Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2014.989911

57 (2016) 3689–3699
February

Taylor & Francis
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Investigation of inhibition kinetics of Zn(II) Ions on the acid phosphatase activity and growth of *R. delemar* and Zn(II) bioaccumulation

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Received 3 February 2014; Accepted 4 November 2014

ABSTRACT

This study investigated the production of acid phosphatase enzyme, which plays a direct role in the metal bioaccumulation and growth of *Rhizopus delemar,* in the presence of increasing concentrations of Zn(II) ions. Inhibition of the organism's growth rate by Zn(II) ions was found to be of the partially competitive type, with an inhibition constant of 301.4 mg Zn(II)/L. In addition, Zn(II) ions exhibited a non-competitive mixed-type inhibition on the activity of acid phosphatase. For the mixed-type inhibition, the inhibition constant (K_I) and the binding affinity (α) were determined as 116.67 mg Zn(II)/L and 0.395, respectively.

Keywords: Wastewater treatment; Bioaccumulation; Heavy metal; Acid phosphatase; Inhibition kinetics

1. Introduction

Heavy metals in wastewater are generated by various industries that work with metals and metal components. Such industries include mining, electronics, electroplating, petrochemicals, production of iron and steel and their alloys, paints, plastics, battery manufacturing, textiles, leather tanning and even paper printing [1]. Industrial wastewater can harbour heavy metals such as Cu^{+2}/Cu, Ni^{+2}, Cr^{+3}/Cr^{+6}, Fe^{+2}/Fe^{+3}, Zn^{+2}, Hg^{+2}, Cd^{+2}, Ca^{+2} and K^{+} as well as various anions such as SO_3^{-2}, PO_4^{-3}, Cl^{-},NO_3^{-}, NO_2^{-}, F^{-}, NH^{+4}, SO_3^{-2} and CN^{-} in the mineral form. Recovery of heavy metals from industrial wastewater has been of interest both for mitigation of toxic effects on human health and the environment and for recycling of precious metals for economic benefit [2]. Traditional metal uptake techniques are filtration, ion exchange, reverse osmosis, chemical oxidation and membrane-based methods [3]. However, the poor efficiency and expense of these methods have prompted studies of alternative techniques, including research with micro-organisms capable of biosorption. Many micro-organisms maintain their vitality in heavy metal-contaminated environments resulting from industrial waste, and these organisms acquire resistance to the metals. Additionally, many of those micro-organisms can bioaccumulate the toxic metals [4].

In recent years, the use of quiescent or non-living micro-organisms has gained prominence because these methods are environmentally friendly and cost-effective, and successful results have been achieved [5,6]. During metal uptake by growing cells, both passive and active metal uptake mechanisms are active simultaneously. Metabolism-independent (i.e. passive) uptake is called "biosorption." In this process, anions

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and cations are entrapped on the cellular surface via electrostatic attraction, complexation, ion exchange, physical adsorption and chemical adsorption [7]. Metabolism-dependent (i.e. active) uptake is achieved only by growing cells, and it transports metal ions across the cellular membrane. In bioaccumulation processes, metal ions accumulate inside the cell for use in some metabolic cycles and essential cellular activities. Thus, more rapid and effective metal uptake is achieved by growing cells than by dead cells [8,9].

Zinc is a fundamental element that stimulates microbial growth, especially at low concentrations. Zinc is a cofactor of some enzymes, and it can also act as an enzyme activator. However, at high concentrations, zinc inhibits microbial growth and enzyme production and eventually causes toxic effects on cellular metabolism [10,11]. *Rhizopus Delemar* is a micro-organism that produces definite enzymes such as lipase and acid phosphatase when its fermentation medium includes certain components and is adjusted to the optimum pH value for each enzyme [12,13]. Acid phosphatase enzyme plays an important role in heavy metal uptake [4]. Phosphatases are phosphomonoesterase enzymes catalyse the hydrolysis of COP bonds, and these enzymes are classified as acid or alkaline phosphatase according to their optimum pH value [14]. Metal removal by the cells is mediated by a cell-bound phosphatase that liberates inorganic phosphate from an inorganic phosphate source to precipitate the metal as cell-bound metal phosphate. Acid phosphatase liberates HPO_4^{-2} from an appropriate inorganic or organic phosphate source. Acid phosphatase-mediated metal bioaccumulation is performed by growing and/or quiescent cells that are harvested from the fermentation medium at the end of the growth period [9]. Hydrogen phosphate precipitates stoichiometrically with Zn(II) and binds tightly to the cellular surface as $ZnHPO_4$ [15].

In this study, acid phosphatase production by *R. delemar* during a growth period in the absence and presence of increasing concentrations of Zn(II) ions was measured in stirred batch bioreactors. The role of acid phosphatase enzyme in the bioaccumulation of Zn(II) ions was investigated along with the effects of Zn(II) ions on the growth rate and bioaccumulation properties of *R. delemar*. The inhibition effects of Zn(II) ions on the specific growth rate and acid phosphatase activity were predicted using the Monod and Michaelis–Menten inhibition models to determine the type of inhibition. Inhibition constant values were calculated for both the growth kinetics and the enzyme kinetics. Bioaccumulation and biosorption capacities of growing, quiescent and dead cells were compared. The role of acid phosphatase in bioaccumulation of Zn(II) and growth of *R. delemar* micro-organisms was also investigated for its use in removal of industrial heavy-metal pollution.

2. Theory

2.1. Mathematical description of the experimental system and data evaluation

2.1.1. Calculation of bioaccumulated metal ion quantity

Metal bioaccumulation was calculated from a metal balance yielding:

$$q_{\text{bioaccumulated}} = \frac{C_i - C_f}{X} = \frac{C_{\text{bioaccumulated}}}{X} \tag{1}$$

where $q_{\text{bioaccumulated}}$ is the quantity of bioaccumulated metal ions per unit weight of dried biomass (mg Zn(II)/g dry weight), C_i is the initial metal ion concentration (mg Zn(II)/L), C_f is the residual metal ion concentration in solution (mg Zn(II)/L), X is the concentration of micro-organisms (g/L) and $C_{\text{bioaccumulated}}$ is the concentration of bioaccumulated metal ions (mg Zn(II)/L).

2.1.2. Mathematical modelling of inhibition kinetics for enzyme activity and micro-organism growth

Several substances may cause a reduction in enzymatic reaction rate, and materials that decrease the enzymatic reaction rate are called inhibitors. Inhibitors are classified as either reversible or irreversible. With regard to enzymatic reaction rate and micro-organism growth, reversible enzyme inhibitors are classified as competitive, non-competitive or partially competitive (alternately, "uncompetitive") inhibitors. In competitive inhibition, the reaction rate remains constant as the substrate affinity decreases. In this case, the apparent affinity of the substrate for binding site decreases as the maximum reaction velocity remains constant and K_m increases [16]. Competitive inhibition can be described as follows:

$$v = \frac{v_m S}{K_m\left(1 + \frac{I}{K_I}\right) + S} \tag{2}$$

where I, S and K_I represent inhibitor and substrate concentrations and inhibition constant, respectively.

In non-competitive inhibition, K_m remains constant as maximum reaction velocity, $v_{m,\text{app.}}$, decreases [16].

$$v = \frac{v_m}{\left(1 + \frac{K_m}{S}\right)\left(1 + \frac{I}{K_I}\right)} \tag{3}$$

In partially competitive (uncompetitive) inhibition, both $K_{m,\text{app}}$ and the maximum reaction velocity ($v_{m,\text{app.}}$) decrease [16].

$$v = \frac{v_m S}{\left(\frac{K_m}{\left(1+\frac{I}{K_I}\right)} + S\right)\left(1+\frac{I}{K_I}\right)} \tag{4}$$

Mixed-type non-competitive inhibition is similar to non-competitive inhibition, except that binding of either the substrate or the inhibitor affects the enzyme's binding affinity for the other component. The change in binding affinity is represented by a term, α, in the chemical equation. In mixed-type non-competitive inhibition, if α is greater than 1, then the binding affinity for the substrate reduces in the presence of inhibitor. For mixed-type non-competitive inhibition, the rate equation is the following:

$$v = \frac{v_m S}{K_s\left(1+\frac{[I]}{K_I}\right) + S\left(1+\frac{[I]}{\alpha K_I}\right)} \tag{5}$$

The Lineweaver–Burk equation for mixed-type non-competitive inhibition is the following:

$$\frac{1}{v} = \frac{K_s}{v_m}\left(1+\frac{I}{K_I}\right)\frac{1}{S} + \frac{1}{v_m}\left(1+\frac{I}{\alpha K_I}\right) \tag{6}$$

Due to the α factor, all of the kinetic constants cannot be determined solely from the Lineweaver–Burk plots. To solve this problem, the values of constants were calculated by determining the y-intercepts of (Eq. (7)) and the slopes of Lineweaver–Burk plots (Eq. (8)) at varying concentrations of inhibitors [17]:

$$Y\text{-intercept} = \frac{1}{v_m \cdot \alpha K_I}[I] + \frac{1}{v_m} \tag{7}$$

$$\text{slope} = \frac{K_m}{v_m \cdot K_I}[I] + \frac{K_m}{v_m} \tag{8}$$

Plotting the y-intercepts from Lineweaver–Burk plots vs. [I] reveals a line with a slope of $1/(V_m \alpha K_1)$ and a y-intercept of $1/v_m$. Using the slope of this plot, the value of α can be calculated. Additionally, plotting the slopes of Lineweaver–Burk plots against [I] yields a line with a slope of $K_m/(v_m K_I)$ and an intercept of K_m/v_m. Using the slope of this plot, K_1 can be determined. By analogy to expressions for enzyme inhibition, the expressions for competitive, non-competitive and uncompetitive inhibition of micro-organism growth rates are the following equations:

$$\text{Competitive inhibition} : \mu = \frac{\mu_m S}{K_S\left(1+\frac{I}{K_I}\right) + S} \tag{9}$$

$$\text{Non-Competitive inhibition} : \mu = \frac{\mu_m}{\left(1+\frac{K_S}{S}\right)\left(1+\frac{I}{K_I}\right)} \tag{10}$$

$$\text{Uncompetitive inhibition} : \mu = \frac{\mu_m S}{\left(\frac{K_S}{\left(1+\frac{I}{K_I}\right)} + S\right)\left(1+\frac{I}{K_I}\right)} \tag{11}$$

3. Materials and methods

3.1. Preparation of nutrient medium for phosphatase enzyme production by R. delemar

R. delemar, a filamentous fungus, was obtained from the US Department of Agriculture Culture Collection (NRRL 2872). Flasks containing nutrient media for inoculation were incubated on an orbital shaker at 150 rpm and 30°C for 24 h. The inoculum was developed in a medium containing starch (20 g/L), corn steep liqueur (20 g/L), peptone (10 g/L), K_2HPO_4 (10 g/L) and $MgSO_4{\cdot}7H_2O$ (1 g/L) [4,15]. The pH of the inoculum was initially adjusted to 4.8, which is the optimum pH value for the micro-organism to produce acid phosphatase enzyme. Inoculation was performed when the cells were at the beginning of the exponential growth phase. The optimum inoculum ratio (i.e. the volume of inoculum/production volume of bioreactor) was determined as 10/1,000 [4]. The fermentation medium contained soluble starch (20 g/L) and corn steep liqueur (40 g/L). To determine the type of Zn(II) inhibition on the growth of *R. delemar*, the starch concentration (S, g/L) was varied between 5 and 30 g/L.

3.2. Preparation of bioaccumulation media containing metal ions

Zn(II) solutions were prepared by diluting 1 g/L of stock solutions consisting of metal ions, obtained by dissolving $ZnSO_4{\cdot}5H_2O$ crystals in distilled water. The range of metal ion concentrations in the prepared fermentation media varied between 10 and 100 mg/L. The pH of the fermentation media was adjusted to the

required value by adding 0.1 M H_2SO_4 solution. After metal ion solutions were added, the prepared nutrient media were subjected to autoclave sterilization at 1.2 atm pressure and at 120˚C for 20 min.

3.3. Bioaccumulation of Zn(II) ions by growing R. delemar

Fermentations were performed in an orbital shaker at 30˚C and stirred at 150 rpm for 126 h. To observe the effect of pH on the Zn(II) bioaccumulation and acid phosphatase activity, the bioaccumulation experiments were performed in the pH range of 4–5.5. Samples were withdrawn aseptically at certain time intervals and were centrifuged at 6,030 × *g* for 5 min. Measurement of the residual metal ion concentration in the solution and of the acid phosphatase activity were performed on the supernatant. The precipitated solid phase was used to determine the dry weight of biomass and the biomass concentration in the sample. Two parallel experiments were conducted for each experimental condition.

3.4. Preparation of R. delemar for biosorption experiments

Biosorption experiments were performed to compare metal accumulation capacities of growing, quiescent and dead *R. delemar* cells. After *R. delemar* cultures were grown at 30˚C in stirred batch reactors, the cells were harvested by centrifugation at the end of the exponential growth phase (48–72 h) and then washed twice with distilled water. To obtain dead cells, wet cells were inactivated using a 1% formaldehyde solution followed by drying in an incubator at 60˚C for 24 h. For biosorption studies, 1 g of dry cell mass was suspended in 100 mL of distilled water, and homogenized for 20 min in a homogenizer set at 8,000 rpm. Before the metal solutions were mixed with the micro-organism suspension, the metal solution pH was adjusted to the desired value by adding 0.1 M H_2SO_4. The micro-organism concentration of the media was adjusted to 1 g/L by adding 10 mL of the homogenized micro-organism solution.

3.5. Biomass analysis

To determine the amount of fungal biomass in samples, mycelia taken from the fermentation medium were centrifuged, washed with distilled water and dried at 60˚C overnight until the sample weight was constant. To measure the fungal biomass concentration, 2 mL sample was taken from the fermentation medium, and centrifuged at 5,000 rev/min for 10 min. The remaining biomass in centrifuge tube was diluted to 2 mL with distilled water. Cell concentration (where 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.

3.6. Determination of acid phosphatase activity

Acid phosphatase activity was measured using a spectrophotometric method with p-nitrophenylphosphate (pNPP) as described previously [4]. The reaction mixture contained 15.2 mM p-nitrophenylphosphate, 90 mM citrate solution and 0.1 mL enzyme solution (either filtrate or cell-free extract). One unit of phosphatase activity was defined as the amount of enzyme solution required to liberate 1 μmol p-nitrophenol per minute at pH 4.8 and at 37˚C [18]. The p-nitrophenol released was measured using a spectrophotometer to determine the absorbance at 410 nm.

3.7. Metal analysis

The concentration of free Zn(II) ions in the sample supernatant was determined by measuring the absorbance at 213.9 nm using an atomic absorption spectrophotometer (GBC Avanta Σ) with an HLC Photron Hollow Cathode Lamp.

4. Results and discussion

In this study, the bioaccumulation and/or biosorption of Zn(II) ions by growing, quiescent and thermally/chemically killed *R. delemar* cells were compared. The inhibition types of Zn(II) ions on the specific growth rate and acid phosphatase production of *R. delemar* were determined, and values of the inhibition constants were calculated.

4.1. R. delemar growth curves, effect of Zn(II) ions on the specific growth rate and simultaneous acid phosphatase production

The growth rate of *R. delemar* rapidly increases after the lag phase, which is observed for a few hours after inoculation. The lag phase may extend up to 4 h with increasing concentrations of Zn(II) ions.

After this adaptation period, *R. delemar* cells multiply rapidly, and biomass concentration increases exponentially with time. After 48 h, the growth phase ends

and the deceleration phase, during which the growth rate increases at a smaller acceleration, begins. The maximum biomass concentration obtained was 3.515 g/L at the end of the deceleration growth phase (48–76 h) in the metal-free medium at pH 5. The stationary phase of growth, which follows the exponential growth phase, occurs between 88 and 120 h. In this phase, the maximum biomass concentration remains constant. Increasing concentrations of Zn(II) ions in the medium cause delays in the growth phases. The maximum biomass concentration in the fermentation medium containing 25 mg/L Zn(II) ions was 2.684 g/L, at the end of deceleration growth phase (48 h) (Fig. 1). The deceleration period in the fermentation medium containing 75 mg/L Zn(II) ions shifted to 72 h. At that time, the maximum biomass concentration was 2.218 g/L.

The production of acid phosphatase by *R. delemar* increased with cultivation time. Maximum acid phosphatase production was obtained at the end of the exponential growth phase (48 h), when the maximum micro-organism concentration was also obtained. Depending on culture conditions, acid phosphatase activity was stable for 72 h until the beginning of the stationary phase.

After the beginning of the stationary phase, enzyme activity decreased rapidly. Increasing concentrations of Zn(II) ions in the medium caused reductions in enzyme stability sooner than anticipated. Both the enzyme activity and stability decreased markedly at 75 mg/L Zn(II) ion concentration. The maximum acid phosphatase activity was 176.4 μmol/L-min in the absence of Zn(II) ions. Zn(II) ion concentrations up to 25 mg/L increased the enzyme activity compared to the absence of Zn(II) ions. Maximum enzyme activity was 703.13 μmol/L-min at the end of the deceleration phase and enzyme stability continued at 48th and 72th h.

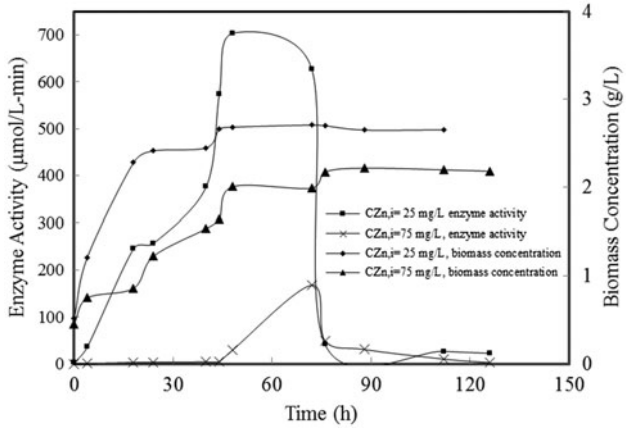

Fig. 1. Time course of acid phosphatase activity and biomass concentration in the fermentation medium containing 25 and 75 mg/L Zn(II) ions (pH 5; C_{starch} = 20 g/L; T = 30°C).

Above 50 mg/L Zn(II) ions, the enzyme activity decreased compared to the activity in the absence of Zn (II) ions. Maximum enzyme activity was 167.77 μmol/L in media containing 75 mg/L Zn(II) ions after 72 h, then, the enzyme activity declined rapidly and stability did not preserve.

The pH of the fermentation medium slightly decreased during growth of *R. delemar*. When the initial pH of the fermentation medium was adjusted to pH 5, maximum acid phosphatase activity was obtained. Then, the pH of the medium decreased from 5 to 4.89 after 48 h of cultivation. The presence of Zn(II) ions in the fermentation medium generally inhibited *R. delemar* growth. Biomass concentration at the end of the exponential growth phase (48 h) decreased from 3.34 to 2.53 g/L with increasing concentrations of Zn(II) ions from 0 to 100 mg/L (Table 1). The presence of 10–25 mg/L Zn(II) ions increased the micro-organism's specific growth rate compared to that in the absence of Zn(II) ions. The biomass concentration and the specific growth rate depend on the concentration of inhibitor, and both the biomass concentration and the specific growth rate decreased as the Zn(II) ion concentration increased. The minimum specific growth rate and biomass concentration at 100 mg/L Zn(II) were 0.0430 h^{-1} and 2.53 g/L, respectively.

4.2. Determination of the inhibition type of Zn(II) ions on the specific growth rate of R. delemar

To determine the effects of Zn(II) ions on the growth of *R. delemar*, the Monod equation was linearized in double-reciprocal form (Fig. 2). A plot of $1/\mu$ vs. $1/S$ gives a line with a slope of K_s/μ_m and a y-intercept of $1/\mu_m$. Table 2 compares the maximum specific growth rate (μ) and the Monod saturation constant (K_s) in the

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 Zn(II) ions (pH 5; C_{starch} = 20 g/L; T = 30°C; $t_{incubation}$ = 48 h)

$C_{Zn,i}$ (mg/L)	Specific growth rate μ (h^{-1})	Biomass concentration (g/L)
0	0.0499	3.34
10	0.0511	2.51
25	0.0582	2.68
50	0.0465	2.57
75	0.0457	2.53
100	0.0430	2.53

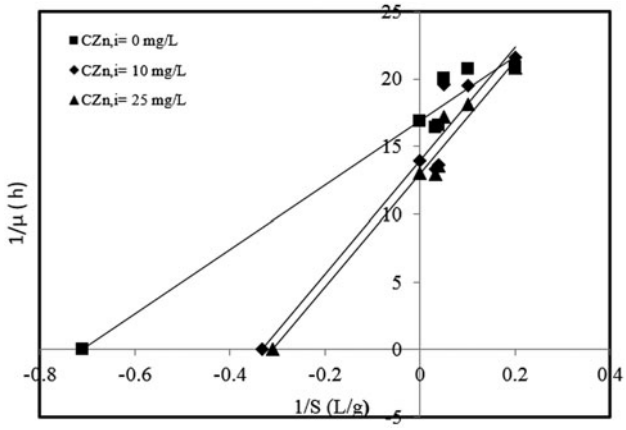

Fig. 2. Double-reciprocal plots of the Monod equation for the growth of *R. delemar* obtained in the Zn(II)-free media and in the presence of increasing concentrations of Zn(II) ions in the range 10–25 mg/L (pH 5; T = 30˚C).

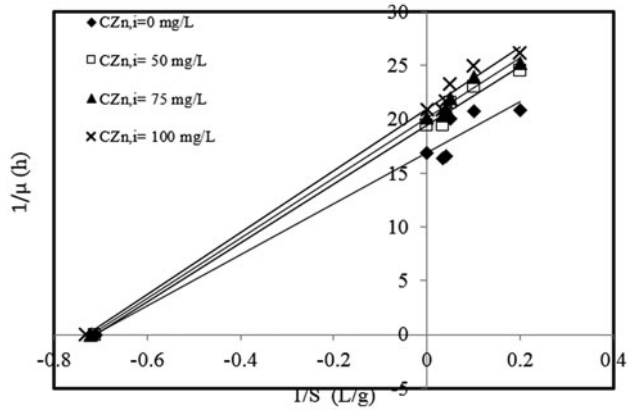

Fig. 3. Double-reciprocal plots of the Monod equation for the growth of *R. delemar* obtained in the Zn(II)-free media and in the presence of increasing concentrations of Zn(II) ions in the range 50–100 mg/L (pH 5; T = 30˚C).

Table 2
Comparison of the maximum specific growth rates and the saturation constants obtained in the Zn(II)-free fermentation medium, with those obtained in the presence of increasing concentrations of Zn(II) ions (pH 5; T= 30˚C)

$C_{Zn,i}$ (mg/L)	$\mu_{m,app}$ (h^{-1})	$K_{s,app}$ (g/L)	R^2
0	0.0649[a]	1.8928[b]	0.982
10	0.0812	4.1007	0.977
25	0.0834	3.9123	0.989
50	0.0534	1.4118	0.953
75	0.0501	1.2674	0.992

[a]Intrinsic maximum specific growth rate.
[b]Intrinsic Monod saturation value.

presence of increasing Zn(II) ion concentrations relative to those obtained using Zn(II)-free medium. Relative to metal-free medium, increased Zn(II) ion concentrations within the 10–25 mg/L range increased both the maximum specific growth rate of the micro-organism and the Monod saturation constant. In this concentration range, the Zn(II) ions can be considered as an activator. From the double-reciprocal plots of the Monod equation, the presence of Zn(II) ions in the concentration range of 50–100 mg/L has a partially competitive (uncompetitive) inhibition effect on the growth of *R. delemar* (Fig. 3).

4.3. Determination of the partially competitive inhibition constant of Zn(II) ions on the specific growth rate of R. delemar

The small value of the saturation constant (i.e. the half-velocity constant), $K_{s,app,}$ shows that the micro-organism has a high binding affinity for the substrate. The net effect of partially competitive inhibition is a decrease in μ_m and in the Monod saturation constant (K_s) that results in a reduced reaction rate. Similarly, decreases in μ_m are more effective in reducing the reaction rate than decreases in K_s. Therefore, the net effect of such an inhibition is a decreased reaction rate. Assuming that the decrease in $K_{s,app}$ with increasing inhibitor concentrations is negligible compared to the decrease in $\mu_{m,app.}$, a plot of $1/\mu_{m,app.}$ vs. inhibitor concentration was drawn and $1/\mu_m$ was estimated from the y-intercept. The value for K_I was estimated from the slope ($1/\mu_m K_1$). The values of μ_m and K_I were determined as 0.0637 h^{-1} and 301.4 mg/L, respectively. The value of μ_m calculated by this method deviated about 1.9% from the experimental (i.e. intrinsic) μ_m obtained in the absence of Zn(II) ions.

4.4. Effect of Zn(II) ions on the acid phosphatase activity of R. delemar

Some heavy metals, including Zn(II), have an activator effect on the enzyme production of micro-organisms. Zn(II) ions affect the acid phosphatase activity of *R. delemar* in two ways. In the lower concentration range (10–25 mg/L), Zn(II) ions act as an activator, while increased Zn(II) ion concentrations (50–100 mg/L) act as an inhibitor. Additionally, the organism's lag phase is extended when Zn(II) ions are added to the medium.

The addition of 10 mg/L Zn(II) ions to the medium increases the acid phosphatase activity visually compared to the activity in absence of Zn(II) (Fig. 4). The activator effect of Zn(II) ions decreases slightly at

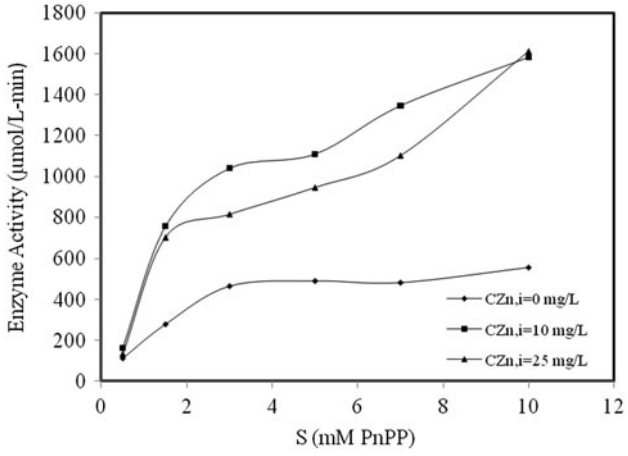

Fig. 4. Change of acid phosphatase activity with p-nitrophenylphosphate concentration in the absence and presence of increasing concentrations of Zn(II) ions (pH 4.8; T = 37˚C).

25 mg/L Zn(II), but the enzyme activity is still higher compared to activity in samples prepared with no added Zn(II). Increasing Zn(II) ion concentrations in the range of 50–100 mg/L leads to inhibition of the enzyme activity. To determine the type of inhibition caused by Zn(II) on the p-nitrophenylphosphate activity of the enzyme in this concentration range, Michaelis–Menten kinetics were applied.

A plot of $1/v$ vs. $1/S$ yields a line with a slope, K_m/v_m, and y-intercept, $1/v_m$. The maximum forward velocities of the reaction, $V_{m,app,}$ were greater for media containing 10 and 25 mg/L Zn(II) ion compared to those obtained in the absence of Zn(II) (Table 3). The value of the Michaelis–Menten constant, $K_{m,app}$, was smaller than that obtained in the absence of Zn(II). The smaller Michaelis–Menten constant indicates a higher affinity of enzyme for the substrate. The values of $V_{m,app}$ and $K_{m,app}$ also affirm the effect of Zn(II) as an activator in the range of 10–25 mg/L.

Conversely, the maximum forward velocities of the reaction, $V_{m,app,}$ decreased as the concentration of Zn(II) was increased to 50–100 mg/L. Additionally, the Michaelis–Menten constants, $K_{m,app}$, increased with increasing Zn(II) ion concentrations (Fig. 5). This type of inhibition is mixed non-competitive inhibition.

Table 3
Comparison of the maximum forward velocities of the reaction and the Michaelis–Menten constants obtained in the Zn(II)-free fermentation medium, with those obtained in the presence of increasing concentrations of Zn(II) ions (pH 4.8; T = 37˚C)

$C_{Zn,i}$ (mg/L)	$v_{m,app.}$ (µmol/L-min)	$K_{m,app.}$ (mM)	R^2
0	833.3[a]	3.17[b]	0.990
10	1,000.0	2.00	0.971
25	909.1	2.73	0.939
50	769.2	3.77	0.933
75	625.0	4.37	0.964
100	588.2	4.88	0.977

[a]Intrinsic maximum forward velocity of the reaction.
[b]Intrinsic Michaelis–Menten constant.

4.5. Determination of the mixed non-competitive inhibition constant for Zn(II) ions' influence on acid phosphatase activity

In mixed non-competitive inhibition, the α factor in Eq. (6) prevents solving for all of the kinetic constants via only the Lineweaver–Burke plot. To solve this problem, re-plots of the slope and the y-intercept at varying concentrations of I were obtained. $K_{m,intrinsic}$ and $v_{m,intrinsic}$ were found from the original double reciprocal plot in the absence of Zn(II) ions in the medium. The value of K_I was determined as 116.67 mg/L from the slope of the re-plot (Eq. (8)). The binding affinity, α, was found to be 0.395 from the slope of the y-intercept re-plot (Eq. (7)).

Huang and Shindo [19] studied the effect of heavy metal ions in soil on free and immobilized acid phosphatase activity [19]. Addition of copper chloride decreased both the K_m values and the v_m/K_m ratios of free and all forms of immobilized enzymes, and the system exhibited mixed-type inhibition kinetics. Abdallah et al. [20] obtained acid phosphatase from *Lactobacillus plantarum* DPC2739, and investigated the

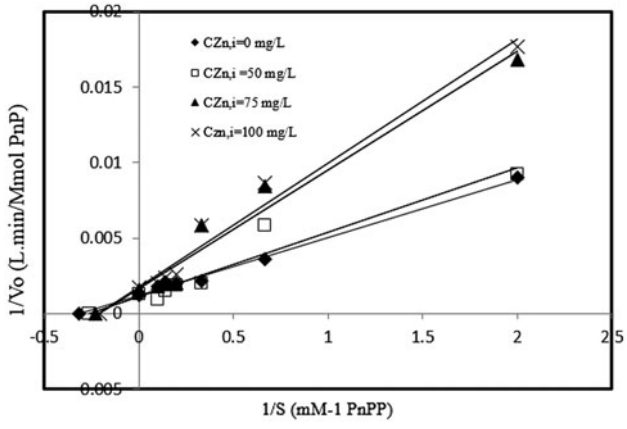

Fig. 5. Lineweaver–Burk plots for acid phosphatase enzyme in the absence and presence of increasing concentrations of Zn(II) ions in the range 50–100 mg/L (pH 4.8; T = 37˚C).

inhibition effects of heavy metals. For enzyme production, the optimum pH range was found to be 3.5–5.5. In this pH range, the investigators determined the inhibition effects of $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, MgO_2, $MnCl_2$ and $ZnCl_2$ on acid phosphatase activity. They found that $ZnCl_2$ and MgO_2 do not change the activity, $CoCl_2$ reduces the activity by 4% and the other metals somewhat increase the activity [20].

4.6. Zn(II) bioaccumulation by R. delemar

The change in bioaccumulated Zn(II) ion concentration (mg/L) and the change in bioaccumulated Zn(II) ion quantities per unit mass of biomass (mg Zn(II)/g dry weight) with changes in pH are shown in Fig. 6. The maximum bioaccumulation of Zn(II) ions was obtained over a pH range of 5–5.5. As micro-organism growth was higher and metal bioaccumulation was lower compared to those observed at pH 5–5.5, a lower value of q was obtained for pH 4.5. Maximum acid phosphatase activity was also obtained at pH 4.5–5, whereas biomass concentration reached its maximum at pH 4.5–5.5 (Fig. 7).

Maximum acid phosphatase activity was also observed at the end of the exponential growth phase for which the maximum biomass concentration was obtained. The bioaccumulated Zn(II) ion concentration increased slowly during lag phase and then, rapidly during exponential growth, reaching a maximum value after 48 h. As acid phosphatase mainly mediates Zn(II) bioaccumulation, this was an expected result. The bioaccumulated Zn(II) ion concentration remained approximately constant through both the deceleration

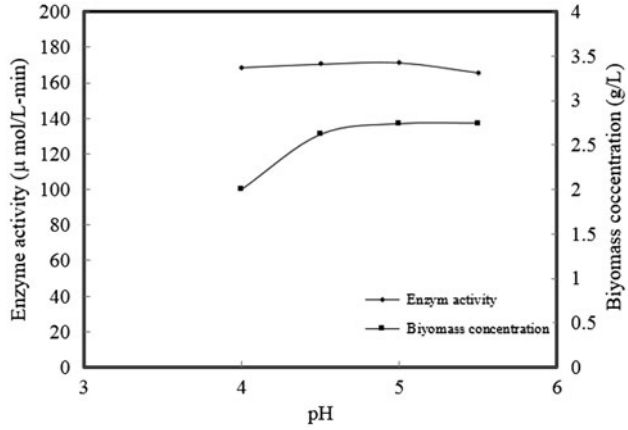

Fig. 7. Change of enzyme activity and biomass concentration with initial pH (C_{starch} = 20 g/L; T = 30°C; $C_{Zn,i}$ = 50 mg/L; $t_{incubation}$ = 48 h).

growth phase and the stationary growth phase. As the concentration of young cells was relatively low, a step-change increase in the bioaccumulated Zn(II) ion quantities per unit mass of biomass was observed in the lag phase and early exponential growth phase. When micro-organism concentration increased and reached an approximately constant value, the increase in bioaccumulated Zn(II) ion quantities per unit mass of biomass with time decelerated (Fig. 8).

4.7. Comparative Zn(II) uptake capacities of growing, quiescent and dead R. delemar cells

The Zn(II) sorption capacities and sorption isotherm compliance of growing, quiescent and dead

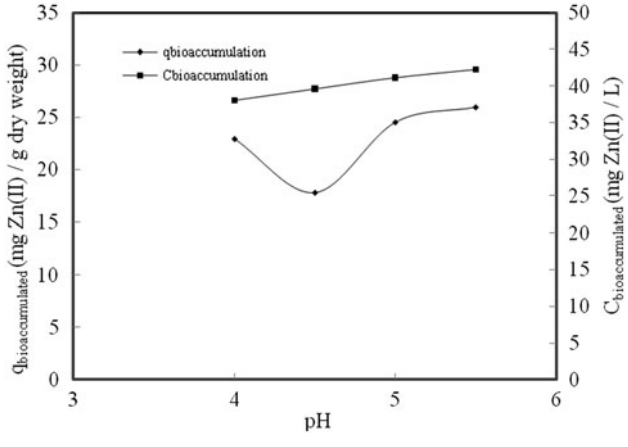

Fig. 6. Change of bioaccumulated Zn(II) ion concentration and bioaccumulated Zn(II) ion quantities per unit mass of biomass on dry weight basis with initial pH (C_{starch} = 20 g/L; T = 30°C; $C_{Zn,i}$ = 50 mg/L; $t_{incubation}$ = 48 h).

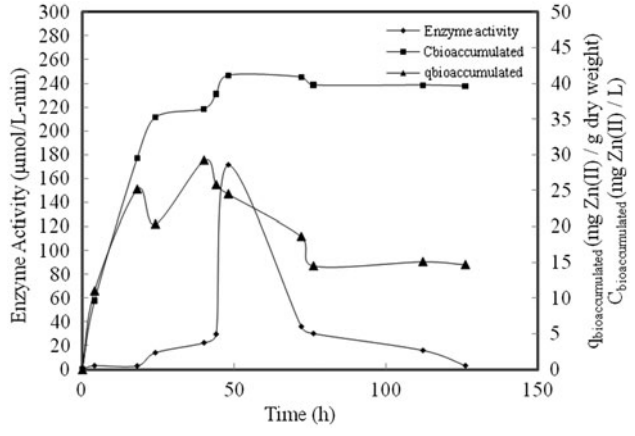

Fig. 8. Change of bioaccumulated Zn(II) ion concentration, bioaccumulated Zn(II) ion quantities per unit mass of biomass on dry weight basis and enzyme activity with time (C_{starch} = 20 g/L; T = 30°C; $C_{Zn,i}$ = 50 mg/L).

Table 4
Comparison of bioaccumulated Zn(II) ion concentration, quantity of bioaccumulated Zn(II) ion concentration per unit mass in the basis of dry weight and bioaccumulation efficiency obtained by acid phosphatase-mediated Zn(II) uptake of growing, resting and dead cells of *R. delemar* (pH 5; T = 30 °C; C_{starch} = 20 g/L; $t_{incubation}$ = 48 h)

	Bioaccumulation of growing cells				Bioaccumulation of resting cells				Biosorption			
$C_{Zn,i}$ (mg/L)	Enzyme activity (μmol/L-min)	$C_{bioaccum}$ (mg/L)	$q_{bioaccum}$ (mg/g)	Y_{eff} (%)	Enzyme activity (μmol/L-min)	$C_{bioaccum}$ (mg/L)	$q_{bioaccum}$ (mg/g)	Y_{eff} (%)	Enzyme activity (μmol/l- min)	$C_{bioaccum}$ (mg/L)	$q_{bioaccum}$ (mg/g)	Y_{eff} (%)
9.99	756.63	7.69	3.06	76.98	11.98	4.74	2.66	48.07	1.671	4.64	4.64	46.4
25.15	703.13	15.02	5.59	74.99	21.40	7.03	4.02	34.89	3.42	11.23	11.23	41.81
50.86	171.39	41.11	24.5	80.83	20.34	15.75	7.25	31. 3	2.62	16.04	16.04	38.94
74.01	167.77	51.82	26.03	69.90	11.70	18.89	8.41	25.52	2.39	25.28	25.28	32.18
100.10	58.52	58.85	26.12	58.82	13.66	23.31	12.15	23.34	1.03	31.22	31.22	31.22

R. delemar cells were evaluated. The bioaccumulation capacity was greatest for growing cells, followed by dead and resting cells. This capacity was evaluated by considering the bioaccumulated Zn(II) ion concentration, the bioaccumulated Zn(II) ion quantity per unit mass (dry basis) and the bioaccumulation efficiency (Table 4). According to these results, Zn(II) removal takes place mainly by bioaccumulation via the acid phosphatase enzyme. Because living cells have independent mechanisms (e.g. physical adsorption on cellular surface, ion exchange, electrostatic interaction, chelation and bioaccumulation processes) from the organism's essential metabolic functions, and dependent mechanisms (e.g. precipitation of metal as cell-bound metal phosphate via acid phosphatase activity of *R. delemar*), the high metal uptake observed is an expected result.

In another study published by Evirgen and Açıkel [21], *R. delemar* was grown in sucrose-molasses medium containing 0.5 g/L KH_2PO_4. The pH of the fermentation media was adjusted to 5. In this medium, *R. delemar* does not produce acid phosphatase enzyme. The Zn(II) ion bioaccumulation capacity of *R. delemar* grown in sucrose-molasses medium containing a few amount of KH_2PO_4 was compared with that of grown in starch-corn steep liqueur medium containing excess amount of KH_2PO_4 (10 g/L KH_2PO_4 was added to inoculum to produce acid phosphatase by *R. delemar*). Higher bioaccumulated Zn(II) ion concentration of 58.85 mg/L and efficiency of 58.79% at 100.1 mg/L initial Zn(II) ion concentration were obtained in starch-corn steep liqueur medium in the present study. On the other hand, the bioaccumulated Zn(II) ion concentration and efficiency at the same initial Zn(II) ion concentration of 100 mg/L decreased to 21.65 mg/L and 21.65% in sucrose-molasses medium.

Although the enzyme activity is high for lower Zn(II) ion concentrations (10–25 mg/L), the high rate of micro-organism growth results in small amounts of bioaccumulated Zn(II) ions per unit mass. By contrast, there are several advantages to work with dead cells. Removal of inhibition by high metal concentrations and increased adsorption capacity via physical and chemical processes as the micro-organism is inactivated are some of these advantages. As *R. delemar* cells are taken from the nutrient medium and centrifuged, the acid phosphatase activity is considerably reduced. Therefore, wet cells have low bioaccumulation capacity.

Fits of Zn(II) sorption equilibrium data with adsorption models were investigated for growing, resting and dead *R. delemar* cells to compare Zn(II) bioaccumulation capacity and intensity. The best correlations between experimental and model-predicted equilibrium uptake data were obtained using the

Table 5
Freundlich sorption constants for the Zn(II) removal of growing, resting and dead cells of *R. delemar* (pH 5; T = 30°C; C_{starch} = 20 g/L; $t_{incubation}$ = 48 h)

	K_F ($mg^n\ g^{-1}\ L^n$)	n
Growing cells	1.8094	1.2771
Resting cells	0.9579	1.7921
Dead cells	1.6703	2.004

Freundlich model ($q_{eq} = K_F C_{eq}^{1/n}$; [22]). In terms of the Freundlich adsorption capacity constant, K_F, the greatest Zn(II) bioaccumulation was obtained by growing cells followed by dead cells and resting cells (Table 5). The slope of the Freundlich adsorption isotherm, $1/n$, shows the effect of concentration on sorption and is a good measure of sorption intensity. The Freundlich adsorption isotherms obtained for well-sorbed Zn(II) ions have a steep rising slope, and sorbed concentrations corresponded to higher $q_{eq,values}$, indicating that growing cells have the highest Zn(II) bioaccumulation intensity.

5. Conclusions

- Double-reciprocal Monod plots were used to determine the inhibition type of Zn(II) ions impacting the growth rate of *R. delemar*. Increased Zn(II) concentration in the range of 50–100 mg/L results in partially competitive inhibition. The partially competitive inhibition constant of Zn(II) ions acting on the specific growth rate of *R. delemar* was 301.4 mg/L.
- The effect of Zn(II) ions on the acid phosphatase activity of *R. delemar* over a Zn(II) concentration range of 0–100 mg/L was investigated, and 50 mg/L Zn(II) concentration was observed to cause inhibition. Inhibition kinetic models were applied for 50–100 mg/L Zn(II) ion concentrations.
- The enzymatic reaction rate decreased as Zn(II) ion concentration increased, and the values of the corresponding Michaelis–Menten constants increased. Such inhibition is known as uncompetitive mixed inhibition. The inhibition constant for Zn(II) ions, K_I, and the change in binding affinity, α, were 116.67 mg/L and 0.395, respectively, for mixed-type inhibition.
- The maximum Zn(II) ion bioaccumulation by *R. delemar* was obtained over a pH range of 5–5.5. For initial Zn(II) ion concentration at 50 mg/L during cultivations at pH 5 and 25°C, bioaccumulation increased slowly during the lag phase, then rapidly during the exponential phase. The maximum Zn(II) bioaccumulation was obtained at 48 h. The best correlation between experimental and model-predicted equilibrium uptake data was obtained using the Freundlich isotherm. Using the values of K_F, Zn(II) bioaccumulation was the highest for growing cells (1.8094), followed by dead cells (1.6702).
- This study proves that acid phosphatase enzyme produced by *R. delemar* enhances the bioaccumulation of Zn(II) ions. Living or dead *R. delemar* cells, which are widely used in food and pharmaceutical industries, will be an important resource for the economical, efficient removal of heavy metal pollution in wastewater.

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