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Investigation of mutual interactions of physicochemical parameters on simultaneous Zn(II) bioaccumulation and lipase production of *R. delemar*

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ABSTRACT

In this study, while *Rhizopus delemar* removes Zn(II) ions in the wastewater with the help of bioaccumulation mechanisms, the effects of increasing concentrations of Zn(II) ions both on micro-organism growth and lipase enzyme production which biodegrades lipids were investigated. The parameters which affect micro-organism growth, lipid biosynthesis, and Zn(II) bioaccumulation were determined as pH, temperature, and initial Zn(II) ion concentration. In single factor experiments, pH and temperature were studied in the ranges of 5.0–8.0 and 25–35°C, respectively, while initial Zn(II) ion concentration was changed between 5 and 200 mg/L. At pH 5.0, and temperature 35°C, maximum Zn(II) removal was obtained as 26.31 mg/L in the bioaccumulation medium containing 30 mg/L Zn(II) initially. The lipase activities and micro-organism concentrations obtained in Zn(II)-free and the preceding media were 656 and 537 U/L, 1.16 and 0.82 g/L, respectively. When the biomass concentration was kept constant at a required level, in order to maximize the bioaccumulation of Zn (II) and lipase biosynthesis, a statistical experimental design was applied. Maximum Zn(II) bioaccumulation was obtained as 32.38 mg/L at pH 6.8, 33.8°C and 41 mg/L initial Zn(II) concentration. At this optimum point, it was determined that lipase activity is 586 U/L and micro-organism concentration is 0.62 g/L.

Keywords: Wastewater treatment; Heavy metal; Bioaccumulation; Lipase enzyme; *Rhizopus delemar*

1. Introduction

Heavy metal ions which cannot be eliminated by spontaneous degradation in nature generate a highly dangerous pollution in terms of both human health and ecosystem. On the other hand, lipid pollution is available particularly in food, milk and milk products industries, domestic and restaurant wastewaters, slaughter house wastewaters, and domestic sewage disposal [1,2]. Lipid films occurred on the surface of water inhabit the oxygen diffusion from air to water and cause the death of several species living in water [3,4]. Heavy metal ions directly mixed with domestic wastewater including high concentration of lipids lead to very serious environmental pollution. This pollution

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contaminates firstly soil and then ground water, thus poses a serious threat to any living form. Moreover, industrial wastewater like wool scouring, oils and lubricants, leather, and textile production includes high concentrations of both lipid and heavy metal ion pollution [5–7].

Rhizopus delemar is a widely used micro-organism in fermentation and food industries. Living and dead cells of *R. delemar* by bioaccumulation and biosorption mechanisms have been used in the removal of heavy metals from aquatic media. From this aspect, it has been the subject of many scientific studies. On the other hand, it is known that *R. delemar* micro-organism produces lipase enzyme. Lipase enzyme also known as triacylglycerol ester hydrolase belongs to the class of serine hydrolases that catalyze the breakdown of triacylglycerol to diacylglycerol, monoglycerol, free fatty acids, and glycerol, which is very important in technological point of view. The use of lipase enzyme which degrades lipids in the wastewater treatment is an issue only recently started to be studied [8–10]. However, the usage of free or immobilized forms of limitedly produced enzymes in wastewater treatment is very expensive and cannot be evaluated as a realistic process [11–13]. Direct use of crude lipase enzyme which was produced during the growth period of *R. delemar* without the need for purification and downstream processes in biodegradation of lipids seems to be more appropriate. Until now, simultaneous lipase production and heavy metal bioaccumulation by *R. delemar* have not been investigated in any scientific researches. In this study, it is firstly investigated, how the bioaccumulation of a specific metal ion affects lipase activity during the growth period of micro-organism and in what pattern the metal bioaccumulation is occurred under the lipase producing conditions of micro-organism.

Metal removal bioprocesses are separated in two categories: (i) biosorptive (passive) removal performed by dead and/or nongrowing (resting cells) biomass or biomass products and (ii) bioaccumulation by living cells [14]. First mechanism contains surface binding occurred on the surface of cell membrane and exterior cellular capsule entrapment, adsorption, precipitation, complexing, and oxidation-reduction reactions [15]. In the second mechanism, metal ions penetrate into the cell passing through the cell membrane and participate in biochemical pathways [16]. Living cells, by using both biosorption and bioaccumulation mechanisms, remove metal ions from the aqueous media [17]. Until today, the removal of metal ions by micro-organisms was performed by dead cells, while biosorption process was deeply investigated; very few studies were carried out by bioaccumulation mechanisms with living cells [18,19]. For metabolic activities, fundamental metals are necessary as co-factors of the enzymatic reactions. These metals also affect as activators for some enzymes; however, at high concentrations, they cause inhibition on metabolic reactions, micro-organism growth and enzyme production, thus have eventually toxic effects on cellular metabolism [20–22]. Zinc, copper, nickel, and cobalt ions are the fundamental metals included into metabolic activities. There are a few studies published in the literature on the effects of heavy metal ions on lipase activity and these studies have results that generally conflict on each other. According to the related literature, an extracellular lipase from *Pseudomonas aeruginosa* KKA-5 to start hydrolysis of castor oil in the presence of various metal chlorides was investigated [23]. It was indicated that $AlCl_3$, $CrCl_3$, and $MgCl_2$ except $CaCl_2$ are frequently used for castor oil hydrolysis and these increase hydrolysis capability. Reported results referring the effect of heavy metals on lipase activity are sometimes exactly opposite of each other. However, Ca(II) and Mg(II) ions are generally reported as lipase activator; and it was recorded that Hg(II) and Fe(II) ions are strong lipase inhibitors and Ag(I), Al(III), Mn(II), Sn(II), Zn(II), Fe(III), and Cu(II) ions show generally inhibition effect on lipase activity [24–26].

In this study, the bioaccumulation of Zn(II) ions by *R. delemar* micro-organism and the effects of Zn(II) ions bioaccumulation on micro-organism growth and lipase activity have been studied at different pH and temperatures while changing the initial metal ion concentration. The first part of this study was performed varying one parameter at a time and keeping the others constant. Using only one-at-a-time method, optimum values of the process conditions and interactive effects between different physiological and nutritional parameters cannot be determined exactly. As the optimization of process conditions in these kind of systems having several parameters directly affects process efficiency and economy, in the second part of the studies, experimental factorial design and response surface methodology (RSM) were applied in order to maximize the Zn(II) ions bioaccumulation and lipase biosynthesis while holding the biomass concentration within a desired level.

2. Materials and methods

2.1. Micro-organism and growth medium

R. delemar which was obtained from US Department of Agriculture with culture code of NRRL 2872 was produced at 30°C in shaking liquid nutrient

mediums. The complex liquid nutrient medium is composed of the following components (g/L): sucrose 5; molasses sucrose 1; K_2HPO_4 0.5; KH_2PO_4 0.5; $MgSO_4.7H_2O$ 0.2, and yeast extract 2. In order to induce lipase enzyme production of the micro-organism and to increase the secretion of fermentation medium, 0.5% (v/v) sunflower oil as inducer and 1.0% (v/v) Tween 80 as surfactant were added to the medium. 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 5/1,000, and all inoculum to the fermentation media was performed at this ratio.

Lipase fermentation and simultaneous bioaccumulation of Zn(II) ions with *R. delemar* were carried out in an orbital shaker at a constant temperature under stirring rate of 150 rpm for 9 d. Samples were taken aseptically at certain time intervals from fermentation media, then centrifuged at 6,030 g for 5 min, and the supernatant liquid was analyzed for Zn(II) ion concentration and lipase activity. The precipitated cells were used for the determination of the dry weight of the biomass and the biomass concentration.

2.2. Analysis of biomass concentration, lipase activity, and free Zn(II) concentration

Samples taken from the fermentation media for the biomass concentration analysis were filtered via filter paper in order to determine the biomass dry weight. Biomass retained on the filter paper was dried in an oven adjusted to 60°C until it reached constant weight. In the second analysis method, samples taken from the fermentation medium were centrifuged. Biomass remaining in the centrifuge tubes was diluted with distilled water until it reached volume of the sample taken. Cell concentration was measured with the spectrophotometric method at 600 nm wave length. By using absorbance/cell concentration and wet weight/dry weight calibration lines, biomass concentration was determined on the basis of dry weight. Lipase activity in the culture filtrate was analyzed spectrophotometrically using p-nitrophenylpalmitate as the substrate that was published previously [27,28]. One international unit of lipase was defined as the amount of enzyme required to release 1 μM of p-nitrophenol or fatty acid per minute at 37°C and pH 8.5. 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 (AAS) (Thermo Scientific ICE 3000 Series) with an air-acetylene flame. For atomic absorption analysis, pH of the supernatant liquid was adjusted to 2.0 with 1% HNO_3. Calibration curve for Zn(II) was constructed using atomic absorption standard solutions that were diluted into 1% HNO_3. Prior to AAS measurement, the supernatant liquid samples were diluted to $3.8–15.3 \times 10^{-3}$ mM for Zn(II) analyses.

2.3. Experimental design

The variation and mutual interactions of three different and independent parameters (initial pH, temperature, and initial Zn(II) ion concentration) were investigated using a central composited design (CCD) method within a RSM [29] in order to manage the optimization of Zn(II) bioaccumulation, lipase production, and *R. delemar* growth simultaneously. Identification of initial pH, temperature, and concentration levels of Zn(II) which have significant effects on Zn(II) bioaccumulation, lipase production and *R. delemar* growth were gathered via one-at-a-time approach earlier with former experiments. The experiments were performed in 250 mL Erlenmeyer flasks containing 200 mL complex growth media while orbitally rotating at 150 rpm. Samples from the fermentation media were taken periodically in order to measure the concentration of unabsorbed Zn(II) ions, lipase activity, and biomass concentration.

A total of 20 experiments were set to obtain maximum bioaccumulation of Zn(II) ions and simultaneous lipase production by *R. delemar*. The five-level-three-factor CCD has been employed in this study, requiring 20 experiments, consisting of three factorial (2^3) designs, six replications at the central points, and six star points. Coding of the parameters were done at five different levels which are $-\alpha$, −1, 0, +1, and $+\alpha$ [30,31]. The ranges of pH, temperature, and initial Zn (II) ion concentration were determined in the preliminary single-factor experiments. In developing the regression equation, the independent variables are coded according to the equation:

$$x_i = \frac{X_i - X_o}{\Delta X_i} \quad (1)$$

where x_i and X_i are the coded and uncoded value of the independent variable i, respectively; whereas uncoded value of the independent variable i at the center point and the step change value are represented by X_o and ΔX_i, respectively [32–34]. The independent variable pH (x_1) has coded in level ranges of 5.00, 5.61, 6.50, 7.39, and 8.00 for $-\alpha$, −1, 0, 1, and α, respectively, while variable T (°C) (x_2) has coded in levels of 24.27, 27.00, 31.00, 35.00, and 37.73, as well as variable

$C_{Zn,i}$ (mg/L) (x_3) has coded in levels as 5.06, 14.20, 27.60, 41.00, and 50.14 for $-\alpha$, -1, 0, 1, and α, respectively.

Experiments were carried out in a random order in duplicate, the mean value of duplicate data obtained from the bioaccumulation and enzyme production media was used in the regression analysis. The responses Y_1, Y_2, and Y_3 were measured at the end of 96 h of growth period for which the maximum values of Zn(II) bioaccumulation, lipase activity, and biomass concentration were obtained.

A mathematical model, following a second-order polynomial equation, was developed to describe the relationships between the predicted response variables, lipase activity (Y_1), bioaccumulated Zn(II) ion concentration (Y_2), and biomass concentration (Y_3) and the independent variables of lipase production, bioaccumulation, and micro-organism growth, as it is shown in Eq. (2):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (2)$$

where Y is the predicted response variable; β_0, β_i, β_{ii}, β_{ij} the intercept, linear, quadratic, and interaction constant coefficients of the model, respectively; x_i, x_j ($i = 1, 3$; $j = 1, 3$; $i \neq j$ represent the coded independent variables.

The software of Design Expert 8.0.0 (Stat Ease Inc. Minneapolis, USA) was used to design and regress the experimental data. The significance of the data was tested using an analysis of variance (ANOVA) statistical test. The lack-of-fit test was used to determine whether the constructed model was adequate to describe the obtained data. Response surfaces and contour plots were obtained using the fitted model, by keeping one independent variable at a constant value while changing the other two variables.

3. Results and discussion

3.1. Simultaneous lipase production and Zn(II) bioaccumulation by growing cells of R. delemar*: single-factor experiments*

3.1.1. Growth curves of R. delemar *in the Zn-free medium and in the presence of increasing concentrations of Zn(II) ions and simultaneous lipase production*

The initial concentrations of Zn(II) ions were varied between 0 and 200 mg/L, while the initial pH of the fermentation medium was held constant at pH 5.0 and 8.0, which was the most appropriate pH range for *R. delemar* growth, lipase production, and Zn(II) bioaccumulation. In the same way, the single-factor experiments were carried out at 25 and 35˚C.

The growth and simultaneous lipase production curves of *R. delemar* at pH 5.0 and temperature 25˚C in the medium containing 30 mg/L initial Zn(II) ion concentration and in the Zn(II)-free fermentation medium are given in Fig. 1(a). The lag phase of *R. delemar* growth was observed immediately after the inoculating and ended within 4–8 h. The lag phase was monitored to prolong until the end of 16th hour with increasing concentrations of Zn(II) ions. After this adaptation period, the growth rate of *R. delemar* showed a rapid increase, and the exponential growth phase began in which biomass concentration or cell number increases exponentially with time. After 48–72 h, the exponential growth phase ended and then deceleration phase began. In the deceleration phase, growth rate increases slowly compared to the exponential growth phase. The stationary phase started at the end of the deceleration phase, when the net growth rate is zero or when the growth rate is equal to the death rate. The stationary phase of *R. delemar* continued between 96 and 168 h. In this phase, the maximum biomass concentration was obtained and remained approximately stable. The maximum biomass concentrations were obtained as 1.09 and 1.16 g/L on the day 4 and 5 of growth, at pH 5.0 and temperature 25 and 35˚C, respectively, in the metal-free medium. In the presence of 5 mg/L Zn(II) ion concentration, a very small increase in biomass concentration was observed. The maximum biomass concentration in the fermentation medium containing 5 mg/L Zn(II) ions at pH 5.0 and 25˚C was obtained as 1.12 g/L at the end of 96th hour. The presence of increasing concentrations of Zn(II) ions in the medium caused delay in growth phases and decrease in biomass concentration. In the fermentation medium containing 200 mg/L Zn(II) ions, the beginning of the stationary phase shifted to the end of 120th hour, and at that time the maximum biomass concentration decreased to 0.43 g/L.

Lipase enzyme production of *R. delemar* started at the beginning of the exponential growth phase, and then increased in a continuous manner with the cultivation time until midway through the stationary phase of growth period. The highest lipase enzyme activity was gained after 4 d of culture. During the stationary phase of growth (96–168 h), lipase activity maintained a stable position, and then showed a rapid decrease at the beginning of the death phase. Lipase can be thought as a growth-associated product produced simultaneously with microbial growth. Maximum lipase activities in metal-free medium at pH 5.0 were determined as 589 and 656 U/L at 25 and 35˚C, respectively. The lipase activity as well as micro-organism growth was inhibited in the presence of increasing concentrations of Zn(II) ions. Maximum lipase activity

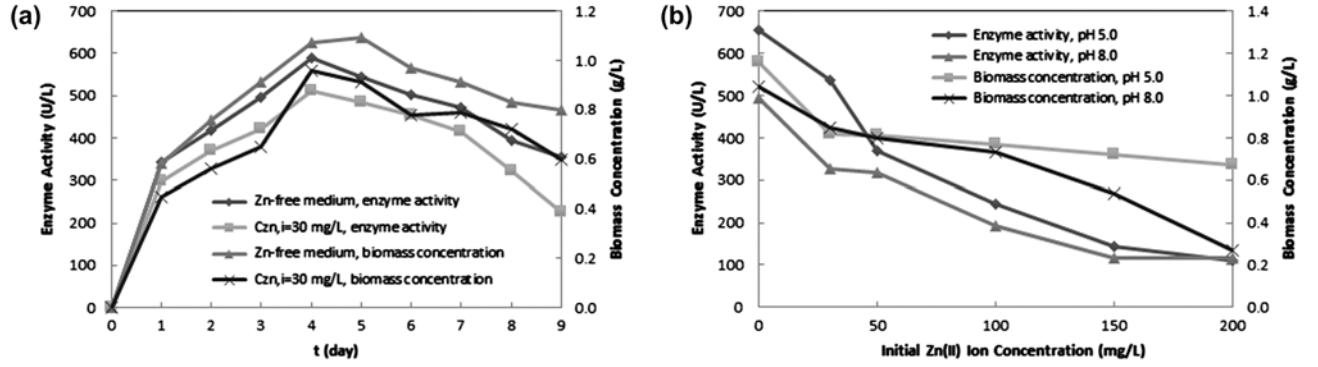

Fig. 1. (a) Growth and simultaneous lipase production curves of *R. delemar* at pH 5.0 and 25˚C in the medium containing 30 mg/L initial Zn(II) ion concentration and in the Zn(II)-free fermentation medium and (b) Change of enzyme activity and biomass concentration with initial Zn(II) ion concentration at pH 5.0 and 8.0 and at 35˚C.

in the presence of 30 mg/L Zn(II) ion concentration at pH 5.0 and 35˚C was obtained as 537 U/L. The lipase activity and biomass concentration at all of the studied pH and temperatures decreased with increasing initial Zn(II) ion concentrations between 30 and 200 mg/L (Fig. 1(b)). As seen in Table 1, both at 25 and 35˚C, higher biomass concentration and lipase activity in the presence of 30 mg/L Zn(II) ion concentration and in the absence of Zn(II) ions obtained at pH 5.0 were compared to those obtained at pH 8.0. On the other hand, it was seen that the lipase activity at pH 8.0 and 25˚C was higher than that of 35˚C, while the maximum lipase activity at pH 5.0 was obtained at 35˚C. It was concluded that the mutual interactions of pH and temperature parameters change the lipase activity and biomass concentration together. Using one-at-a-time method, it is not possible to determine a precise pH and temperature optimum maximizing both the lipase activity and biomass concentration. It should be also noted that the main dependent variable requested to investigate the effect on the lipase activity and growth of *R. delemar* was Zn(II) bioaccumulation.

3.1.2. Zn(II) bioaccumulation and simultaneous lipase production and growth of R. delemar

The bioaccumulated Zn(II) ion concentration increased during lag phase slowly and exponential growth phase rapidly, and reached a maximum value at the beginning of the stationary phase (Fig. 2). Then, the bioaccumulated Zn(II) ion concentration remained approximately stable through stationary growth phase. A bioaccumulation equilibrium between the residual Zn(II) concentration in solution and the sorbed Zn(II) concentration on surface and inside of *R. delemar* cells was reached through stationary growth phase. Maximum metal bioaccumulation was acquired in the 96th hour of the fermentation when both maximum enzyme activity and maximum micro-organism concentration were also achieved and stayed approximately stable until the end of 144th hour. In the beginning of death phase of growth (168 h), the bioaccumulated Zn(II) ion concentration began to decrease, the release of Zn(II) ions was detected following the metal uptake and stationary phase of growth or equilibrium stage.

Table 1
Comparison of biomass concentration and enzyme activity in bioaccumulation media containing Zn(II) ions and Zn (II)-free at different pH and temperatures

	Biomass concentration (g/L)		Enzyme activity (U/L)	
	$C_{Zn,i} = 0$ mg/L	$C_{Zn,i} = 30$ mg/L	$C_{Zn,i} = 0$ mg/L	$C_{Zn,i} = 30$ mg/L
pH 5.0—25˚C	1.07	0.96	589	510
pH 8.0—25˚C	0.95	0.61	566	488
pH 5.0—35˚C	1.16	0.82	656	537
pH 8.0—35˚C	1.04	0.85	495	326

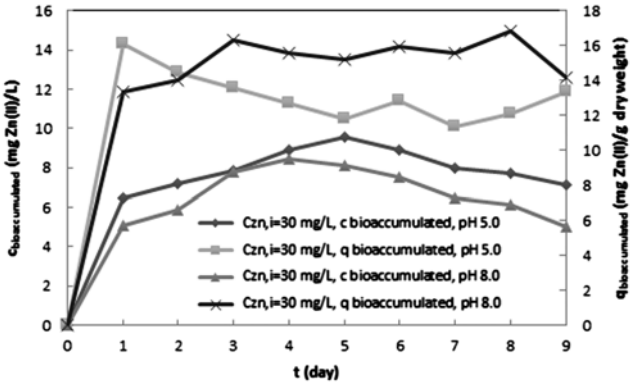

Fig. 2. Time course of bioaccumulated Zn(II) ion concentration (mg/L) and bioaccumulated Zn(II) ion quantities per unit mass of biomass on dry weight basis (mg Zn(II)/g dry weight) at pH 5.0 and 8.0 and at 25˚C.

The bioaccumulated Zn(II) ion quantities per unit mass of biomass on dry weight basis (mg Zn(II)/g dry weight) with time increased rapidly in the exponential growth phase. In this phase, as the bioaccumulated Zn (II) ion concentration was relatively high and young biomass concentration was low, the bioaccumulated Zn(II) ion quantities per unit mass of biomass was high. The bioaccumulated Zn(II) ion quantities per unit mass of biomass reached the maximum value in the stationary phase of growth in which the bioaccumulated Zn(II) ion concentration was substantially high and the biomass concentration was also high. In the death period, the bioaccumulated Zn(II) concentration decreased together with the loss of biomass, so that the bioaccumulated Zn(II) ion quantities per unit mass of biomass remained a stable value.

The bioaccumulated Zn(II) ion concentration and the bioaccumulated Zn(II) ion quantities per unit mass of biomass increased with increasing initial Zn(II) ion concentration up to 30–150 mg/L (Fig. 3). In the 30–150 mg/L concentration interval, the bioaccumulated Zn(II) ion concentration and the bioaccumulated Zn(II) ion quantities per unit mass of biomass stayed stable reaching to a saturation value. When the initial Zn(II) ion concentration increased to 200 mg/L, a significant decrease in metal bioaccumulation was observed. At this concentration, the significant reduction of biomass concentration shows that high initial Zn(II) ion concentration has an inhibition effect on micro-organism growth. At lower initial Zn(II) ion concentrations, the bioaccumulation efficiency was within the range of 95.1 to 87.7%, then it declined rapidly with increasing initial Zn(II) ion concentrations up to 30 mg/L. However, depending on the pH and temperature studied, the effect of initial Zn(II) ion concentration on Zn(II) bioaccumulation also changes. It was understood that the mutual interactions between pH and initial Zn(II) ion concentration, between temperature and initial Zn(II) ion concentration altered the Zn(II) bioaccumulation together. On the other hand, the amount of Zn(II) bioaccumulated within the cells up to saturation concentration level (26.3 mg/L, 32.1 mg/g, at pH 5.0 and 35˚C) did not cause a significant decrease in the lipase enzyme activity, stability, and biomass concentration (Fig. 4(a)).

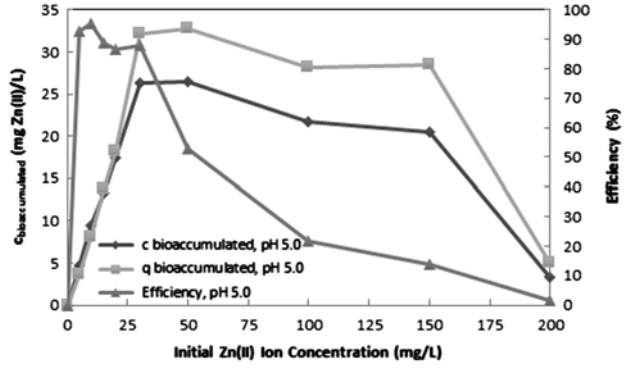

Fig. 3. Change of bioaccumulated Zn(II) ion concentration (mg/L), bioaccumulated Zn(II) ion quantities per unit mass of biomass on dry weight basis (mg Zn(II)/g dry weight), and bioaccumulation efficiency at pH 5.0 and at 35˚C.

Comparing Fig. 4(a) and (b), it is observed that the lipase activity and metal bioaccumulation at 35˚C and pH 5.0 were higher than those at pH 8.0; however, in Fig. 2, at 25˚C, the metal bioaccumulation at pH 5.0 and 8.0 was very close to each other. The mutual interactions of pH and temperature as well as the lipase activity and micro-organism concentration affect Zn(II) bioaccumulation together. As well as seen in the lipase activity and micro-organism concentration, interactive responses of pH and temperature influence Zn(II) bioaccumulation. Varying one independent parameter at a time and keeping the others constant, it was not possible to determine the optimum process conditions maximizing the values of dependent variables. That is why the optimal studying conditions were determined by using RSM.

3.2. Mutual interactions of pH, temperature, and initial Zn(II) ion concentration on lipase biosynthesis of R. delemar

For optimizing the lipase production by *R. delemar* in the presence of Zn(II) ions, RSM was employed with three independent variables such as pH (x_1), temperature (x_2), and initial Zn(II) ion concentration (x_3). The five-level-three-factor CCD matrix in coded units along with the observed responses, lipase activ-

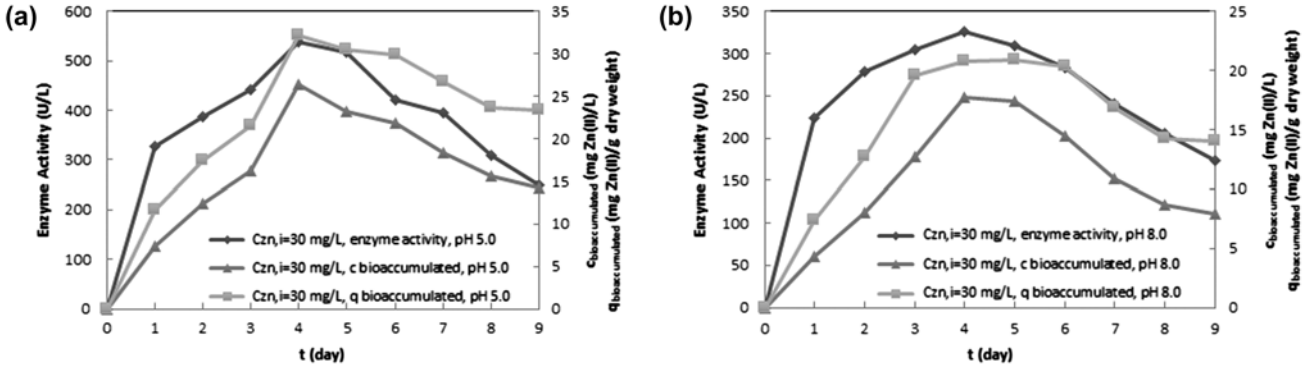

Fig. 4. (a) Time course of lipase activity, bioaccumulated Zn(II) ion concentration (mg/L), and bioaccumulated Zn(II) ion quantities per unit mass of biomass on dry weight basis (mg Zn(II)/g dry weight) at pH 5.0 and at 35°C and (b) Time course of lipase activity, bioaccumulated Zn(II) ion concentration (mg/L), and bioaccumulated Zn(II) ion quantities per unit mass of biomass on dry weight basis (mg Zn(II)/g dry weight) at pH 8.0 and at 35°C.

Table 2
CCD matrix along with the experimental and predicted values of bioaccumulated Zn(II) ion concentration, enzyme activity, biomass concentration, and relative percentage errors

	Independent variables			Enzyme activity (U/L)			Bioaccumulation (mg/L)			Biomass concentration (g/L)		
Runs	x_1	x_2	x_3	Exp.*	Pre.†	RPE (%)‡	Exp.	Pre.	RPE (%)	Exp.	Pre.	RPE (%)
1	$+\alpha$	0	0	502	492	2	17.87	18.41	3	0.54	0.59	9
2	0	0	0	486	507	4	24.22	25.38	5	1.03	1.02	1
3	0	$+\alpha$	0	513	516	1	22.06	22.26	1	1.21	1.26	4
4	1	−1	−1	357	370	4	5.13	4.73	8	0.91	0.90	1
5	1	−1	1	539	534	1	27.76	27.59	1	0.16	0.14	11
6	0	0	0	523	507	3	25.56	25.38	1	1.04	1.02	2
7	−1	1	−1	403	405	0	15.17	15.08	1	1.45	1.44	0
8	1	1	1	549	574	5	32.73	32.01	2	0.68	0.61	10
9	−1	−1	1	479	495	3	25.97	25.57	2	0.54	0.53	2
10	0	0	$+\alpha$	613	600	2	31.77	32.34	2	0.06	0.10	71
11	1	1	−1	439	421	4	5.69	5.83	2	1.33	1.31	1
12	$-\alpha$	0	0	432	446	3	24.71	24.52	1	1.05	1.03	2
13	0	0	0	502	507	1	25.9	25.38	2	1.12	1.02	9
14	0	0	0	498	507	2	26.05	25.38	3	0.97	1.02	5
15	−1	−1	−1	345	317	8	14.97	15.43	3	1.39	1.43	3
16	0	0	$-\alpha$	304	321	6	1.96	1.77	10	1.47	1.45	1
17	0	$-\alpha$	0	406	407	0	18.65	18.83	1	0.87	0.85	2
18	0	0	0	524	507	3	25.21	25.38	1	0.94	1.02	8
19	0	0	0	512	507	1	25.39	25.38	0	1.01	1.02	1
20	−1	1	1	589	573	3	28.43	28.57	0	0.61	0.60	2

*Experimental values.†Model predicted values.‡Relative percentage error.

ity (U/L), bioaccumulated Zn(II) ion concentration (mg/L), and biomass concentration (g/L) is represented in Table 2. The mathematical model which represents a second-order polynomial for lipase activity is given by Eq. (3), where the variables pH, temperature, and initial Zn(II) ion concentration take their coded values.

$$Y_1\,(\mathrm{U/L}) = 507.38 + 13.60x_1 + 32.21x_2 + 82.86x_3 - 9.50x_1x_2 - 3.50x_1x_3 - 2.50x_2x_3 - 13.56x_1^2 - 16.21x_2^2 - 16.56x_3^2 \quad (3)$$

Table 3
ANOVA for the fitted quadratic polynomial models of lipase activity, bioaccumulated Zn(II) ion concentration and biomass concentration as a function of independent variables of pH, temperature, and initial Zn(II) ion concentration

Model	Source	Sum of squares	DF	Mean square	*F*-value	Probability (p) > F
Y_1	Model	120,000	9	13,337	30.55	<0.0001
	Residual	4,365	10	437		
	Lack of fit	3,250	5	650	2.91	0.1327
	Pure error	1,155	5	223		
	Corrected total	124,000	19			
	$R^2 = 0.9649$, $R^2_{Adj} = 0.9333$, $R^2_{Pred} = 0.7643$, adequate precision = 19.16					
Y_2	Model	1,440	9	160.1	398.69	<0.0001
	Residual	4.02	10	0.4		
	Lack of fit	1.89	5	0.38	0.89	0.5499
	Pure error	2.13	5	0.43		
	Corrected total	1,444	19			
	$R^2 = 0.9972$, $R^2_{Adj} = 0.9947$, $R^2_{Pred} = 0.9873$, adequate precision = 63.19					
Y_3	Model	2.88	9	0.32	92.47	<0.0001
	Residual	0.035	10	3.46×10^{-3}		
	Lack of fit	0.015	5	3.02×10^{-3}	0.78	0.6066
	Pure error	0.019	5	3.90×10^{-3}		
	Corrected total	2.91	19			
	$R^2 = 0.9881$, $R^2_{Adj} = 0.9774$, $R^2_{Pred} = 0.9505$, adequate precision = 32.34					

The *F*-test was applied to prove the statistical significance of Eq. (3), the results of the second-order regression model fitting in the form of ANOVA are given in Table 3. The model *F*-value of 30.55 and the infinitesimal *p*-value of < 0.0001 demonstrated that Eq. (3) was highly significant. The *F*-value is defined as the ratio of the mean square due to regression to the mean square due to error. The R^2 value provided a measure of how much of the variability in the observed response values could be explained by the experimental factors and their interactions. The R^2 values above 0.9 are considered very well and a good model explains most of the variation in the response. The value of determination coefficient, R^2 (= 0.9649), shows that 96.49% of the sample variation in the lipase activity is attributed to the independent variables and the model cannot explain only 3.51% of the total variations, i.e. model fits quite well. The adjusted determination coefficient, $R^2_{Adj,}$ corrects the R^2 values for the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the R^2_{Adj} may be noticeably smaller than the R^2. In this case, the R^2_{Adj} value is very close to the R^2 value. The R^2_{Adj} (= 0.9333) is also high, showing a high applicability of the model. The predicted determination coefficient, R^2_{Pred}, of 0.7643 indicates a good agreement between the experimental and predicted values for lipase production and is also in reasonable agreement with the R^2_{Adj} of 0.9333.The coefficient of variation (CV = 4.39%) is also sufficiently low indicating high precision and reliability of experiments. The lack of fit measures the failure of the model to represent data in the experimental domain at points which are not included in the regression. The value of lack of fit (*F*-value: 2.91) for regression of Eq. (3) is not significant. Nonsignificant lack of fit is desired and indicates that the model equation was adequate for predicting the lipase activity under any combination of values of the variables. There is a 13.27% chance that a "lack of fit *F*-value" could occur due to noise. The purpose of RSM is to determine which experimental factors generate signals, which are large in comparison to the noise. Adequate precision measures signal-to-noise ratio, a ratio greater than 4 is desirable [30]. The ratio of 19.16 for lipase activity designates an adequate signal.

The *p*-values indicate the significance of linear, quadratic, and interaction constant coefficients of the model and are used for understanding the pattern of the mutual interactions between the parameters. A value of Prob > *F* less than 0.05 denotes that the model terms are significant. The smaller the *p*-value, the more significant the corresponding coefficient. The main parameters that affect the lipase activity were the linear coefficients of pH (x_1) ($p = 0.037$), temperature (x_2) ($p = 0.0002$), and initial Zn(II) ion concentration (x_3) ($p < 0.0001$) and the quadratic terms x_1^2(p = 0.0335), x_2^2($p = 0.0147$), x_3^2($p = 0.0131$). The linear terms

with positive coefficients indicate an increase in lipase activity. According to the calculated coefficients, all independent variables had positive effects on lipase activity. First-order effect of temperature is highly significant (x_2) ($p = 0.0002$), whereas its quadratic effect x_2^2 ($p = 0.0147$) is much less significant. This means that temperature has a strong effect on the activity of lipase enzyme, but its small variation in the temperature range studied is not going to change the activity to a large extent. A similar comment can also be made for the effect of initial Zn(II) ion concentration on lipase activity. The interaction coefficient between pH and temperature x_1x_2 ($p = 0.2274$) was not significant. Insignificant interaction coefficient between pH and temperature means that the change of lipase activity with pH is not affected by the change of temperature and vice versa. In other words, for the lipase activity of *R. delemar*, the pH optimum is the same at all temperatures in the studied range, and vice versa. The interaction effects of pH-initial Zn(II) ion concentration ($p = 0.6458$) and temperature-initial Zn(II) ion concentration ($p = 0.742$) had also no significant. This situation shows that each of independent variable affects the lipase activity separately; however, mutual interactions of these variables are not important for lipase activity.

The three-dimensional response surface graph and two-dimensional contour plot of the quadratic model for the lipase activity as a function of pH and temperature are given in Fig. 5(a) and (b). Fig. 5 shows the interaction relationship between two independent variables, pH-temperature, and their effects on the response variable lipase activity, while the initial Zn (II) ion concentration was held at 41.0 mg/L. The shapes of the contour plots, circular or elliptical, specify if the mutual interactions between the independent variables are significant. In case of the mutual interactions between pH and temperature on lipase activity, the contour plots are not perfectly elliptical. This reveals that there are fewer interactions between the pH and temperature corresponding to the response surface. The lipase activity increased with increasing temperature. An increase in pH up to 6.8 led to an enhancement in lipase activity, but then the lipase activity reached a plateau value. Lipase activity increased steeply with increasing temperature rather than increasing pH. Increase in both initial Zn(II) ion concentration and temperature results in a sharp increase in lipase activity. Optimum physicochemical conditions generated from the model proposed for the lipase activity of *R. delemar* in the presence of Zn(II) ions were determined as pH 6.8, temperature 33.8˚C, and 41.0 mg/L initial Zn(II) ion concentration. At these conditions, a maximum lipase activity of 586 U/L was reached.

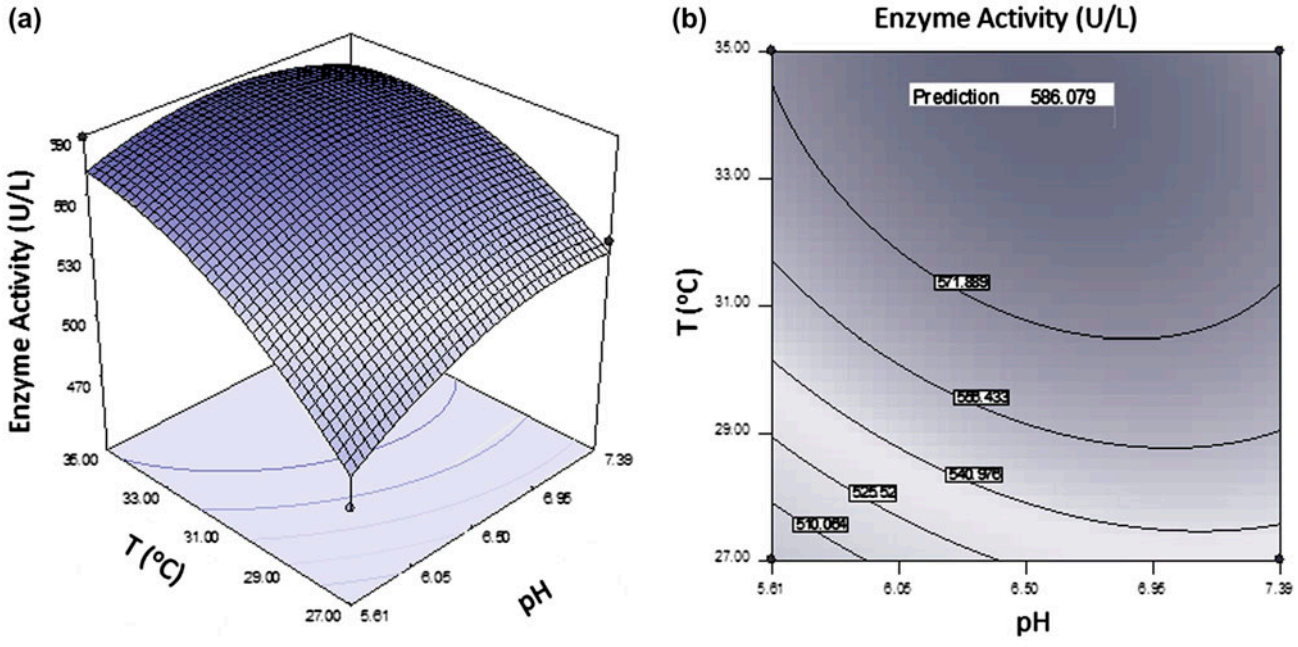

Fig. 5. (a) Response surface plot described by the model Y_1, which represents the effect of pH and temperature and their mutual effects on lipase activity and (b) response surface contour plot of lipase activity showing interactive effect of pH and temperature.

3.3. Mutual interactions of pH, temperature, and initial Zn(II) ion concentration on Zn(II) bioaccumulation by R. delemar

The Zn(II) bioaccumulation by *R. delemar* in the presence of extracellular lipase enzyme in the fermentation medium was expressed as a nonlinear function of the input process parameters in coded form as follows:

$$Y_2\,(\mathrm{mg/L}) = 25.38 - 1.81x_1 + 1.02x_2 + 9.08x_3 + 0.36x_1x_2 + 3.18x_1x_3 + 0.83x_2x_3 - 1.38x_1^2 - 1.71x_2^2 - 2.94x_3^2 \quad (4)$$

The statistical significance of the second-order model equation was evaluated by the *F*-test ANOVA which revealed that this regression is statistically significant ($p < 0.0001$) at 99% of confidence level (Table 3). The coefficients of determination, R^2 (0.9972), adjusted R^2_{Adj} (0.9947) and predicted R^2_{Pred} (0.9873) which suggested that there were excellent correlations between the independent variables. The *p*-values obtained from the regression analysis results showed that all of the coefficients of the linear ((X_1) ($p < 0.0001$), (X_2) ($p = 0.0001$), (X_3) ($p < 0.0001$)), interaction ((x_1x_3) ($p < 0.0001$), (x_2x_3) ($p = 0.004$)) except (x_1x_2) ($p = 0.1404$), and quadratic terms ((x_1^2) ($p < 0.0001$), (x_2^2) ($p < 0.0001$), (x_3^2) ($p < 0.0001$)) had a significant effect on Zn(II) bioaccumulation. The interaction of initial Zn(II) concentration with pH ((x_1x_3) ($p < 0.0001$)) and temperature ((x_2x_3) ($p = 0.004$)) is more pronounced than that between pH and temperature ((x_1x_2) ($p < 0.1404$)). This means that the optimum pH for Zn(II) bioaccumulation is not impressed singly by the change of temperature and vice versa. However, the optimum initial Zn (II) ion concentration for Zn(II) bioaccumulation can be affected significantly by both the change in pH and temperature. Metal solubility strongly depends on pH and temperature. As can be seen from Fig. 6(a) and (b), the Zn(II) bioaccumulation increased with increase of initial solution pH ranging from 5.0 to 6.8 as well as with initial Zn(II)ion concentration ranging from 5.1 to 41.0 mg/L, then reaching a plateau value. Fungal biomass contains high content of ionizable groups: polysaccharides of the cell wall, often complexed with proteins, lipids, and other substances (e.g. pigments), which suggest that the bioaccumulation process could be affected by changes in the solution pH. At lower pH below the isoelectric point, the surface of fungi may acquire a positive charge leading to decreased metal cations uptake due to the electrostatic force of repulsion. As the pH of the medium is increased, the number of negatively charged sites increases and the number of positively charged sites decreases. An increase of Zn(II) bioaccumulation by increasing initial metal ion concentration is a result of the increase in the driving force of the concentration gradient. In the

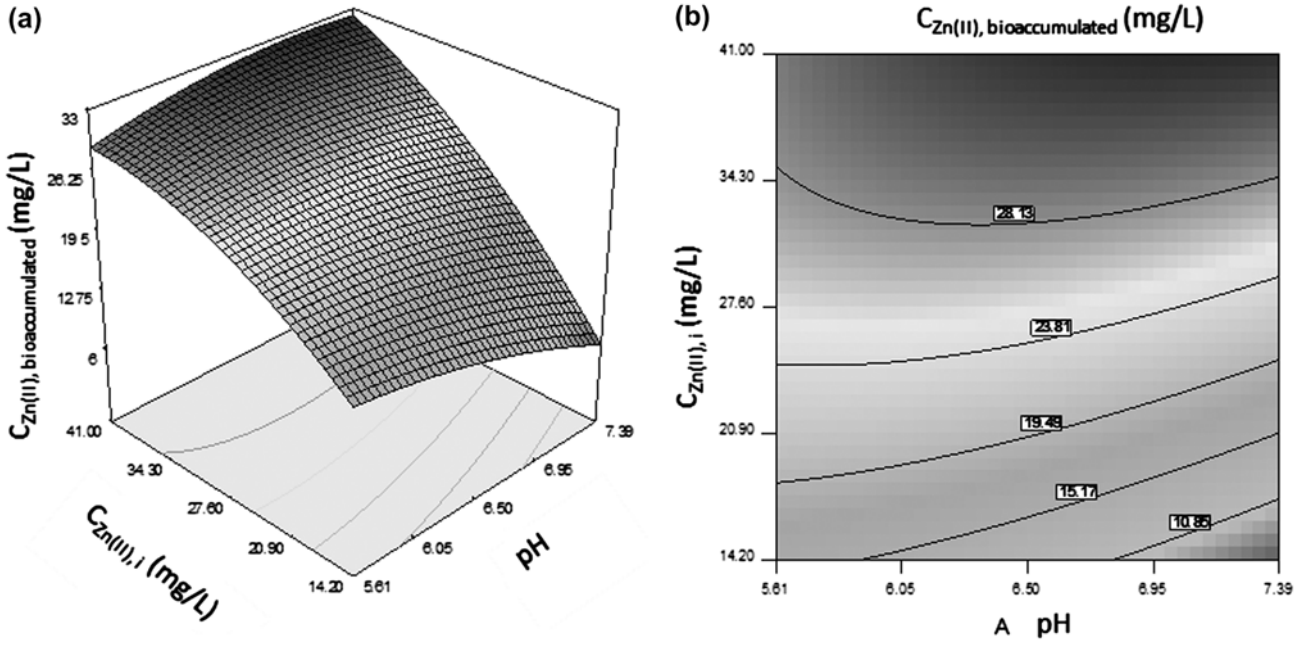

Fig. 6. (a) Response surface plot described by the model Y_2, which represents the effect of pH and initial Zn(II) ion concentration and their mutual effects on Zn(II) bioaccumulation and (b) response surface contour plot of Zn(II) bioaccumulation showing interactive effect of pH and initial Zn(II) ion concentration.

same condition, if the concentration of metal ions in the solution was higher, the active sites of *R. delemar* would be surrounded by more metal ions, and the bioaccumulation process would be carried out more sufficiently. In living cells, metal removal occurs in two ways: adsorption in surface molecules, accumulation via auxiliary proteins that cells usually use for the incorporation of basic elements such as phosphorous and sulfur, and/or reduction by enzymatic processes. If the process is metal bioaccumulation, an efflux mechanism can be functioning at a certain metal concentration preventing more metal bioaccumulation. The increase of the Zn(II) bioaccumulation with increasing temperature indicated that the bioaccumulation of Zn(II) ions to *R. delemar* is endothermic in nature. The optimum ranges of pH, temperature, and initial Zn(II) ion concentration for the maximum Zn(II) bioaccumulation of 32.38 mg/L lie in 6.81, 33.78°C, and 41.0 mg/L, respectively. The bioaccumulation efficiency of Zn(II) ions is 79% under above optimum conditions.

3.4. Mutual interactions of pH, temperature, and initial Zn(II) ion concentration on growth of R. delemar

According to the statistical evaluations shown in Table 3, the obtained quadratic equation for the dependent variable, response, in the terms of independent variables was determined as:

$$Y_3\,(\mathrm{g/L}) = 1.02 - 0.13x_1 + 0.12x_2 - 0.4x_3 + 0.10x_1x_2 + 0.04x_1x_3 + 0.01x_2x_3 - 0.07x_1^2 + 0.01x_2^2 - 0.08x_3^2 \quad (5)$$

where the observed response Y_3 is biomass concentration (g/L) and the independent variables are pH (x_1), temperature (x_2), and initial Zn(II) ion concentration (x_3).

The ANOVA of the quadratic polynomial model for biomass concentration showed low p-values and both high determination coefficients (R^2) and high adjustment of the determination coefficients (R^2_{Adj}). The model was statistically significant ($p < 0.0001$). The first-order effects of independent factors ($p < 0.0001$), quadratic main effects of pH, and initial Zn(II) ion concentration ($p = 0.0008$ and $p = 0.0003$, respectively) and the interaction coefficient between pH and temperature (x_1x_2, $p = 0.0007$), which indicated that the micro-organism growth was strongly affected by the changes of pH and temperature, were statistically significant.

There is an optimum pH for the biomass concentration as well as lipase activity and Zn(II) bioaccumulation around pH 6.81, then the micro-organism

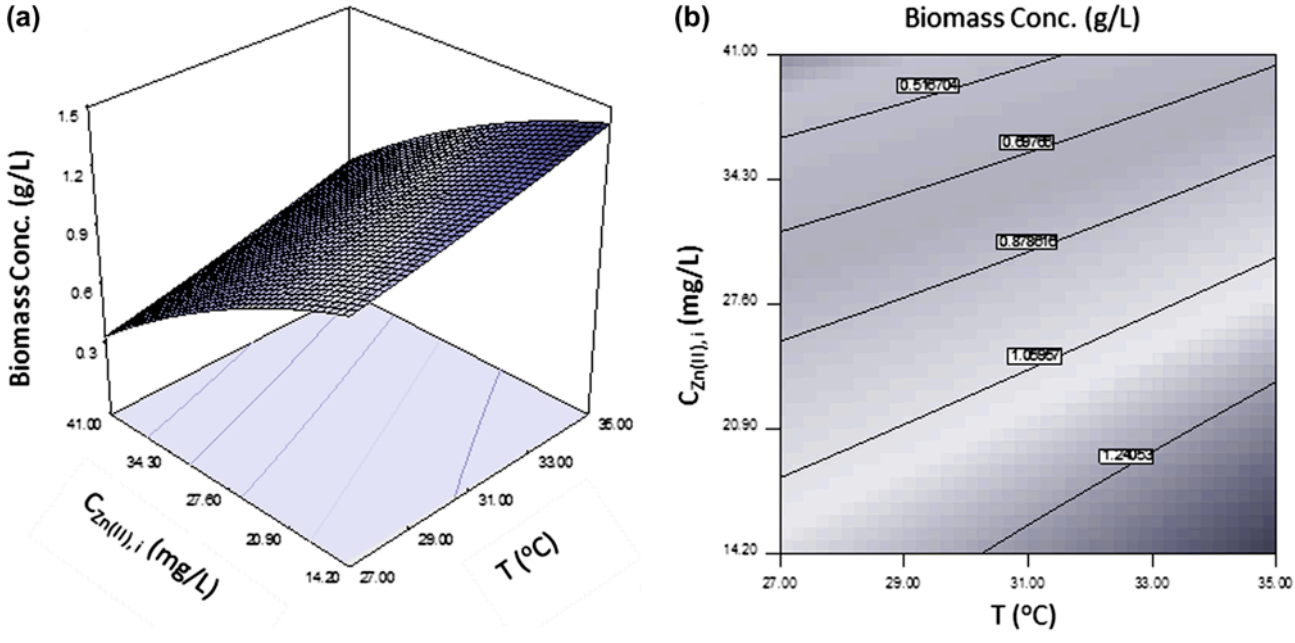

Fig. 7. (a) Response surface plot described by the model Y_3, which represents the effect of temperature and initial Zn(II) ion concentration and their mutual effects on biomass concentration and (b) response surface contour plot of biomass concentration showing interactive effect of temperature and initial Zn(II) ion concentration.

growth began to decrease. Biomass concentration increased with increasing temperature, however, decreased in the presence of increasing concentrations of Zn(II) ions (Fig. 7(a) and (b)). High concentrations of Zn(II) ions inhibit micro-organism growth and can show a toxic effect on metabolic enzymes and functions of living cells. A minimum biomass concentration was obtained close to 41 mg/L Zn(II) ion concentration. However, Zn(II) ions at high concentrations did not alter the lipase activity very much with respect to Zn-free medium. Previous studies performed in metal-free medium marked that the lipase activity decreased with increasing biomass concentration. Therefore, to obtain high lipase activity, the amount of inoculum was optimized and kept constant at 5/1,000 (volume of inoculum/production volume of bioreactor). At low biomass concentrations, high enzyme activity can be obtained, in this way the inhibition effect of Zn(II) ion concentrations on micro-organism growth becomes insignificant. As cellular growth decreased in presence of metal in comparison with metals-free culture, this implied less available binding sites for bioaccumulation. Although the bioaccumulated Zn(II) ion concentration increased with increasing amount of biomass, the bioaccumulated Zn (II) ion quantities per unit mass of biomass on dry weight basis (mg Zn(II)/g dry weight) decreased. Because of an inverse relationship between lipase activity, Zn(II) bioaccumulation, and biomass concentration for the initial Zn(II) ion concentration as independent variable, the main objective was defined as Zn(II) bioaccumulation and lipase activity as high as possible. The optimal process conditions were described to attain maximum Zn(II) bioaccumulation and lipase activity, but a definite micro-organism concentration maximizing lipase activity and Zn(II) bioaccumulation. A biomass concentration of 0.62 g/L was predicted by the quadratic polynomial model under the conditions specified above at pH 6.81, 33.78˚C, and 41 mg/L initial Zn(II) ion concentration.

3.5. Validation of the models

The simultaneous Zn(II) bioaccumulation and lipase production by *R. delemar* was performed at the predicted optimum values (pH 6.81; 33.78˚C; 41.00 mg/L initial Zn(II) concentration) for the critical physicochemical parameters of the fermentation medium. The verification experiments were done in duplicate. According to the model Y_1, Zn bioaccumulation was predicted as 32.38 mg/L, and the duplicate experimental results had results of 30.94 and 31.69 mg/L of which relative percent errors for the variation (RPE) were 4.65 and 2.18% respectively. The predicted value of the model Y_2 had a lipase activity value of 586 U/L, while the experimental validation results were 566 and 573 U/L with the RPE of 3.53% and 2.27%, respectively. In addition to these, the biomass concentration which was calculated via model Y_3 had a response value of 0.62 g/L and the duplicate experimental results of biomass concentration were 0.64 g/L (RPE: 3.13%) and 0.67 g/L (RPE: 7.46%). These well correlations between predicted and measured values of these experiments justified the validity of the response models and the existence of an optimum point. The experimental values of lipase activity and Zn(II) bioaccumulation are little lower than the predicted values. On the other hand, the experimental biomass concentration values showed the positive deviation from the predicted value.

4. Conclusion

In this study, lipase enzyme production and Zn (II) bioaccumulation of *R. delemar* were investigated simultaneously. In the experiments in which changing a single parameter while holding the others as constant, maximum Zn(II) bioaccumulation and lipase activity were obtained in the stationary phase of growth period of *R. delemar*, on the fourth day of fermentation. Maximum lipase activity in the medium including 30 mg/L Zn(II) concentration at pH 5.0 and 35˚C was obtained as 537 U/L, whereas maximum lipase activity in Zn(II)- free medium at pH 5.0 and 35˚C was acquired as 656 U/L. At both 25 and 35˚C, the lipase activity and biomass concentration in the medium containing 30 mg/L Zn(II) concentration were higher at pH 5.0 than those at pH 8.0. The similar trend was also observed in the Zn-free medium. On the other hand, the lipase activity at pH 8.0 was higher at 25˚C than that 35˚C, while the maximum lipase activity at pH 5.0 was obtained at 35˚C. It was observed that the Zn (II) bioaccumulation at 35˚C was higher at pH 5.0 than that at pH 8.0, whereas the Zn(II) bioaccumulation at 25˚C was very close at pH 5.0 and pH 8.0. As the initial Zn(II) ion concentration was increased, the bioaccumulated Zn(II) ion quantity increased in the range of 30–150 mg/L. Within this concentration interval, the bioaccumulated Zn(II) ion concentration in the cells reached at a saturation concentration of 26.3 mg/L. The pH, temperature, and initial Zn(II) ion concentration both individually and interactively affect on Zn(II) bioaccumulation, lipase activity, and micro-organism growth. Depending on this, in order to determine the optimum experimental conditions, the implementation of RSM was decided. With using the CCD, an optimum process operating point was

determined at pH 6.81, 33.78°C, and 41 mg/L initial Zn(II) ion concentration. At this point, a stable enzyme activity of 586 U/L at 0.62 g/L micro-organism concentration was acquired, while Zn(II) bioaccumulation and efficiency were 32.38 mg/L and 79%, respectively.

This paper is principally an attempt to prove the applicability of statistical design to optimize simultaneous lipase production and heavy metal bioaccumulation of *R. delemar*. RSM methodology is more practical compared to the single-factor approaches, as it takes into account interactive effects among the variables, it demonstrates the overall effects of the parameters on the process and, eventually, it gives a more precise and accurate results. In the present study, using the optimum experimental conditions determined by the RSM method, growth of *R. delemar* will be kept at a required level for lipase production as well as Zn(II) bioaccumulation. When process engineer gets *R. delemar* to produce lipase in order to biodegrade lipids during the growth period, the Zn(II) ion concentration which will not inhibit the production of lipase, will even increase, was determined. Thus, wastewater-containing lipid and heavy metal pollution together will be treated simultaneously. We hope that the optimum conditions determined by RSM will be tested in the scale-up of the bioreactor operated in batch or continuous mode in pilot treatment plant, and the proposed process can be applied to the real industrial or domestic wastewater treatment. In the future, *R. delemar* biomass coming from food or pharmaceutics industries' wastes will be combined with wastewater of leather, textile, and similar industries including high concentrations of lipid and heavy metal pollution. Moreover, the treatment plant of these factories would be operated as a closed-cycle process.

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