs by purple nonsulfur bacteria (PNB) (Giotta et al. 2006; Seki et al. 1998; Smiejan et al. 2003; Watanabe et al. 2003) mainly because PNB are normally used to treat many kinds of wastewater (Kantachote et al. 2005; Nagadomi et al. 2000). The unique advantages of PNB are that they can use solar radiation as an energy source under anaerobic-light conditions and organic matter as sources of energy and carbon under aerobic-dark conditions (Imhoff and Truper 1989). Therefore, the PNB isolated from various HMs contaminated shrimp ponds may be good candidates to remove HMs by immobilization. Hence, the aims of this work were to investigate the potential of PNB strains isolated from contaminated shrimp ponds to immobilize HMs and also the ability of other factors to have an effect on their immobilization.

Materials and methods

Preparation of cell pellets of PNB for immobilization of HMs

Two PNB strains *Rhodobium marinum* NW16 and *Rhodobacter sphaeroides* KMS24, used in this study were isolated in our laboratory from water and sediment samples collected from HMs contaminated shrimp ponds. Our previous work showed that both strains grew well in GM medium (Lascelles 1956) and were resistant to HMs at the highest concentrations that were found in the sediments from the shrimp ponds (0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}). A 10% inoculum from a growing culture (NW16 and KMS24) was added to GM medium and incubated with their optimal growth conditions under microaerobic-light and also aerobic-dark conditions. Conditions included an initial medium pH of 7; shaking speed, 150 rpm; incubating temperature, 30°C and their optimal light intensity was 3,000 lux. Cells grown with microaerobic-light and aerobic-dark conditions were used to test for immobilization of HMs when incubated with microaerobic-light and aerobic-dark conditions, respectively. These experiments were designed because both PNB strains will be expected to treat water in shrimp ponds, after harvesting, where both sets of conditions can prevail. Cells in the log phase (48 h) unless otherwise stated were harvested by centrifugation at 8,000 rpm for 15 min and were washed twice with sterile 0.1% peptone water to obtain cell pellets for testing the immobilization of HMs.

Preparation and analysis of HMs ions

The following HMs tested in this study; Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} were prepared from $CuCl_2$, $ZnCl_2$, $CdCl_2$, and $PbCl_2$, respectively. Stocks of each heavy metal (HM) ion were prepared in deionized water and then sterilized by a (0.22 μm) filter membrane. They were stored at 4°C until used. The HM concentrations were analyzed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (PerkinElmer, Germany).

Immobilization of HMs ions by PNB

Metabolism-independent

In this experiment cell pellets of each culture harvested from a late log phase culture (60 h) was suspended in 1 M sodium azide (NaN_3) solution for 45 min to inhibit metabolic activity (Gourdon et al. 1990). Cell suspensions were centrifuged then washed with the same processes as previously mentioned. The wet cell pellet equivalent to 0.625 mg dry cell weight (DCW)/ml was resuspended in the mixed HMs solution (Highest concentrations detected in shrimp sediments as previously mentioned) containing 3% NaCl, at a pH of 5.8. The 3% NaCl was added to equate with the average concentration that was detected in the water from shrimp ponds. The cell suspensions were incubated at 30°C on a shaking incubator with a speed of

100 rpm for 30 min with aerobic-dark conditions and the same condition was set for the microaerobic-light conditions but with the light intensity adjusted to 3,000 lux. After centrifugation, the remaining HMs in the supernatant was analyzed using ICP-OES. Two control sets were also prepared; negative control (without addition of cell suspension) and positive control (without treatment by 1 M sodium azide). The percentage of each HM removed was finally calculated based on their initial concentrations.

Metabolism-dependent

Wet cell suspensions of NW16 and KMS24 were prepared as previously described except that peptone, yeast extract, Na_2SO_4, and KH_2PO_4 were added into the mixed HMs solution at concentrations of 5.0, 5.0, 0.20, and 0.02 g/l, respectively (Gourdon et al. 1990) and a control set had no added supplement of nutrients and produced a clear solution. As some turbidity appeared after adding a set of nutrients, this precipitate was removed by centrifugation and the supernatant was used to measure the initial concentration of each HM before inoculation. In addition, the initial concentration of each HM in a control set was adjusted by using the measured concentrations found in the supernatant of the set with added nutrients. Immobilization of HMs by both bacterial strains was conducted as previously described.

Factors affecting immobilization of HMs

The effects of the biomass or cell properties (cell growth phase, biomass dose) and environmental factors (pH, temperature, contact time and presence of other cationic ions) were determined consecutively on the immobilization capacity of HMs by both PNB strains with both standard microaerobic-light and aerobic-dark conditions as previously mentioned. After each experiment, optimum conditions were adjusted before carrying out the next series of tests and measurements of HMs by ICP-OES.

Effect of the cell growth phase

A ten percent inoculum size of each culture was grown in GM medium under the optimal conditions as previously mentioned and their growth were observed every 6 h. The cells were harvested at log phase (48 h), late log phase (60 h), and stationary phase (72 h). The cell pellets were prepared as previously described and then HMs immobilization studies were carried out as previously described. However, to achieve a higher immobilization of HMs in this experiment the wet cells equivalent to 2.5 mg DCW/ml was used instead of 0.625 mg DCW/ml.

Effect of biomass dose

Cell pellets of each PNB isolate that were harvested at the log phase (optimum growth phase for removing of HMs) were prepared by varying wet cell concentrations of 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 mg DCW/ml for HMs immobilization tests.

Effect of pH

The pH of the mixed HMs solution was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0.

Effect of temperature

The temperature was adjusted to 20, 25, 30, 35, and 40°C.

Effect of contact time

The time of incubation with the mixed HMs solution varied from 0, 5, 10, 15, 20, 30, 45, and 60 min.

Effect of other cationic ions

The effect of the presence of other cationic ions, 85 mg/l Ca^{2+} ($CaCl_2$) and 160 mg/l Mg^{2+} ($MgSO_4$) on immobilization from the mixed HMs solution was investigated. This included the presence of a control set (HMs solution in 3% NaCl) without the addition of both cationic ions. The amounts of cationic ions used were determined by reference to the concentrations that were found in the water from shrimp ponds. Cell pellets were prepared and incubated based on the optimal conditions of each factor obtained from the previous experiments.

Data presentation and statistical analysis

All experiments in this study were conducted in three replicates. Means and standard deviations are presented. Statistical analysis using one way ANOVA to analyze statistical differences at a P-value < 0.05 and mean comparisons were performed by the Duncan' s multiple range test.

Results

Immobilization of HMs by PNB

Metabolism-independent

Results of the effect of the metabolic inhibitor sodium azide, with both incubating conditions (microaerobic-light

and aerobic-dark conditions) show that it strongly inhibited the immobilization of standard HMs concentrations (0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}) in 3% NaCl by both PNB strains (NW16 and KMS24) (Fig. 1). The percentage reduction of immobilization by strains NW16 and KMS24 under both incubating conditions was between 45–58% and 38–57%, respectively when compared to a set of untreated cells (Fig. 1). The relative efficiency of removal of HMs by both PNB strains was in the order of Pb > Cu > Zn > Cd with both of the incubating conditions. HMs removed by strain NW16 with microaerobic-light (% ions removal: Pb, 33.21; Cu, 16.15; Zn, 13.36; Cd, 9.89) was only slightly different from that with the aerobic-dark conditions, except for Pb^{2+} (Pb, 37.80; Cu, 16.15; Zn, 13.04; Cd, 9.33). A similar result was also found with strain KMS24, except that in this case the removal of Pb^{2+} was not different.

Metabolism-dependent

Results of immobilization of HMs by both PNB strains under all conditions tested (microaerobic-light and aerobic-dark conditions) show that both strains did remove HMs in the presence of nutrients in g/l (5 peptone, 5 yeast extract, 0.2 Na_2SO_4, and 0.02 KH_2PO_4) when compared with those of no added nutrients. HMs did interact with the nutrients as the turbidity increased slightly when the HMs were added to the medium containing nutrients before inoculation. However, this precipitation had been removed by centrifugation. Therefore, the loss of HMs from the medium with added nutrients was due to the presence of bacterial cells. Both PNB strains removed ≥83% of Pb^{2+}, when the medium was supplemented with nutrients under the conditions tested, 31–34% for Cu^{2+}, 22–26% for Zn^{2+} and 17–20% for Cd^{2+} (Fig. 2). In the absence of nutrients the removal capacity of; Cd^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+} by the strain NW16 was decreased by about 43, 59,47 and 43%, respectively under microaerobic-light and about 53, 56, 49 and 51% under aerobic-dark conditions. A similar result was also found for the strain KMS24.

Factors affecting HMs immobilization

Cell age or cell growth phase

In all conditions tested both strains were more effective in immobilizing HMs in the log phase of growth (Fig. 3). Differences were not significant when comparing cells in the late log and stationary phases. The biggest removal of Pb^{2+} detected was about 80% by KMS24 with both conditions tested and also by the strain NW16 but in this case only with aerobic-dark conditions. In contrast, the removal percentage of Pb^{2+} was only 67% by strain NW16 under microaerobic-light conditions. Both strains under all conditions tested

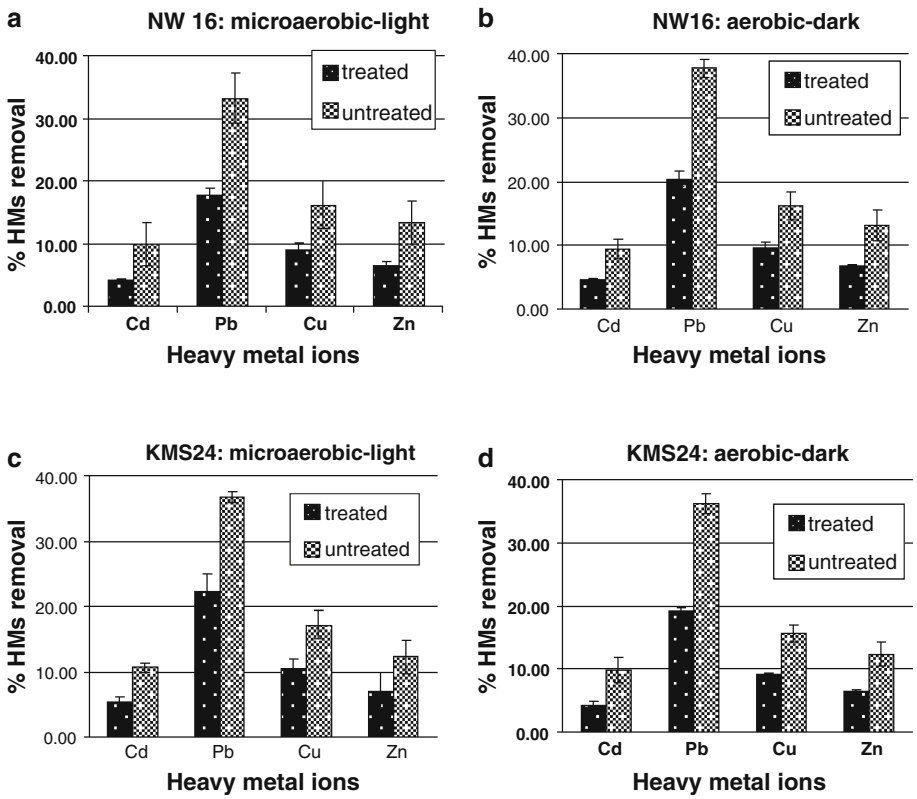

Fig. 1 Effect of metabolic inhibition using 1 M sodium azide on the immobilization of mixed HMs (0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: 0.625 mg DCW/ml from late log phase, pH 5.8, 30 min; NW16 strain with microaerobic-light (**a**) and aerobic-dark (**b**), KMS24 strain with microaerobic-light (**c**) and aerobic-dark (**d**)

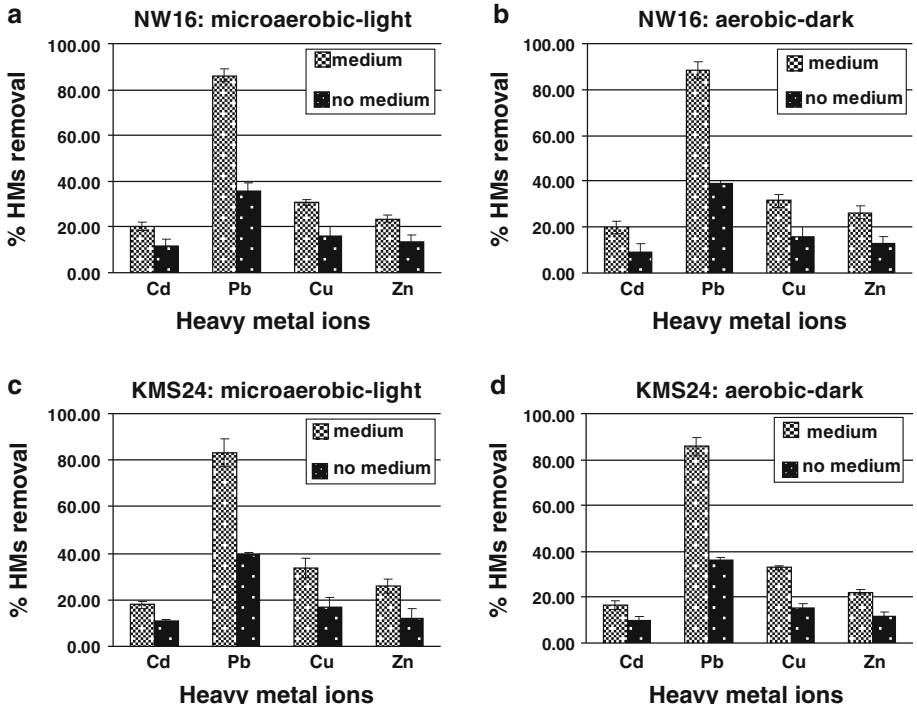

Fig. 2 Effect of adding nutrients on the immobilization of HMs (0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: 0.625 mg DCW/ml from late log phase, pH 5.8, 30 min; NW16 strain with microaerobic-light (**a**) and aerobic-dark (**b**), KMS24 strain with microaerobic-light (**c**) and aerobic-dark (**d**)

showed removal percentage of Cu^{2+}, Zn^{2+} and Cd^{2+} in a range of 30–50, 10–20 and $\leq$10, respectively.

Biomass dose

The removal capacity of HMs by both strains, harvested in the log phase, increased with increasing biomass from 2.0 to 5.0 mg DCW/ml (Fig. 4). However, a wet biomass dose equivalent to 4.5 mg DCW/ml of the strain NW16 under both incubating conditions provided the best removal percentage of all HMs. With strain KMS24 under both incubating conditions there was no difference when the dose of biomass increased from 4.5 to 5.0 mg DCW/ml for Cu^{2+} and Pb^{2+} but the removal capacity of Cd^{2+} and Zn^{2+} was highest at 5.0 mg DCW/ml. Therefore, the optimum amount of biomass for NW16 and KMS24 was 4.5 and 5.0 mg DCW/ml, respectively. These concentrations were selected for further studies.

pH

The removal capacity of HMs by strain NW16 in the pH range from 5.0 to 9.0, with all conditions tested, significantly increased with increasing pH, in the range of 5.0–6.0, with an optimum pH of pH 6.0 for removal of all HMs by log phase cells at biomass doses of 4.5 and 5.0 mg DCW/ml for NW16 and KMS24, respectively (Fig. 5). However, for the strain KMS24 under all conditions tested the optimum pH was 5.5. Therefore, the selected optimal pH for further studies with strain NW16 was 6.0 while for strain KMS24 a pH of 5.5 was chosen.

Temperature

The highest removal ability of each HM by both strains under all incubating conditions was between 30 and 35°C (Fig. 6). However, removal of Zn^{2+} by strain NW16 under both incubating conditions decreased significantly at 35°C. Hence, the optimal temperature for HMs removal by strain NW16 was 30°C. In the case of strain KMS24 removal of Pb^{2+} significantly increased at 35°C under both incubating conditions hence 35°C was chosen as the optimal temperature for the strain KMS24. In addition, the removal ability of HMs particularly Zn^{2+} and Cd^{2+} by the strain KMS24 was higher than the strain NW16 under both incubating conditions (Fig. 6).

Contact time

The removal of all HMs by cells of both strains harvested in the log phase under both new standard incubating conditions at a biomass dose of 4.5 mg DCW/ml, a pH of 6.0 and 30°C for strain NW16 and at a biomass dose of 5.0 mg DCW/ml, a pH of 5.5 and 35°C for strain KMS24 increased at a significant rate from $t = 0$ but only until 30 min for the strain NW16 and 45 min for the strain KMS24 (Fig. 7). Therefore, the optimal contact times to remove HMs under both incubating conditions for the strains NW16 and KMS24 were 30 and 45 min, respectively.

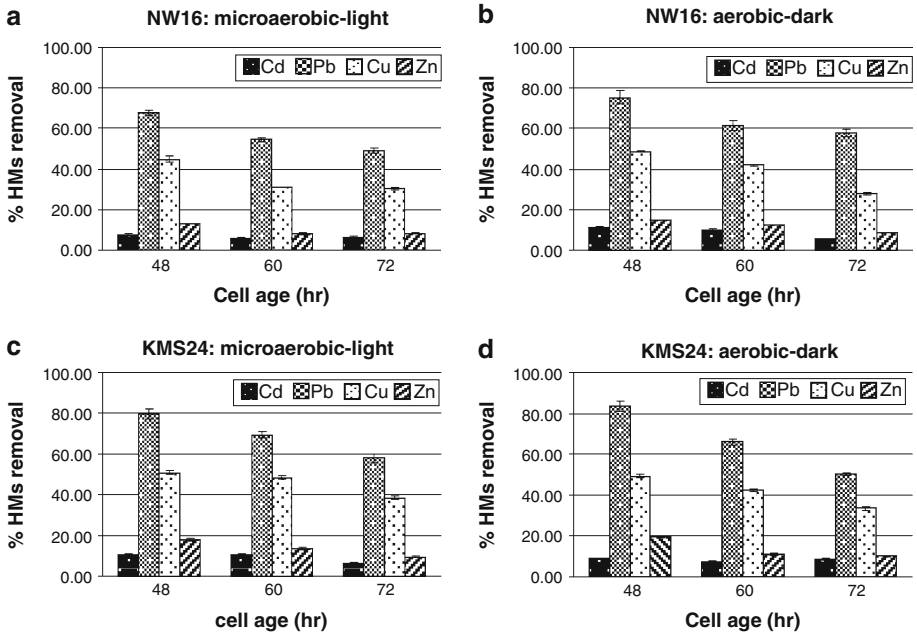

Fig. 3 Effect of cells age on the immobilization of HMs (0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: 2.5 mg DCW/ml, pH 5.8, 30 min; NW16 strain with microaerobic-light (**a**) and aerobic-dark (**b**), KMS24 strain with microaerobic-light (**c**) and aerobic-dark (**d**)

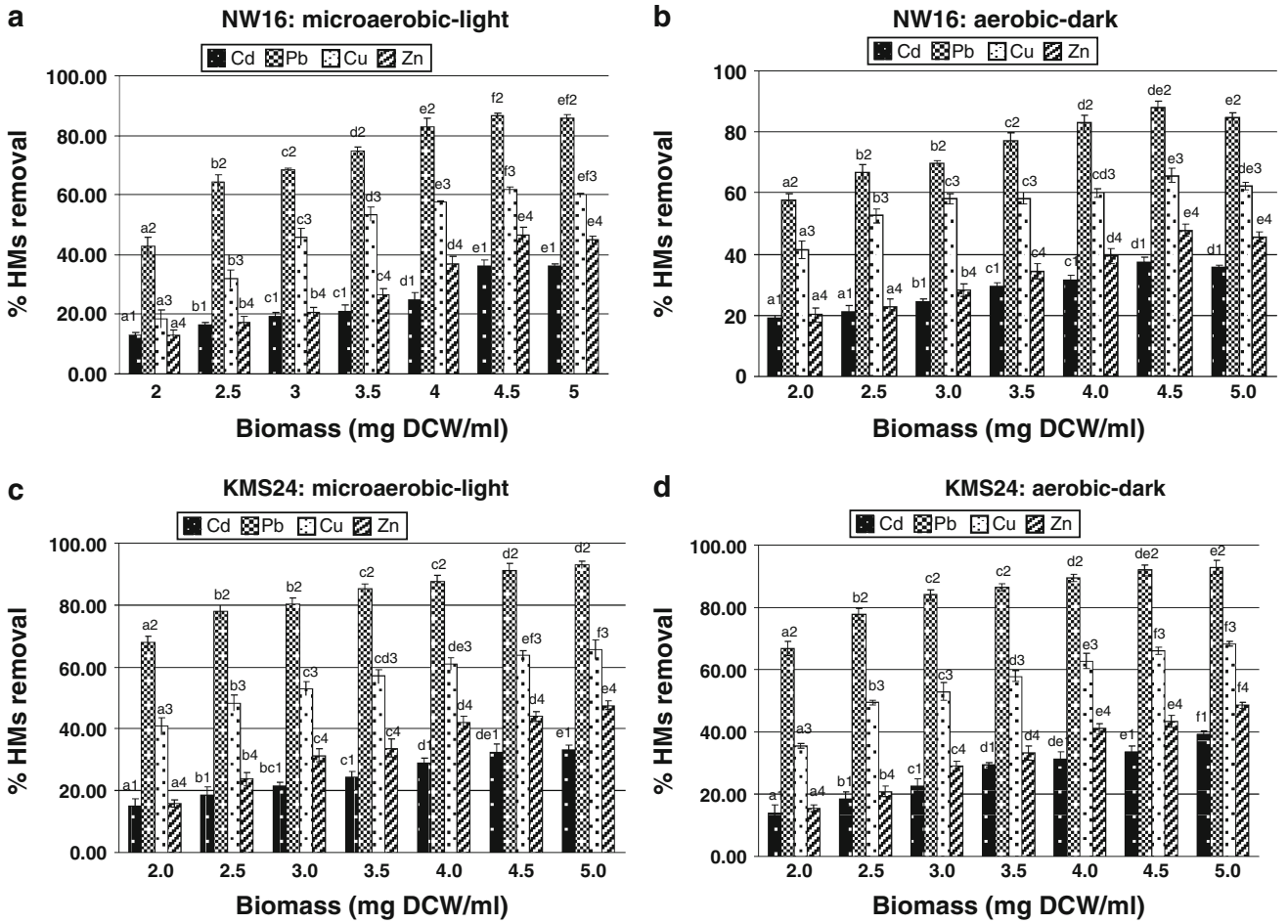

Fig. 4 Effect of biomass dose on the immobilization of HMs (0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: 2.5 mg DCW/ml from log phase, pH 5.8, 30 min; NW16 strain with microaerobic-light (**a**) and aerobic-dark (**b**), KMS24 strain with microaerobic-light (**c**) and aerobic-dark (**d**). *Lowercase letters* with *numbers* above *bars* with *different letters* indicate significant differences ($P < 0.05$)

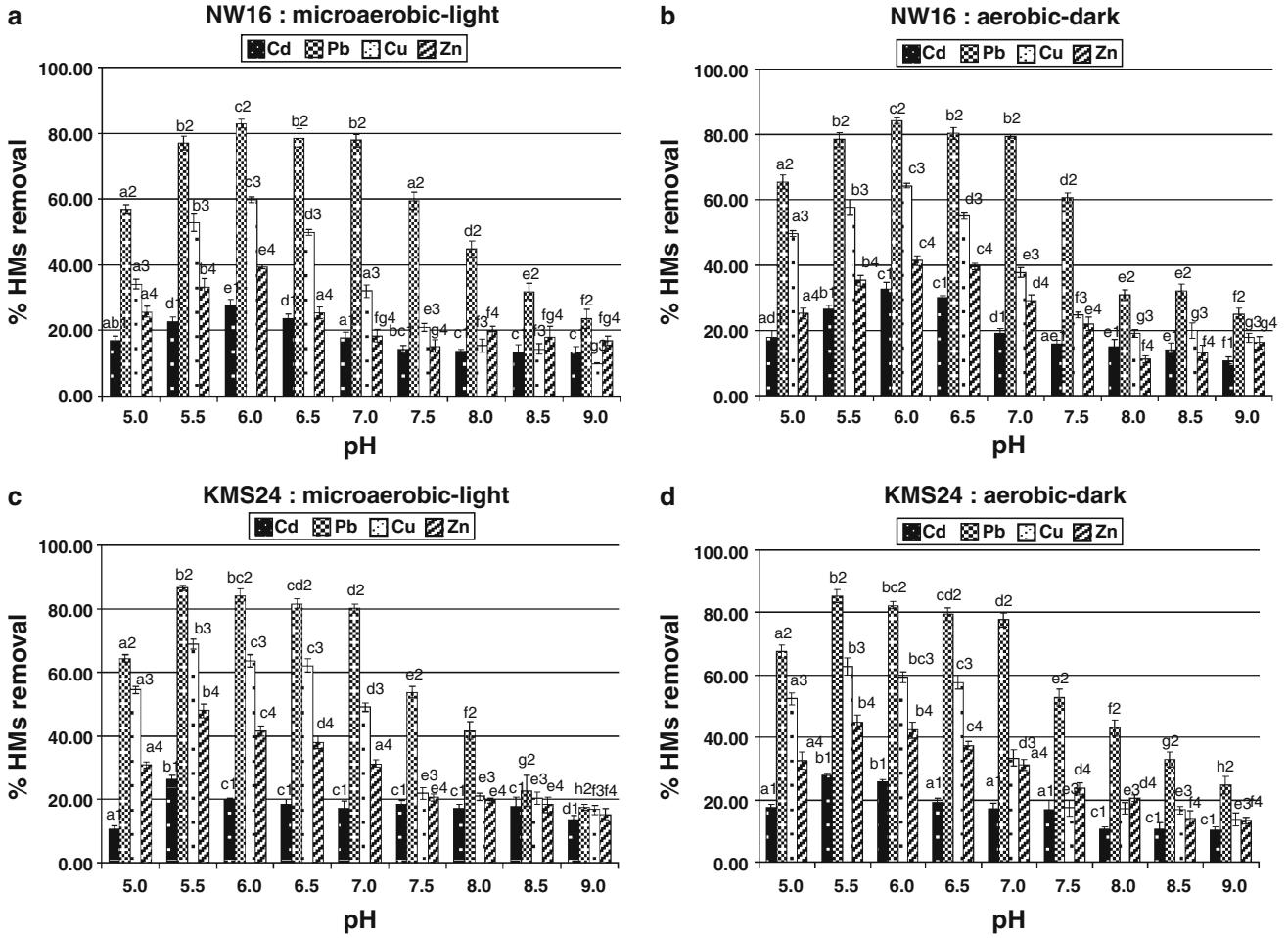

Fig. 5 Effect of pH on the immobilization of HMs (0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: cells from log phase, 30 min; NW16 strain (4.5 mg DCW/ml) with microaerobic-light (**a**) and aerobic-dark (**b**), KMS24 strain (5.0 mg DCW/ml) with microaerobic-light (**c**) and aerobic-dark (**d**). *Lowercase letters* with *numbers* above *bars* using a *different letter* indicate significant differences ($P < 0.05$)

Other cationic ions

In the presence of calcium and magnesium ions at 85 mg/l Ca^{2+} and 160 mg/l Mg^{2+}, respectively, the immobilization of the mixed HMs in 3% NaCl by both PNB strains under both incubating conditions was significantly decreased (Fig. 8). For example, removal of HMs by strain NW16 with optimal conditions (control set) with aerobic-dark conditions was Pb, 90%; Cu, 69%; Zn, 46%; Cd, 28% whereas with microaerobic-light conditions it was Pb, 83%; Cu, 59%; Zn, 39%; Cd, 23%. The average percentage reduction of HMs by the strain NW16 under both incubating conditions was roughly 32, 40, 44 and 52 for Pb, Cu, Zn and Cd, respectively. A similar trend for a reduced removal of HMs was observed in the presence of both cations by the strain KMS24 under both incubating conditions (Pb, 26%; Cu, 36%; Zn, 50%; Cd, 55%). In contrast, no such differences for removal of HMs was found between microaerobic-light and aerobic-dark conditions when the average removal percentages in the control set were 96, 75, 46 and 30 for Pb, Cu, Zn and Cd, respectively.

Discussion

Immobilization of HMs by PNB

The present study demonstrates that the PNB cells (NW16 and KMS24) with microaerobic-light and aerobic-dark conditions can effectively remove HMs from an aqueous solution (mixed solution containing 3% NaCl, 0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}). The metabolic inhibition and metabolic-dependent studies (Figs. 1, 2) reveal that the processes of HMs immobilization by PNB cells may involve intracellular uptake (bioaccumulation) and surface binding (biosorption). It is clear that 1 M sodium azide strongly decreased HMs removal by both strains with both incubating conditions

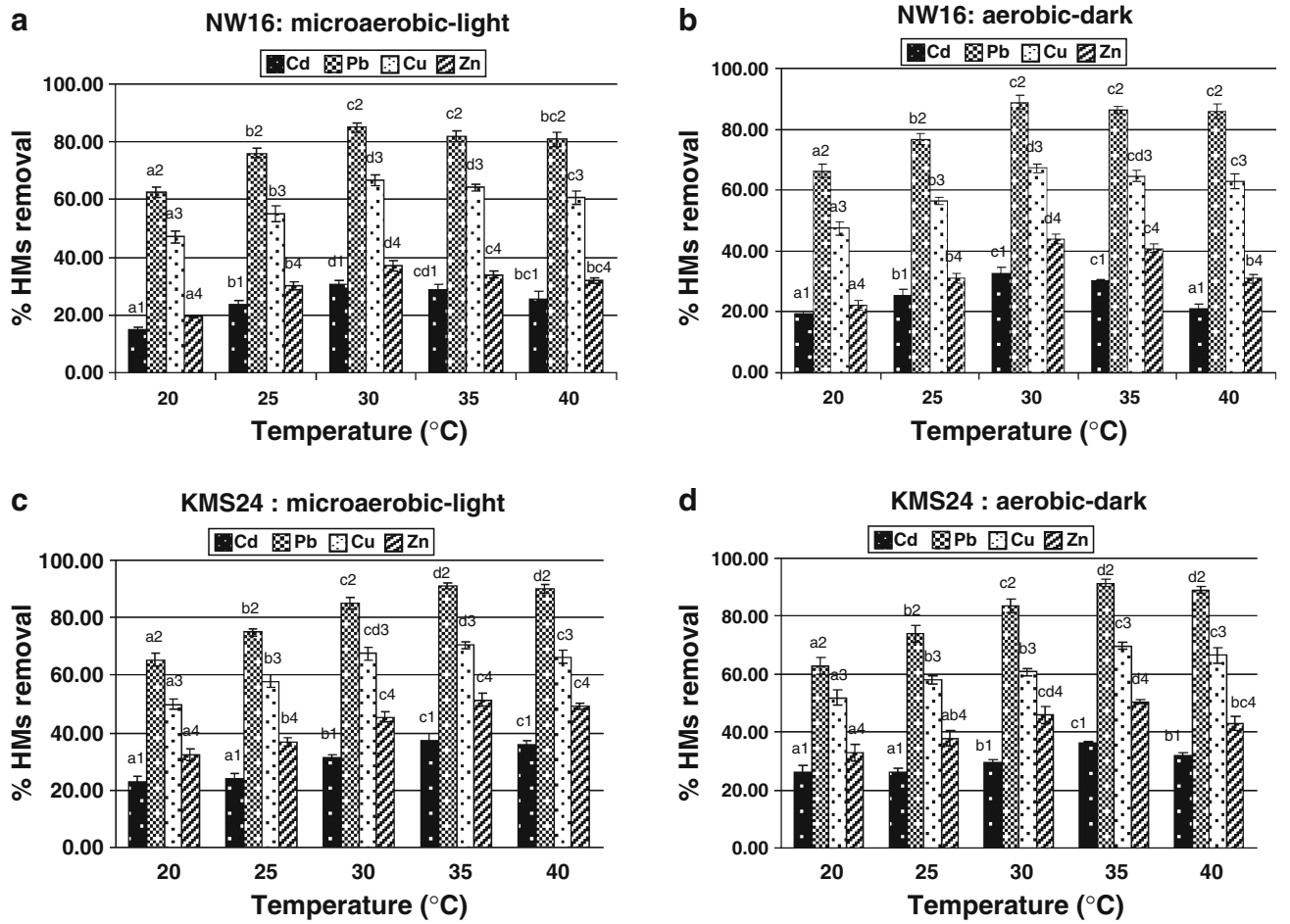

Fig. 6 Effect of temperature on the immobilization of HMs (0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}) in 3% NaCl by PNB isolates. Conditions used: cells from log phase, 30 min); NW16 strain (4.5 mg DCW/ml, pH 6.0) with microaerobic-light (**a**) and aerobic-dark (**b**), KMS24 strain (5.0 mg DCW/ml, pH 5.5) with microaerobic-light (**c**) and aerobic-dark (**d**). *Lowercase letters* with *numbers* above *bars* using a *different letter* indicate significant differences ($P < 0.05$)

(Fig. 1) and as live cells were used that means bioaccumulation is also involved with removal of HMs. The intracellular accumulation of HMs is governed by energy dependent transport systems. This is also supported by the increase of the HMs removal capacity after adding nutrients into the mixed HMs solution (Fig. 2). This indicates that there were some metabolic processes that facilitated the uptake of HMs into the cells. These mechanism-dependent processes are also energy dependent, requiring an active energy generating system by the cells and probably through specific transport systems (Acosta et al. 2005).

The problem caused by the precipitation after adding nutrients was eliminated by first centrifuging the medium as described in the "Methods" section. This indicates that the metabolically dependent accumulation led to the remarkable removable of ≥83% of the Pb^{2+}. Metal precipitation through the formation of Pb-phosphates may be possible as one of the added nutrients was 20 mg/l KH_2PO_4 although the pH of the system tested was 5.8 and precipitation is normally accelerated at a more alkaline pH. It has long been recognized that degradation of organophosphates to orthophosphate by microbes can lead to metal precipitation as metal-phosphates, especially above pH 7 do precipitate (Gazso 2001). Therefore, it could be concluded that a higher removal of metal ions (Pb^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+}) in the solution with added nutrients was mainly caused by both biosorption and bioaccumulation. As no precipitation occurred when azide inhibited metabolism and in the control with no nutrient supplements in the metabolic- dependent experiment and also because the testing time was for only 30 min we suggest that immobilization of HMs by both PNB strains is governed by bioaccumulation and biosorption but not by precipitation. To date the accumulation of HMs by living PNB cells such as *Rhodobacter sphaeroides* has been restricted to the case of tellurite, selenite and rare earth metal oxides (Bebien et al. 2001; Moore and Kaplan 1992) and this study is the first to report the accumulation of HMs from a mixed solution of Cd^{2+},

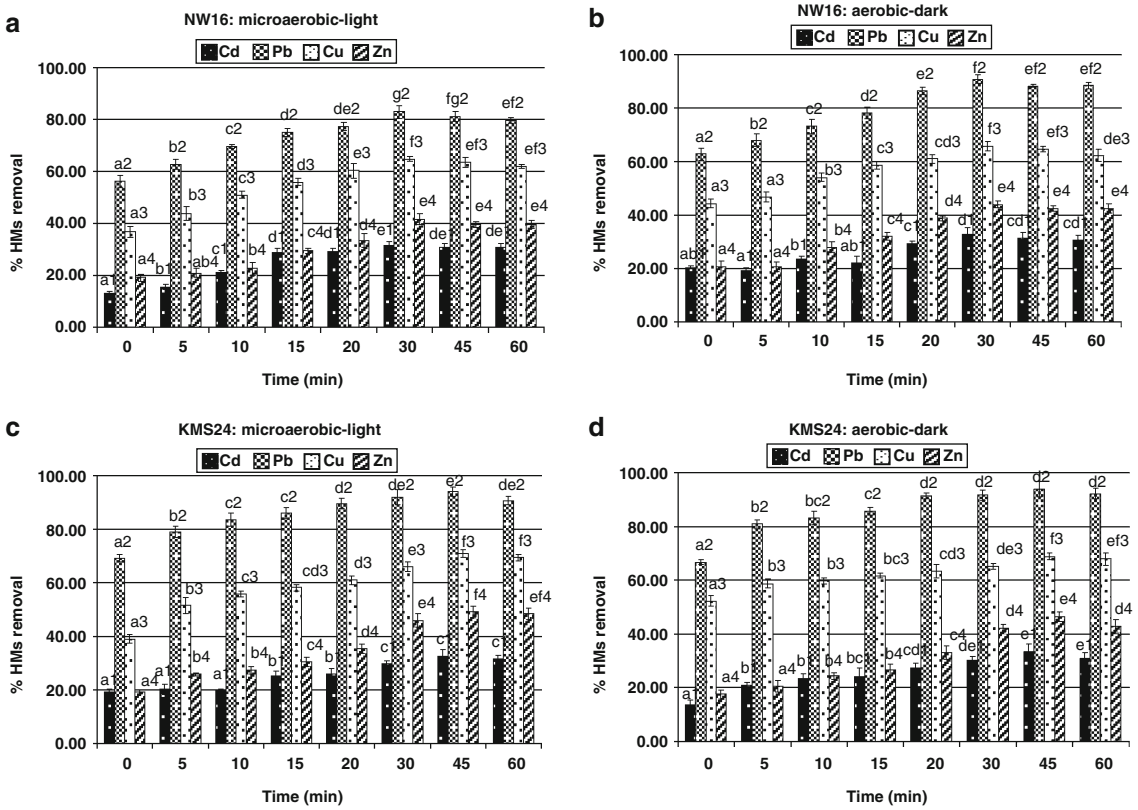

Fig. 7 Effect of contact time on the immobilization of HMs (0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: cells from log phase; NW16 strain (4.5 mg DCW/ml, pH 6.0, 30°C) with microaerobic-light (**a**) and aerobic-dark (**b**), KMS24 strain (5.0 mg DCW/ml, pH 5.5, 35°C) with microaerobic-light (**c**) and aerobic-dark (**d**). *Lowercase letters* with *numbers* above *bars* using a *different letter* indicate significant differences ($P < 0.05$)

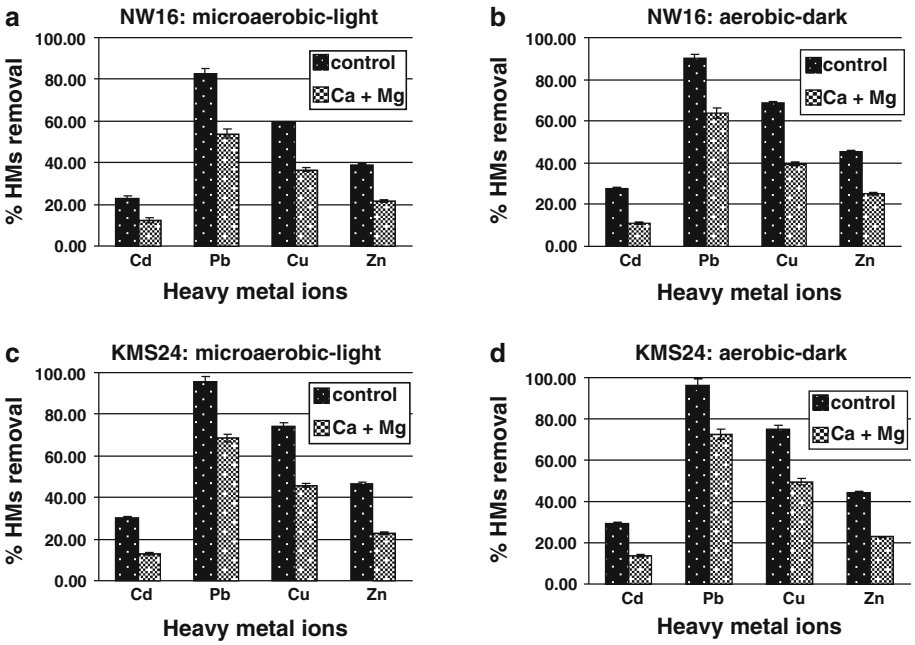

Fig. 8 Effect of other cationic ions on the immobilization of HMs (0.75 mg/l Cd^{2+}, 62.63 mg/l Pb^{2+}, 34.60 mg/l Cu^{2+} and 58.50 mg/l Zn^{2+}) in 3% NaCl by PNB strains. Conditions used: cells from log phase; NW16 strain (4.5 mg DCW/ml, pH 6.0, 30°C, 30 min) with microaerobic-light (**a**) and aerobic-dark (**b**), KMS24 strain (5.0 mg DCW/ml, pH 5.5, 35°C, 45 min) with microaerobic-light (**c**) and aerobic-dark (**d**)

Cu^{2+}, Pb^{2+} and Zn^{2+} in 3%NaCl by *Rhodobium marinum* NW16 and *Rhodobacter sphaeroides* KMS24.

Factors affecting HMs immobilization

It is well recognized that the removal capacity of HMs is mainly influenced by micro-environmental factors of the contact solution including pH, temperature, and interaction with other ions. Additionally, biomass itself, like cell age and the biomass dose, also affect the efficiency of removal of HMs. Cells in their log phase gave the best removal capacity but there were no significant differences found for the cells in their late log and stationary phases (Fig. 3). These results are similar to reports from previous studies (Goyal et al. 2003; Simmons and Singleton, 1996) that cells at an early stage of growth have a higher biosorptive capacity for HMs than do those of stationary phase cultures.

As removal of HMs by both PNB strains was catalyzed by both biosorption and bioaccumulation (Figs. 1, 2) therefore removal of HMs from aqueous solution by both PNB strains was increased as the cell concentration increased (Fig. 4). Increasing biomass provides an increase of surface area and of functional groups on the cell wall for binding HMs. An increase of the biomass dose in the biosorption system resulted in increasing the sorption site interactions and thus an increased rate of immobilizing of the HMs occurred. In addition, the efficiency of removing HMs increased with an increasing initial concentration of HMs when the amount of biomass was constant. This agrees with the findings of Monteiro et al. (2009). Hence, the biosorptive capacity of HMs is related to the ratio of the concentration of initial HMs to the concentration of biomass and this is the main reason why Pb^{2+} was the most efficiently removed HM by both strains of PNB as its initial dose was 62.63 mg/l whereas the initial dose of Cd^{2+} was only 0.75 mg/l and this was the HM that was least efficiently used (Fig. 4). However, in case of Zn^{2+} although its initial concentration was 58.50 mg/l, its removal efficiency was lower than that found in Cu^{2+} with an initial concentration of 34.60 mg/l. One possible reason for this may be that Zn^{2+} has more adverse effects than Cu^{2+} on living cells (Balsalobre et al. 1993) and therefore has reduced bioaccumulation. The order of the biosorption capacity found in yeast cells was Pb > Cu > Cd (Goksungur et al. 2005).

In addition, more viable biomass accounts for more bioaccumulation of HMs ions. The largest percentage of HMs removal was with the viable biomass equivalent to $\leq$5 mg DCW/ml and this indicates the effectiveness of PNB to remove HMs from solution. For biosorption of HMs, pH is one of the more important environmental factors, as this parameter affects the protonation state of the functional groups on the cell wall of the biomass (Bayramoglu and Arica 2008). The surface charge of the cell wall at a low pH level is more positive and when the pH is raised, there is more affinity for metal ions as more ligands bearing negative charges increase (Gupta and Rastogi 2008). This phenomenon was also found in this study (Fig. 5). The most suitable pH values for removal of all HMs by both PNB strains were 5.5–7.0. The results of this study agree with a study of Blackwell et al. (1995) that the optimal pH ranged from 4.0 to 8.0 for metal uptake for almost all types of biomass. The reason for this can be explained by the extra protons at the low pH value tends to compete with the metal ions for the binding sites as previously mentioned. On the other hand, at a high pH value metal complexes will precipitate. Hence, the optimal pH for strains NW16 and KMS were 6.0 and 5.5 as depicted in Fig. 5.

The removal capacity of HMs with both incubating conditions significantly increased when the temperature increased from 20 to 30°C for the strain NW16 and up to 35°C for the strain KMS24 (Fig. 6). This can be explained as living cells were used and thus in addition to biosorption, bioaccumulation is also involved with the removal efficiency of HMs. Both strains grew well in a range of temperature from 20 to 30 or 35°C. Therefore, it will be possible to use both strains for removing HMs in shrimp ponds water without any temperature control. The optimum biomass dose of the strain KMS24 (5.0 mg DCW/ml) was higher than for strain NW16 (4.5 mg DCW/ml); however, strain KMS24 gave higher efficiencies for the removal of Zn^{2+} and Cd^{2+} than the strain NW16 in all conditions tested. Additionally, the incubating conditions had no additional adverse effects for the strain KMS24. Hence, in order to achieve the optimum removing HMs contaminated in shrimp ponds, mixed cells of both strains might be even more effective.

The removing of HMs by both PNB strains with both incubating conditions occurred very quickly at the start of the incubation and this amount significantly increased until 30 and 45 min for strains NW16 and KMS24, respectively (Fig. 7). The fast binding of HMs at the starting time of contact was most likely associated with adsorption of HMs onto the cell surface. This process is usually completed rapidly at around 5 min (Gaber et al. 2008; Kadukova and Vircikova 2005). However, the additional rapid uptake of HMs absorption over the next 30 or 45 min indicates that this process was due to a continuous metabolic uptake of HMs after the initial physical adsorption (Figs. 1, 2, 7). Hence, the advantage of using living cells is due to their ability to remove HMs continuously through bioaccumulation.

As shrimp water contains many additional ionic components, that includes metal cations like Na^{+}, Ca^{2+} and Mg^{2+} and anions like Cl^{-} and SO_4^{2-} normally the biosorption of any HM might be modified by their presence.

The observed decrease of HMs uptake in competitive conditions is believed to be a response to increased competition between similar charged species for binding onto the cell surface (Goksungur et al. 2005). This explains the reduced biosorption capacity of PNB cells in the presence of the bivalent cations, Ca^{2+} and Mg^{2+} (Fig. 8) as both calcium and magnesium ions will increase competition for binding sites on the cell surface.

Conclusion

These results have proved that both PNB strains (NW16 and KMS24) can effectively remove HMs in the 3% NaCl aqueous system with either microaerobic-light or aerobic-dark conditions and thus have a good potential for use to remove HMs present in contaminated shrimp ponds. Removal efficiency is governed by both energy independent (biosorption) and dependent (bioaccumulation) processes and the removal efficiency is mainly influenced by cell age, biomass dose, properties of the target metals, pH and competition with other bivalent cations. Consequently, removal of HMs from shrimp pond water using both these promising strains is now being economically assessed in our laboratory.

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