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Acid phosphatase production by *Rhizopus delemar*: A role played in the Ni(II) bioaccumulation process

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ABSTRACT

The microbial growth and activity of acid phosphatase enzyme during the growth of *Rhizopus delemar* in the presence or absence of Ni(II) ions were investigated. An increase in initial Ni(II) ion concentration inhibited both growth rate of *R. delemar* and acid phosphatase activity. The maximum-intrinsic specific growth rate (μ_m) and Monod constant (K_s) of microorganism in Ni(II)-free medium were found as 0.0649 h^{-1} and 1.8928 g/L, respectively. The inhibition of Ni(II) ions on growth rate of *R. delemar* was found to be a competitive inhibition and *the inhibition constant was found to be 67.11 mg Ni(II)/L*. The intrinsic Michaelis–Menten constant (K_m) and maximum forward velocity of the reaction (v_m) were determined as 3.17 mM and 833.3 μmol/L min, respectively, in Ni(II)-free medium. In the presence of Ni(II) ions, the activity of acid phosphatase was inhibited. Addition of Ni(II) ions decreased the maximum reaction velocity, v_m, showed noncompetitive-type inhibition kinetics and *the inhibition constant was determined as 50 mg Ni(II)/L*. Maximum Ni(II) uptake was obtained by the growing cells of *R. delemar*, while the uptake capacity of resting cells was lowest. This study proved that acid phosphatase enzyme participated in the Ni(II) bioaccumulation mechanism of growing *R. delemar*.

1. Introduction

Heavy metal pollutants are being released into the environment by various industrial processes, mining, smelting, domestic contaminants and waste materials. Due to permanent pollution effects, heavy metals accumulate throughout the foodchain and may affect human beings, animals, the aquatic and terrestrial ecosystems. Ni(II) is used in the metallurgical industry, for the production of high quality iron-based alloys, in the chemical and food industry as catalysts, for the production of paints and batteries as prime materials, and in the electroplating industry. Wastewaters from paint-ink formulation and porcelain enamelling industries contain Ni(II) concentrations ranging from 0–40 mg/L to 0.25–67 mg/L, respectively. In plating plants, Ni(II) concentrations can approach 2–205 mg/L and 2–900 mg/L (rinse waters). In mine drainage, Ni(II) concentrations can approach 0.19–0.51 mg/L, 0.46–3.4 mg/L (acidic) and 0.01–0.18 mg/L (alkaline) [1]. On the other hand, nickel is an essential element stimulated the microbial growth at relatively low concentrations, and component in a number of enzymes. Nickel takes part in important metabolic reactions such as ureolysis, hydrogen metabolism, methane biogenesis and acitogenesis. It has been specified as a vital element for some microorganisms like the cyanobacterium *Oscillatoria* sp. and *Pseudomonas flava* (chemilithotropic microorganisms) [2]. Nickel is also considered as toxic (e.g. in concentrations of 15 mg/L) particularly to activated sludge bacteria, and its presence is detrimental to the operation of anaerobic digesters used in waste water treatment plants [1].

The increasing problem of heavy metal contamination of soil and water has stimulated scientific researches for new mechanisms to remove these pollutants. Conventional methods for removing heavy metal ions from aqueous solutions are chemical precipitation, oxidation–reduction processes, filtration, ion exchange, electrochemical treatment, membrane technologies, adsorption on activated carbon, evaporation [3]. These methods present several disadvantages like high energy and chemical requirements, low efficiency and usually produce large amounts of sludge, difficult separation of precious metals from sludge, high cost, interference by other wastewater constituents [4]. Biological treatment methods, based on growing, resting or non-living microorganisms and sorbents of biological origin provide the reduction of toxic metal levels to acceptable limits at an international level in an efficient, cost-effective and environmentally friendly manner [5,6]. According to the dependence on the cells' metabolism, bioaccumulation mechanisms fall in two large categories:

(i) *Metabolism dependent uptake (also known as active metal uptake)*: metal ions are transported across cell membrane, pre-

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cipitated inside or outside the cell by enzymatic mechanisms. They interact with the metabolic cycle inside the cell. Active metal uptake is an energy-driven process. Live cell-type bioaccumulation also causes valency state alterations to yield a more readily precipitable metal form, associated with intrinsic metabolic processes [7–9].

(ii) *Metabolism-independent uptake (passive metal uptake, also known as bioadsorption, biosorption)*: metal ions are entrapped by binding sites present on the cellular surface. Passive metal uptake is a relatively rapid, generally reversible, non-specific uptake. Although mechanisms responsible for biosorption have been understood to a limited extent, they may be one or combination of several mechanisms such as physical or chemical adsorption, ion exchange, coordination, electrostatic interaction, chelation and microprecipitation [10–15]. As the passive metal uptake is carried out by growing, resting and dead cells, the active metal uptake is performed only by growing cell cultures and is slower than and subsequent to biosorption.

An important advance in the heavy metal uptake process is the identification of the enzyme catalyzing the reactions responsible for metal removal [16]. Metal removal by the cells is mediated by a cell-bound phosphatase which liberates inorganic phosphate from an inorganic phosphate source to precipitate metal as cell-bound metal phosphate [17]. Metal bioaccumulation is mediated via an acid-type phosphatase produced during pregrowth that functions also in resting and immobilized cells to liberate HPO_4^{2-} from appropriate inorganic or organic phosphate that precipitates stoichiometrically with M^{2+} to form $MHPO_4$ tightly bound at the cell surface [18]. The bacteria *Citrobacter* sp. is capable of accumulating heavy metals via enzymically mediated precipitation as insoluble metal phosphates. Enzyme is associated with the outer membrane and found extracellularly. Strains of *Citrobacter* sp. were reported to accumulate Cd, U, Sr and Pb ions when resuspended in metal-containing solution [18–21]. However, little information is available about the effect of enzymatic mechanisms and role of phosphatases in heavy metal uptake by fungi and yeasts. The fungi *Rhizopus delemar* and *Aspergillus niger* were reported to produce extracellular and cellular acid phosphatase activities during growth in the presence or absence of Cu ions in the medium [22,23]. Since the acid phosphatases are located near the cell walls, low rates of intracellular metal uptake were recorded. Phosphate groups have an important role in not only enzymically mediated metal uptake but also biosorptive metal uptake. Metal binding to cell wall phosphate residues was reported to cause uranium and lead uptake by *Streptomyces longwoodensis*. Uranium uptake mechanism of denitrifying bacteria is explained as complexation with polyphosphate groups on cell wall [17]. The structure of *R. delemar* cell walls contains positively charged chitosan and negatively charged phosphate above pH 3.0 and glucuronic acid residues. Large quantities of phosphate and glucuronic acid and chitin–chitosan complex existing in these cell walls offer extensive possibilities for binding metals through ion exchange and coordination [10].

In this study, acid phosphatase production by *R. delemar* during growth period in the absence and presence of Ni(II) ions was followed in batch stirred reactors. The role of acid phosphatase enzyme in the bioaccumulation of Ni(II) ions was investigated. The effects of Ni(II) ions on the growth rate and bioaccumulation properties of *R. delemar* cells were investigated. The inhibition effects of Ni(II) ions on the specific growth rate and acid phosphatase activity were modelled using the Monod and Michaelis–Menten inhibition models, the inhibition types were determined, and the inhibition constants were calculated for both growth and enzyme kinetics. Bioaccumulation and biosorption capacities of growing, resting and dead cells were compared. One of objectives of the present study was also to show that bioremediation using growing microorganisms is a feasible alternate to biosorptive removal of metals from industrial effluents.

2. Materials and methods

2.1. Growth of microorganism and preparation for bioaccumulation

R. delemar, a filamentous fungus, was obtained from the US Department of Agriculture Culture Collection (NRRL 2872). *R. delemar* was grown at 30 °C in agitated liquid media. For acid phosphatase production by *R. delemar*, a complex medium defined by Tsekova and Galabova [22] was used. The inoculum was developed in the medium containing soluble starch 20 (g/L); corn steep liqueur 20 (g/L); peptone 10 (g/L); KH_2PO_4 10 (g/L); $MgSO_4{\cdot}7H_2O$ 1.0 (g/L). The flasks containing inoculum were incubated on an orbital shaker at 150 rpm, at 30 °C for 24 h. The pH of inoculum was adjusted initially to pH 4.8 with HNO_3. Inoculum was carried out aseptically with the cells at the beginning of exponential growth phase. The optimum inoculum ratio (volume of inoculum/production volume of bioreactor) was determined as 10/1000, and all inoculum to the fermentation media was performed at this ratio. The composition of fermentation medium contained soluble starch 20 (g/L) and corn step liqueur 40 (g/L). To determine type of Ni(II) inhibition on the growth of *R. delemar*, starch concentration (S, g/L) was varied between 5 and 30 g/L.

2.2. Preparation of bioaccumulation media containing Ni(II) ions

Ni(II) solutions were prepared by diluting 1.0 g/L of stock solutions of Ni(II), obtained by dissolving $Ni(NO_3)_2{\cdot}6H_2O$ in distilled water, respectively. The range of Ni(II) ion concentrations of prepared fermentation media varied between 10 and 200 mg/L. The pH of the fermentation media was varied between 4.0 and 5.5 by adding HNO_3, prepared by diluting 1 mol/L of stock solution. The sterile metal ion solutions were added to the fermentation media after sterilization at 120 °C for 20 min.

2.3. Bioaccumulation experiments

Fermentations were carried out in an orbital shaker at 30 °C under stirring rate of 150 rpm for 126 h. Samples (2 mL) were taken aseptically at certain time intervals from fermentation media, then centrifuged at 6030 × g for 5 min and the supernatant liquid was analysed for metal ions and acid phosphatase activity. The precipitated cells were used for the determination of the dry weight of the biomass and the biomass concentration. Two parallel experiments were conducted at each experimental condition. Arithmetic mean of results of two parallel experiments was used in data evaluation.

Metal bioaccumulation was calculated from a metal balance yielding:

$$q_{\text{bioaccumulated}} = \frac{C_i - C_f}{x} = \frac{C_{\text{bioaccumulated}}}{x} \quad (2.1)$$

where $q_{\text{bioaccumulated}}$ is bioaccumulated metal ion quantity per unit weight of dried biomass (mg Ni(II)/g dry weight), C_i shows the initial metal ion concentration (mg Ni(II)/L), C_f represents residual metal ion concentration in solution (mg Ni(II)/L), x denotes microorganism concentration (g/L) and $C_{\text{bioaccumulated}}$ (mg Ni(II)/L) indicates bioaccumulated metal ion concentration.

2.4. Preparation of microorganism for biosorption experiments

To compare metal accumulation capacities of growing, resting and dead cells of *R. delemar*, biosorption experiments were also

performed. In the absence of Ni(II) ions, *R. delemar* was grown in soluble starch and corn step liqueur medium in batch culture at 30 °C and at pH 5.0. Batch experiments were conducted with 100 mL cultures, agitated at 150 rpm on a shaker for 72 h. At the deceleration phase of the growth, agitation was stopped. After the growth period, *R. delemar* was separated from fermentation medium by centrifugation, and washed twice with distilled water. For biosorption studies, 1.87 g (1.0 g dry cell weight) of resting cells was suspended in 100 mL of distilled water. To obtain dead cells, *R. delemar* was harvested from fermentation medium, washed twice with distilled water, inactivated using 1% formaldehyde and then dried in a sterilizer at 60 °C for 24 h. For biosorption studies, 1.0 g of dried cells was suspended in 100 mL of distilled water and homogenized for 20 min in a homogenizer at 8000 rpm. Initial metal ion concentrations were varied between 10 and 200 mg/L while the dry cell weight in each sample was constant at 1.0 g/L. Before the solutions were mixed with the fungal suspension, pH was adjusted to 4.0–5.5 by adding 1 mol/L of HNO_3.

2.5. Analysis of heavy metal ions and microorganism concentration

The concentrations of free Ni(II) ions in the sample supernatant were determined using an atomic absorption spectrophotometer (GBC Avanta $\sum$) with an air–acetylene flame. Ni(II) ions were measured at 351.5 nm. PHOTRON hollow cathode lamp was used. The amount of fungal biomass was determined by centrifuging mycelia, washing with distilled water and drying to constant weight at 60 °C for overnight. Cell concentration (cell concentration = 0.505 OD) was measured spectrophotometrically at 600 nm and the obtained values were converted to g cell dry wt. L^{-1} using a factor previously determined from a calibration curve relating the wet weight of the biomass to the dry weight of the biomass at 30 °C.

2.6. Enzyme assay

The activity of acid phosphatase was measured using a spectrophotometric method with p-nitrophenyl phosphate (pNPP) as a substrate. The substrate solution was prepared by adding 0.50 mL of solution A (90 mM citrate buffer, adjusted to pH 4.8 at 37 °C) to 0.50 mL of solution B (15.2 mM p-nitrophenyl phosphate) under intense stirring. After the substrate solution was mixed and equilibrated to 37 °C, 0.10 mL of enzyme sample (culture filtrate or cell-free extract) was added. The preparation of blank solution was the same with reaction mixture, but enzyme solution was not added in this stage. The reaction mixture was immediately mixed under intense stirring and incubated at 37 °C for exactly 10 min. Then, 4.00 mL of 100 mM NaOH solution was added to both the reaction mixture and the blank solution. In this stage, 0.10 mL of enzyme solution was added to blank solution. The p-nitrophenol released was measured at 410 nm in spectrophotometer. One unit of phosphatase activity was defined as the amount of enzyme solution liberating 1 μmol p-nitrophenol per minute at pH 4.8 and at 37 °C [24].

To investigate effects of Ni(II) ions on the activity of acid phosphatase, enzyme solution was harvested from metal-free fermentation media at pH 5.0 and at the end of 48 h, and then, was mixed with the substrate solution in the presence of $Ni(NO_3)_2{\cdot}6H_2O$ solution at 25 and 50 mg/L concentration, and the assay method was applied as mentioned above [25]. The inhibition kinetics of enzyme was examined using substrate (p-nitrophenyl phosphate pNPP) concentrations in the range of 0.5–10 mM.

2.7. Mathematical models for R. delemar growth kinetics and enzyme kinetics

Kinetics of simple enzyme-catalyzed reactions is often referred to as Michaelis–Menten kinetics or saturation kinetics [26–28]. Michaelis–Menten equation is given as follows:

$$v = \frac{v_m S}{K_m + S} \tag{2.2}$$

where the maximum forward velocity of the reaction and the Michaelis–Menten constant are v_m and K_m, respectively. K_m relates to the substrate concentration, giving the half-maximal reaction velocity. A number of substances may cause a reduction in the rate of an enzyme-catalyzed reaction. The three major classes of reversible enzyme inhibitors are competitive, noncompetitive and uncompetitive inhibitors, and the rate equations are given as follows [26–28]:

$$\text{Competitive enzyme inhibition}: \quad v = \frac{v_m S}{K_m(1 + (1/K_I)) + S} \tag{2.3}$$

$$\text{Non-competitive enzyme inhibition}: \quad v = \frac{v_m}{(1 + (K_m/S))(1 + (I/K_I))} \tag{2.4}$$

$$\text{Uncompetitive enzyme inhibition}: \quad v = \frac{v_m S}{(K_m/(1 + (I/K_I)) + S)(1 + (I/K_I))} \tag{2.5}$$

In the presence of inhibitory substances such as heavy metal ions in the fermentation medium, microorganism growth also becomes inhibited, and growth rate depends on inhibitor concentration. The following rate expressions are used for competitive, noncompetitive, and uncompetitive inhibition of growth in analogy to enzyme inhibition [26,27].

$$\text{Competitive inhibition}: \quad \mu = \frac{\mu_m S}{K_S(1 + (I/K_I)) + S} \tag{2.6}$$

$$\text{Noncompetitive inhibition}: \quad \mu = \frac{\mu_m}{(1 + (K_S/S))(1 + (I/K_I))} \tag{2.7}$$

$$\text{Uncompetitive inhibition}: \quad \mu = \frac{\mu_m S}{(K_S/(1 + (I/K_I)) + S)(1 + (I/K_I))} \tag{2.8}$$

3. Results and discussion

3.1. Acid phosphatase production by R. delemar in the absence and presence of Ni(II) ions

The lag phase occurred immediately after inoculation and prolonged until the end of 4 h with increasing concentrations of Ni(II) ions. After this adaptation period, *R. delemar* cells multiplied rapidly, and biomass concentration increased exponentially with time. The deceleration growth phase followed the exponential phase and continued between 48 and 72 h. The maximum biomass concentration was obtained as 3.515 g/L at the end of deceleration growth phase (72–76 h) and at pH 5.0, then the maximum biomass concentration remained approximately constant (Fig. 1). The production of acid phosphatase increased steadily with cultivation time. Maximal enzyme activity was reached after two days of culture. Most of the acid phosphatase by *R. delemar* was produced

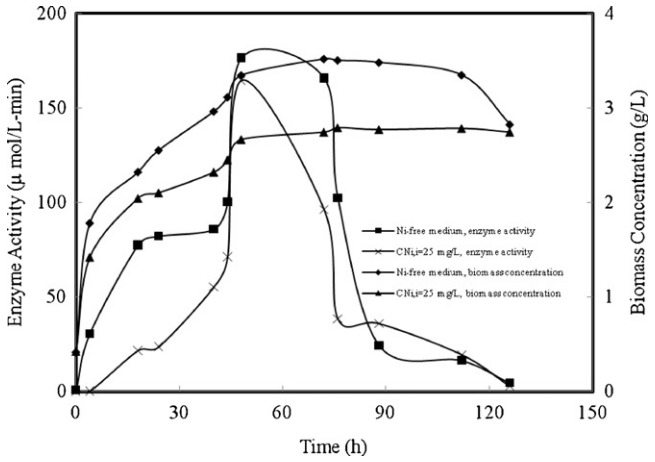

Fig. 1. Time course of acid phosphatase activity and biomass concentration in the fermentation medium containing 25 mg/L Ni(II) ions and in the Ni(II)-free medium (pH = 5.0; starch concentration = 20 g/L; temperature = 30 °C).

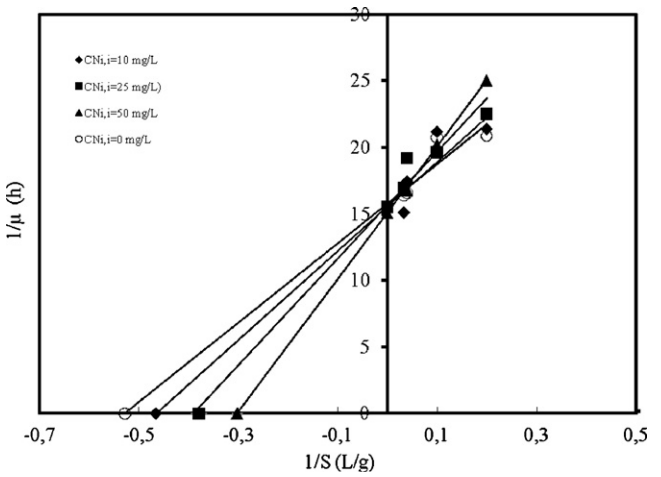

Fig. 2. Double-reciprocal plots of the Monod equation for the growth of *R. delemar* obtained in the Ni(II)-free media and in the presence of increasing concentrations of Ni(II) ions in the range 10–50 mg/L (pH = 5.0; temperature = 30 °C).

in the late exponential phase of the growth (48 h). Depending on culture conditions, enzyme production prolonged until the end of 72 h of growth. Maximum acid phosphatase activity was obtained as 176.4 μmol/L min at pH 5.0 in the absence of Ni(II) ions. In the presence of Ni(II) ions, maximum acid phosphatase activity was also obtained in the late exponential phase of the growth (48 h). However, the acid phosphatase remained stable relatively in a short time period, and the enzyme activity decreased rapidly. The pH of the fermentation medium slightly decreased during the growth of *R. delemar*. When the initial pH of the fermentation medium was adjusted to pH 5.0, pH decreased from pH 5.0 to pH 4.6 at the 126th hour of growth. When the maximum acid phosphatase activity was obtained at 48th hour of cultivation, the pH of the fermentation medium was recorded as 4.72.

3.2. Effect of Ni(II) ions on the growth of R. delemar

The presence of Ni(II) ions in the fermentation medium inhibited *R. delemar* growth, and biomass concentration at the end of exponential growth phase (48 h) decreased from 2.884 to 1.677 g/L with increasing concentrations of Ni(II) ions in the range 10–50 mg/L (Table 1). Specific growth rate obtained at pH 5.0 in the absence of Ni(II) ions at 20 g/L starch concentration and at 48 h was found to be 0.0499 h^{-1}. The lowest specific growth rate was determined as 0.0478 h^{-1} in the media containing 50 mg/L Ni(II) ions. Specific growth rate became dependent on inhibitor concentration. The inhibition pattern of microbial growth is analogous to enzyme inhibition. The effect of inhibitors on specific growth rate of microorganisms can be three types: competitive, noncompetitive and uncompetitive as defined in the literature [26,27].

To determine type of Ni(II) inhibition on the growth of *R. delemar*, Monod equation was linearized in double-reciprocal form. A plot of $1/\mu$ versus $1/S$ gives a linear line with a slope of K_s/μ_m and y-axis intercept of $1/\mu_m$. The maximum specific growth rate (μ_m) and the saturation constant (K_s) of microorganism in metal-free media were determined as 0.0649 h^{-1} and 1.8928 g/L, respectively, from the linearized form of Monod equation. The presence of Ni(II) ions in the range of 10–50 mg/L seems to be a competitive inhibition effect on the growth of *R. delemar* (Fig. 2). The maximum specific growth rates (μ_m) and the saturation constants (K_s) in the presence of increasing concentrations of Ni(II) ions are compared with that obtained in the Ni(II)-free medium in Table 2. The net effect of competitive inhibition is an increased value of $K_{s,app}$ and, therefore, reduced reaction rate. The maximum specific growth rates seemed to remain constant approximately at 0.0652 h^{-1}, and this value was the same with the maximum specific growth rate obtained in Ni(II)-free medium, whereas the Monod constants increased in the media containing the Ni(II) ions at increasing concentrations. A low value of the saturation constant or half velocity constant, K_s, suggests that the microorganism has a high affinity for the substrate. As the saturation constant is equal to the concentration of the rate-limiting substrate when the specific rate of growth is equal to one-half of the maximum, competitive inhibition can be overcome by high concentrations of substrate.

The apparent $K_{s,app}$ values calculated from the plots of Fig. 2 were themselves plotted against inhibitor concentrations (Ni concentrations) to give a straight line from which the slope is K_s/K_I and the intercept is K_s. The inhibition constant for Ni(II) ions on specific growth rate of *R. delemar* was found to be 67.11 mg Ni(II)/L. The inhibition constant in the competitive inhibition was greater than the inhibitor concentration. The contribution of I_{Ni}/K_{Ni} inhibition term at lower inhibitor concentrations on the increase of apparent Monod constant was lower than that at higher inhibitor concentrations.

Table 1
Comparison of the specific growth rates and maximum biomass concentrations with the metal-free medium and in the presence of increasing concentrations of Ni(II) ions (pH = 5.0; starch concentration = 20 g/L; temperature = 30 °C; incubation time = 48 h).

Concentration of the metal ions (mg/L)	Specific growth rate μ (h^{-1})	Biomass concentration (g/L)
0	0.0499	3.3427
10	0.0497	2.8838
25	0.0487	2.6648
50	0.0478	1.6770

Table 2
Comparison of the maximum specific growth rates and the saturation constants obtained in the Ni(II)-free fermentation medium, with those obtained in the presence of increasing concentrations of Ni(II) ions, and the Ni(II) inhibition constants for the specific growth rates calculated from the competitive Monod inhibition model (pH = 5.0; temperature = 30 °C).

$C_{Ni,i}$ (mg/L)	μ_m (h^{-1})	K_s (g/L)	R^2
0	0.0649	1.8928	0.9827
10	0.0654	2.1442[a]	0.9721
25	0.0644	2.6407[a]	0.9835
50	0.0662	3.3102[a]	0.9990

[a] Apparent Monod constants.

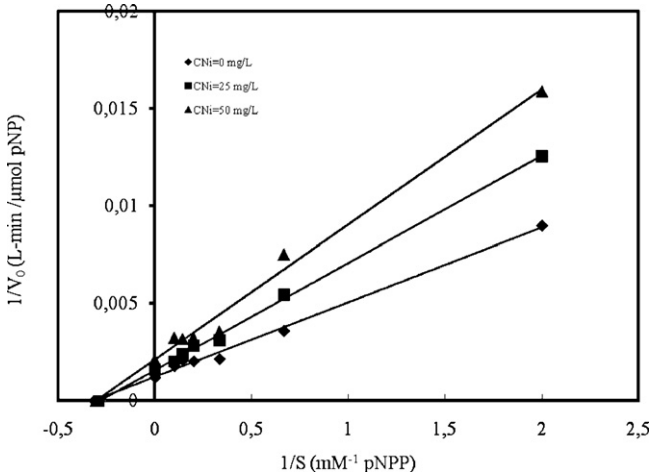

Fig. 3. Lineweaver–Burk plots for acid phosphatase enzyme in the absence and presence of increasing concentrations of Ni(II) ions in the range 10–50 mg/L (pH = 5.0; temperature = 30 °C).

3.3. Effect of Ni(II) ions on the acid phosphatase activity of R. delemar

Acid phosphatase activity slightly decreased in the presence of Ni(II) in the fermentation medium. Maximum phosphatase activity was determined as 164.4 μmol/L min at the end of exponential growth (48 h), and at 25 mg/L of Ni(II) ions (Fig. 1). Then, phosphatase activity began to decrease with increasing concentrations of Ni(II) ions. The lag phase in the biosynthesis of acid phosphatase enzyme by *R. delemar* did not occur in the presence of Ni(II) ions, in other words, the biosynthesis of acid phosphatase was not repressed, and the maximum enzyme activity was observed at the same time with Ni(II)-free medium.

The inhibition effect of Ni(II) ions on the p-nitrophenylphosphate activity of the enzyme was searched by increasing the Ni(II) ion concentrations from 25 to 50 mg/L in the reaction mixture. To determine type of Ni(II) inhibition on the p-nitrophenylphosphate activity of the enzyme, Michaelis–Menten kinetics was applied. A plot of $1/v$ versus $1/S$ yields a linear line with a slope of K_m/v_m and y-axis intercept of $1/v_m$ as depicted in Fig. 3. The Michaelis–Menten constant, K_m, and the maximum forward velocity of the reaction, v_m, were determined in Ni(II)-free medium, and found to be 3.17 mM and 833.3 μmol/L min, respectively (Table 3). The Michaelis–Menten constant for the phosphatase production by *A. niger* was determined as 4.7 mM by Tsekova et al. [23]. The acid phosphatases of *Humicola lutea* demonstrated a similar affinity for pNPP and a K_m value of 1.3 mM was determined [29]. A relatively higher affinity for cellular acid phosphatase of *R. delemar* was recorded by Tsekova and Galabova [22] and a K_m value of 0.325 mM of pNPP was estimated. Relatively low values of K_m for the activities of acid phosphatases of *A. niger* and *A. nidulans* were also reported to be 0.38 and 0.47 mM, respectively [25,30].

Table 3
Comparison of the maximum forward velocities of the reaction and the Michaelis–Menten constants obtained in the Ni(II)-free fermentation medium, with those obtained in the presence of increasing concentrations of Ni(II) ions, and the Ni(II) inhibition constants for the acid phosphatase enzyme calculated from the non-competitive Michaelis–Menten inhibition model (pH = 5.0; temperature = 30 °C).

$C_{Ni,i}$ (mg/L)	v_m (μmol/L min)	K_m (mM)	R^2
0	833.3	3.17	0.9904
25	625.0[a]	3.44	0.9984
50	476.2[a]	3.29	0.9943

[a] Apparent maximum forward velocity of the reaction.

The expected phosphatase inhibition (%) in the presence of 25 mg/L Ni(II) ions was 10.8%. The measured phosphatase inhibition with a decreasing enzyme activity from 176.4 μmol/L min in the Ni-free medium to 164.4 μmol/L min in the presence of 25 mg/L Ni(II) ions (Fig. 1) was 6.8% in accordance with the expected phosphatase inhibition. The addition of increasing concentrations of Ni(II) ions decreased the maximum reaction velocity significantly but did not affect the affinity of the enzyme complex and the substrate. Thus the Michaelis–Menten constants remained approximately constant. This type of inhibition is known as noncompetitive inhibition. Noncompetitive inhibitors bind to an inhibitor site which is remote from the active site. They inhibit the enzyme by causing a conformational change which prevents enzyme from converting substrate to product. To prevent noncompetitive inhibition, other reagents need to be added to block binding of the inhibitor to the enzyme [26–28]. Compounds containing heavy metals such as lead, mercury, copper or silver are poisonous. This is because ions of these metals are reported to be noncompetitive inhibitors for several enzymes [26,31]. The two valance-numbered ions such as silver, mercury, copper or lead react with –SH groups in the side groups of cysteine residues in the protein chain [32,33]. Tsekova and Galabova [22] reported that the presence of Cu(II) in the enzyme reaction mixture caused threefold increase of phosphatase activity of *R. delemar*. However, these data are in contrast to those reported for the other acid phosphatases, which activities were decreased in the presence of Cu(II) ions significantly [25]. Very little information is really available on enzyme kinetics in the presence of heavy metals. Inconsistent results about the effect of heavy metals on enzyme kinetics were reported in the related literature. Increased, decreased or no change in K_m and v_m values were recorded for various enzymes in the presence of heavy metal ions. Huang and Shindo [25] observed that the addition of Cu(II) as copper chloride decreased significantly the maximum reaction velocity of acid phosphatase, but increased the affinity of the enzyme complex and the substrate (i.e. lower K_m values). Therefore, the mixed inhibition by Cu(II) of the acid phosphatase was considered as noncompetitive–uncompetitive inhibition.

The $1/v_{m,app}$ values plotted against inhibitor concentrations gave a straight line with $1/(v_m K_I)$ as slope and $1/v_m$ as intercept. The inhibition constant for Ni(II) ions on enzymatic reaction rate was determined as 50 mg Ni(II)/L. The inhibition constants for Cu(II)–enzyme (K_I) and Cu(II)–enzyme–substrate (K_{-I}) for the noncompetitive–uncompetitive inhibition of Cu(II) ions on free acid phosphatase activity were calculated by Huang and Shindo [25], and determined as 0.42 and 0.10 mM, respectively.

3.4. Ni(II) bioaccumulation by R. delemar

The optimum initial pH for the bioaccumulation of Ni(II) ions by growing *R. delemar* was obtained in the range pH 5.0–5.5. This pH range is compatible with the pH range in which the maximum enzyme activity was observed (Figs. 4 and 5). At 50 mg/L initial Ni(II) ion concentration, $C_{Ni,i}$, at pH 5.0 and at 25 °C, the change of bioaccumulated Ni(II) ion concentration (mg/L), the bioaccumulated Ni(II) ion quantities per unit mass of biomass on dry weight basis (mg Ni(II)/g dry weight) and acid phosphatase activity with time are given in Fig. 6. The change of bioaccumulated Ni(II) ion concentration increased during lag phase slowly and exponential growth phase rapidly, and reached a maximum value at the 48th hour of growth and at the end of the exponential growth phase. Maximum acid phosphatase activity was also obtained in the same stage of *R. delemar* growth. As acid phosphatase mainly mediated Ni(II) bioaccumulation, this was an expected result. Then the bioaccumulated Ni(II) ion concentration remained approximately constant

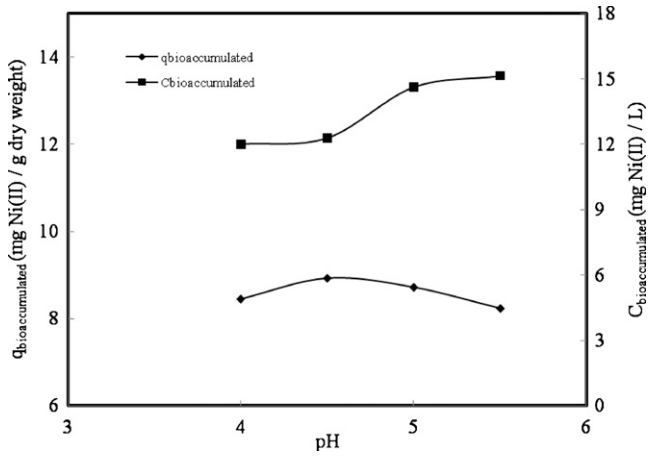

Fig. 4. Change of bioaccumulated Ni(II) ion concentration (mg/L) and bioaccumulated Ni(II) ion quantities per unit mass of biomass on dry weight basis (mg Ni(II)/g dry weight) with initial pH (starch concentration = 20 g/L; temperature = 30 °C; $C_{Ni,i}$ = 50 mg/L; incubation time = 48 h).

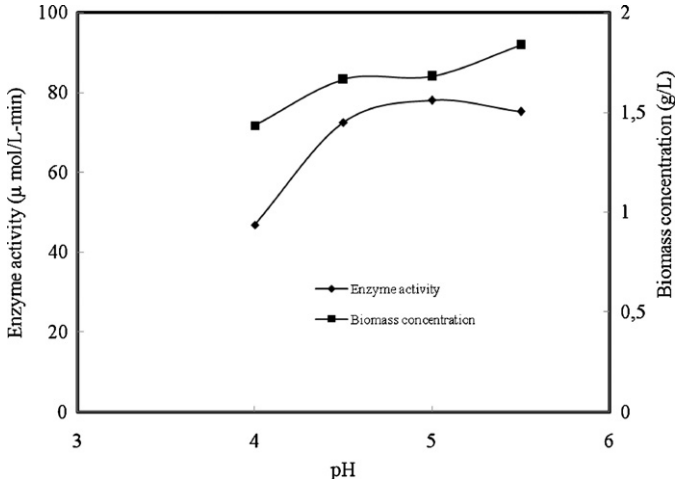

Fig. 5. Change of enzyme activity (μmol/L min) and biomass concentration (g/L) with initial pH (starch concentration = 20 g/L; temperature = 30 °C; $C_{Ni,i}$ = 50 mg/L; incubation time = 48 h).

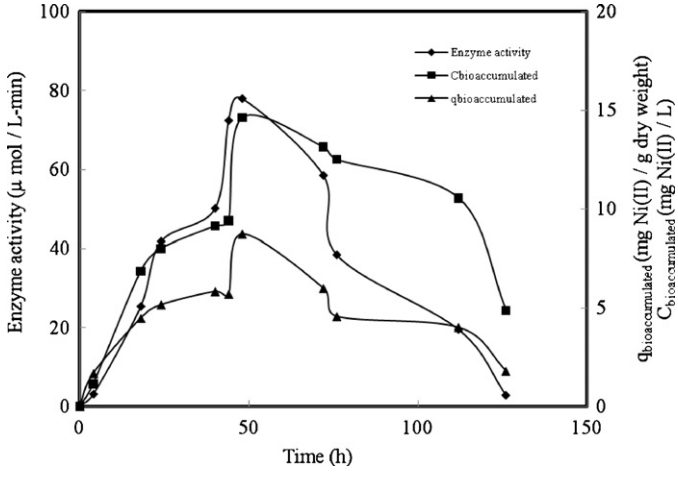

Fig. 6. Change of bioaccumulated Ni(II) ion concentration (mg/L), bioaccumulated Ni(II) ion quantities per unit mass of biomass on dry weight basis (mg Ni(II)/g dry weight) and enzyme activity with time (starch concentration = 20 g/L; temperature = 30 °C; $C_{Ni,i}$ = 50 mg/L).

Table 4
Freundlich sorption constants for the Ni(II) removal of growing, resting and dead cells of *R. delemar* (starch concentration = 20 g/L; pH = 5.0; temperature = 30 °C; incubation time = 48 h).

	K_F	n
Growing cells	0.3188	0.9299
Resting cells	0.1461	0.9207
Dead cells	1.3578	0.5669

through deceleration growth phase and stationary growth phase. At the beginning of death phase of growth (126 h), the bioaccumulated Ni(II) ion concentration began to decrease, the release of Ni(II) ions was detected following the metal uptake and stationary phase of growth or equilibrium stage. As regards the change of bioaccumulated Ni(II) ion quantities per unit mass of biomass with time, the appearance of bioaccumulation curve was slightly altered. As young cell concentration was relatively low, a step increase in the bioaccumulated Ni(II) ion quantities per unit mass of biomass was observed in the lag phase and early exponential growth phase. When microorganism concentration increased and reached approximately a constant value, increase in the bioaccumulated Ni(II) ion quantities per unit mass of biomass with time decelerated.

The Ni(II) sorption capacities of growing, resting and dead cells of *R. delemar* were evaluated in terms of sorption isotherms and the best correlations between experimental and model predicted equilibrium uptake were obtained using the Freundlich model ($q_{eq} = K_F C_{eq}^n$). K_F and n values were obtained by evaluating the isotherms and are presented in Table 4. The intercept K_F is an indication of the sorption capacity; the slope n indicates the effect of concentration on the sorption capacity and represents the sorption intensity. Ni(II) ions were more effectively sorbed by dead cells at low initial metal ion concentrations. The bioaccumulated Ni(II) ion quantities per unit mass of biomass by growing cells at lower concentrations of Ni(II) ions were lower than those by dead cells, because of high microorganism growth of *R. delemar* at the end of 48 h. Comparing sorption isotherms for growing, resting and dead cells shows that well-sorbed Ni(II) ions have steep slopes and were located in the upper sorbed concentration q_{eq} values (Fig. 7). Biosorption is a metabolism-independent process and thus can be carried out by both living and dead cells. The uptake of Ni(II) ions by resting cells of *R. delemar* harvested from the fermentation media at the end of exponential growth phase appeared to be low compared with

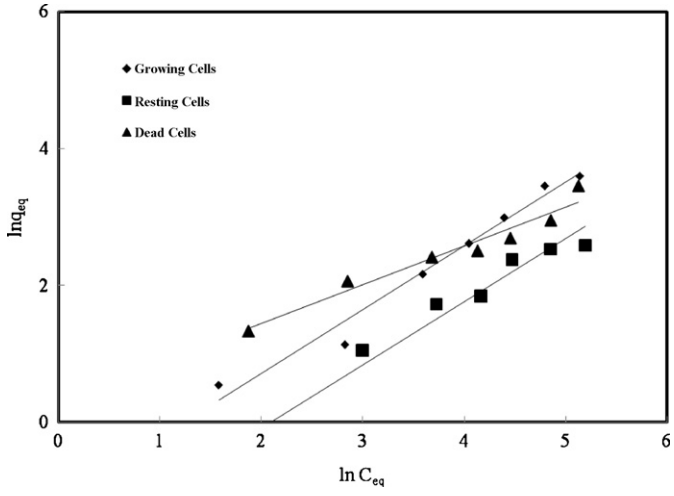

Fig. 7. Freundlich sorption isotherms for the Ni(II) removal of growing, resting and dead cells of *R. delemar* (starch concentration = 20 g/L; pH = 5.0; temperature = 30 °C; incubation time = 48 h).

Table 5
Comparison of the bioaccumulation efficiencies obtained by acid phosphatase mediated Ni(II) uptake of growing cells, resting and dead cells of *R. delemar* (starch concentration = 20 g/L; pH = 5.0; temperature = 30 °C; incubation time = 48 h).

$C_{Ni,i(II)}$ (mg/L)	Bioaccumulation of growing cells		Bioaccumulation of resting cells		Biosorption
	Enzyme activity (μmol/L min)	$Y_{eff.}$ (%)	Enzyme activity (μmol/L min)	$Y_{eff.}$ (%)	$Y_{eff.}$ (%)
10.29	162.70	52.77	10.87	36.15	36.93
25.15	164.40	32.96	9.87	20.36	31.33
50.86	78.00	28.76	7.52	18.54	22.02
74.57	55.46	23.37	6.31	13.80	16.48
100.86	45.47	19.83	5.85	13.08	14.66
147.43	43.47	18.23	5.85	13.18	13.00
200.29	40.41	15.12	3.34	9.98	15.85

the bioaccumulation of growing and dead cells. In this case, Ni(II) removal can also be mediated via the activity of acid phosphatase enzyme, that functions also in non-growing, resuspended cells of *R. delemar*. However, the acid phosphatase activity of *R. delemar* decreased after *R. delemar* was harvested from the growth media. Metal uptake patterns of growing, resting and dead cells of *R. delemar* were almost the same. Although the bioaccumulated Ni(II) ion quantities per unit mass of biomass were increased with increasing Ni(II) ion concentration, higher Ni(II) ion uptake efficiencies were obtained at lower concentrations of Ni(II) ions for all types of biomass. Over 25 mg/L of Ni(II) ion concentration, a decrease in Ni(II) accumulation efficiencies was observed (Table 5).

4. Conclusion

The acid phosphatase mediated bioaccumulation of Ni(II) ions by *R. delemar* during the growth phase and by harvested cells from fermentation media at the end of exponential growth phase were studied. The Ni(II) uptake capacity of growing and resting cells of *R. delemar* was compared with that of dead cells. When the Ni(II) ion concentrations were increased in the range 0–50 mg/L, the specific growth rates of *R. delemar* decreased significantly. The intrinsic kinetic parameters of the Monod growth kinetics in Ni-free medium were found to be $\mu_m = 0.0649\ h^{-1}$ and $K_s = 1.8928$ g/L. The maximum specific growth rates remained the same with the maximum specific growth rate obtained in the absence of the Ni(II) ions while the apparent Monod saturation constants increased in the fermentation media containing the Ni(II) ions at increasing concentrations. For that reason, the inhibition effect of Ni(II) ions on the growth rate of *R. delemar* was found to be a competitive inhibition. The increase of Ni(II) ion concentration to 200 mg/L caused approximately fourfold decrease of acid phosphatase activity. The Michaelis–Menten constant, K_m, and the maximum reaction velocity, v_m, in the absence of Ni(II) ions were determined as 3.17 mM and 833.3 μmol/L min, respectively. The addition of Ni(II) ions decreased significantly the maximum reaction velocity, v_m, but did not affect the affinity of the enzyme complex and the substrate (i.e. K_m values remained approximately constant). Therefore, the inhibition by Ni(II) ions of the acid phosphatase enzyme was considered as noncompetitive inhibition. The bioaccumulation process caused by acid phosphatase enzyme of growing and resting cells and the biosorption by dead cells of *R. delemar* to uptake of Ni(II) ions were compared. Active uptake of Ni(II) ions using growing cells was more efficient than passive uptake using dead cells and resting cells. Although the resting cells show acid phosphatase activity, enzymatic activity was significantly lower than the growing cells. The enhanced Ni(II) uptake of growing *R. delemar* may be due to the participation of acid phosphatases in precipitation of Ni(II) ions away from the responsive cellular sites. The enzyme has resistance to metal inhibition; this is especially pronounced at lower initial Ni(II) ion concentrations. Increasing Ni(II) ion concentrations up to 25 mg/L had little effect on phosphatase activity. As a result, an important advance in the heavy metal removal process may be the identification of the enzyme catalyzing the reactions responsible for metal removal. Although the supply of substrate under physiologically permissive conditions is necessary, these requirements may be less stringent than for metabolizing or growing cells. Furthermore, an enzymic process is easier to optimize and quantify. The principles of Michaelis–Menten kinetics can be applied in a defined system that can be applied to large-scale processes.

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