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Original Research

Optimization of Pb Biosorption from Aqueous Solution Using Genetically Engineered *Saccharomyces cerevisiae* by Response Surface Methodology

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Abstract

Recombinant *Saccharomyces cerevisiae* expressing metallothionein was used for biosorption of lead (Pb). The Plackett-Burman design (PBD) and Box-Behnken design (BBD) were applied to optimize adsorption conditions of Pb by genetically engineered *S. cerevisiae*. The PBD and its subsequent statistical analysis indicate that the initial concentration of Pb, pH and concentration of biosorbent were the three critical factors influencing Pb biosorption by genetically engineered *S. cerevisiae*. The Path of steepest ascent was then used to approximate the optimal levels of the main factors. Then, the response surface method was used to optimize the adsorption conditions further. Considering biosorption quantity of Pb as the response objective, a quadratic model was obtained by BBD for 3 factors. The optimal biosorption conditions were as follows: the initial concentration of Pb (81.12 mg/L), pH 5.05 and the concentration of biosorbent (0.15 g/L). Under optimal conditions, the maximum adsorption quantity of Pb is 129.60 mg/g. Model validation experiments showed good correlation between the predicted values and experimental values. The performance of adsorption of Pb by genetically engineered *S. cerevisiae* is clearly improved by response surface methodology optimization. Genetically engineered *S. cerevisiae* show higher adsorption capacity than other reported biosorbents and may therefore be an effective biosorbent for industrial use.

Keywords: Pb, genetically engineered *S. cerevisiae*, Plackett-Burman design, Box-Behnken design, response surface optimization

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Introduction

Heavy metal pollution problems have become more serious with the rapid development of modern agriculture and industry [1-3]. According to statistical analysis, approximately 21,000 tons of heavy metals was released into the environment in China in 2014 [4]. All types of heavy metals can seriously damage the ecological environment and human health. It is largely because heavy metal is not biodegradable and tends to accumulate in living organisms [5-6]. Pb is one of the toxic heavy metals that is released into the environment from a variety of industrial activities, including smelting, refining, municipal waste, paint and pigment production, and battery production [7-8]. Pb can enter the human body through the digestive tract, respiratory tract, or skin and accumulate in living tissue, such as bone, brain, muscle, and kidneys [9-10]. Pb can cause many diseases, such as anemia, kidney disease, and blood brain and other disorders [11].

At present, the treatment methods for wastewater containing Pb include primarily chemical precipitation, ion exchange, electrolysis precipitation and activated carbon adsorption, among others. However, these methods have many disadvantages such as complicated operating techniques, high operating costs, energy intensive technologies and secondary pollution [12-13]. Biosorption has obvious advantages, including wide adaptability, high selectivity, regenerating ability, low operating costs, and no secondary pollution [14-18]. All types of biological materials, including bacteria, fungi, algae and agriculture and forestry waste, have been considered to be effective biosorption materials for toxic substance removal [19-22]. However, because the binding capacity of wild type microorganisms for heavy metal is not sufficiently high, recent research has focused on the development of new biological material to increase the binding capacity for metal ions [23-24].

Metallothionein is a type of low molecular weight metal-binding protein that can bind all types of heavy metals, including Pb [25-26]. The present research describes the Pb adsorption characteristics of genetically engineered *S. cerevisiae* expressing metallothionein, whose construction we described in previous articles [27].

PBD and RSM have been widely used for determining optimal adsorption conditions [28]. PBD can quickly screen for the main effect factors from numerous variables [29]. Response surface method (RSM) is an efficient method for analyzing the level of independent variables affecting biosorption [30]. In this study, PBD was first used to determine the main factors influencing Pb adsorption by recombinant *S. cerevisiae*. We then determined the center point of the main factors by using the path of steepest ascent, and finally we used the Box-Behnken design to determine the optimum adsorption conditions.

Materials and Methods

Strains and culture conditions *S. cerevisiae* expressing metallothionein were constructed in our previous work [31]. The strains were grown in YPD medium. Cultivation was carried out at 30ºC and 200 rpm.

Preparation of Biosorbents

The strains were incubated for 72 h in YPD medium. We collected the strains by centrifugation (three times) and washed the strains with deionized water. The strains were dried at 65ºC for 48 h, and then bacterial particulates were produced by precision grinding.

Preparation of Stock Solution

Stock solutions were prepared by dissolving 1000 mg of $Pb(NO_3)_2$(analytic grade) in one L of deionized water. The pH of the solution was adjusted with 0.1 M HNO_3 or 0.1 M NaOH.

Biosorption Experiments

For these studies, the desired concentration of $Pb(NO_3)_2$ at the designated pH and appropriate amount of biosorbent were added to Erlenmeyer flasks and shaken in a constant temperature incubation shaker. Samples were taken at scheduled times for analysis by centrifugation at 1000 rpm and were filtered through filter paper. The concentration of Pb was measured using atomic absorption spectrophotometry at 283.8 nm. The amount of Pb adsorbed on genetically engineered *S. cerevisiae* was calculated using Eq. (1):

$$q = (c_0 - c_e)v / m \tag{1}$$

where, q is the adsorption quantity (mg/g), c_0 is the concentration of Pb before biosorption (mg/L), c_e is the concentration of Pb at the desired period (mg/L), v is the volume of the solution (L), and m is the amount of adsorbent (g).

Plackett-Burman Design (PBD)

To assess the effects of pH, the initial concentration of Pb, biosorbent dosage, temperature, speed and time on the biosorption process, we used Plackett-Burman design to screen for the main effect factors from among numerous variables. Twelve groups of experiments were designed for PBD analysis. Each independent variable consisted of two levels and included a low level(-1) and a high level(+1) (Table 1).

The values of the variables were chosen on the basis of published literature [32]. The experimental results of PBD are displayed in Table 2. All the experiments were carried out in duplicate.

Table 1. Levels of the variables tested in Plackett-Burman design for the biosorption of Pb by genetically engineered *S. cerevisiae*.

Designation	Variable	Range and level	
		-1	1
X1	pH	3	6
X2	Concentration of Pb (mg/L)	10	100
X3	Temperature (ºC)	20	40
X4	Adsorbent dosage (g/L)	0.1	1
X5	Contact time (min)	20	60
X6	Speed of agitation (r/min)	100	200

A Linear function (Eq. 2) was applied to screen the factors. The interactions between various were ignored.

$$Y = \beta_0 + \sum \beta_i X_i (i = 1 \cdots k) \quad (2)$$

where Y is the adsorption quantity (mg/g), X_i is the real value of investigation factors, and β_i is the regression coefficient, which reflects the impact degree of X_i.

The influence of each factor was calculated by Eq. (3):

$$E(X_i) = \frac{2\sum(M_i^+ - M_i^-)}{N} \quad (3)$$

where $E(X_i)$ is the effect of the tested variable (X_i); M_i^+ and M_i^- are the responses (biosorption) of trials at which the variable is at its high or low level, respectively; and N is the total number of trials.

Path of Steepest Ascent (PSA)

PSA is a method for analyzing the maximum increase in the response. The PSA analysis began from the zero level of variables in the PBD experiment. Experiments were then carried out along the steepest ascent path until the response value no longer increased. This point would be considered the center point, in order to further optimize the process using BBD.

Response Surface Methodology (RSM)

The adsorption conditions of Pb by genetically engineered *S. cerevisiae* were further optimized by three-factor-three-level Box-Behnken design and response surface analysis. Three key parameters (pH, the initial concentration of Pb and biosorbent dosage) influencing adsorption of Pb were selected as the independent variables. The adsorption capacity of Pb was considered to be the dependent variables. The experimental range and the level of independent variables for Pb adsorption are given in Table 5. The experiment results were analyzed by Design Expert 8.1 (Stat-Ease Inc, Minneapolis USA), and a regression model was proposed.

The quadratic model is shown in Eq. (4).

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon \quad (4)$$

where β_0 is the constant coefficient; β_i, β_{ii} and β_{ij} are the regression coefficients; and X_i and X_j are independent variables. The parameter ε represents random error.

Seventeen experiments were performed in a random order for this study. The suitability of the proposed model was assessed by analysis of variance. The performance of the fitting polynomial is represented by the coefficient of determination R^2. The analysis was carried out by Fisher 's F-test.

The factors were coded according to Eq. (5):

$$X_i = \frac{(X_i - X_0)}{\delta X} \quad (5)$$

where X_0 is the value of X_i at the center point and X represents the step change.

Results and Discussion

Screening of the Parameters by Plackett-Burman Design

Plackett-Burman design is an economical and efficient design method that includes two levels capable of quickly screening the most important factors from among several possible factors.

This research consisted of 12 experiments and six types of affecting factors: pH, the initial concentration of Pb, temperature, speed, biosorbent dosage and time. Each variable was examined at two levels: -1 for the low level and +1 for the high level (Table 1).

The experimental results of PBD are shown in Table 2. Analysis of variance (ANOVA) was carried out by Design Expert 8.0.6 and the results are shown in Table 3. From Table 3, it is apparent that the main factors influencing the results include pH, the initial concentration of Pb and biosorbent dosage, and the impact values were 2.11%, 78.78% and 18.55%, respectively. However, the other influence factors were not significant, which indicates that their values are close to the optimal level. A linear multivariate regression equation as follows, was determined by regression analysis.

$$Y = 53.36 + 5.22{*}X_1 + 31.96{*}X_2 - 1.29X_3 - 15.51{*}X_4 + 1.16X_5 + 0.54X_6$$

As shown in Table 3, the model is statistically significant (P<0.0001). There is a high correlation between the predicted and observed values (R^2 = 0.9969).

Table 2. Plackett-Burman design of 12 trials for six variables in high level(+) and low level(-) along with observed and predicted values of Pb (mg/g).

Run no.	Coded values						Pb(mg/g)	
	X_1	X_2	X_3	X_4	X_5	X_6	Experimental	Predicted
1	6	100	20	1	60	200	79.6	78.03
2	6	10	40	1	60	100	8.8	10.44
3	6	10	20	0.1	60	100	47.3	44.04
4	3	10	20	0.1	20	100	30.1	31.28
5	6	10	40	1	20	200	8.1	9.21
6	6	100	20	0.1	20	200	104.2	106.73
7	3	100	20	1	60	100	63.3	66.49
8	3	10	40	0.1	60	200	30.7	32.09
9	3	100	40	1	20	100	63.9	61.59
10	6	100	40	0.1	20	100	103.5	103.06
11	3	10	20	1	20	200	3.4	1.34
12	3	100	40	0.1	60	200	97.4	96.01

Table 3. Linear multiple regression analysis of Plackett-Burman design experiments for the biosorption of Pb by genetically engineered *S. cerevisiae*.

Variables	Coefficient estimate	Steized effects	Contribution%	P-value	Significance
Intercept	53.36			<0.0001	Significance
pH	5.23	10.45	2.11	0.0021	Significance
Concentration of Pb (mg/L)	31.96	63.92	78.78	<0.0001	Significance
Temperature (ºC)	-1.29	-2.58	0.13	0.2107	Not significance
Adsorbent dosage (g/L)	-15.51	-31.02	18.55	<0.0001	Significance
Contact time (min)	1.16	2.32	0.10	0.2544	Not significance
Speed of agitation (r/min)	0.54	1.08	0.023	0.5735	Not significance

$R^2 = 0.9969$, $R^2_{Adj} = 0.9931$, adequate precision = 44.25.

The value of Adjusted R^2 is 0.9931, indicating that 99.31% of the experimental data can be explained by the regression model. The correlation coefficient of 5.84% shows that the PBD experiment exhibits good reliability and accuracy. The value of Adeq precision in this study is 44.25. The value for this parameter of effective signal-to-noise ratio is substantially higher than the value of 4.0, which is considered reasonable.

Path of Steepest Ascent

The purpose of the path of steepest ascent is to fit equation to provide the best approximated value [33]. Three main influencing factors were chosen according to the experimental results of PBD. The concentration of biosorbent decreased successively because it has negative effects on biosorption. The initial concentration of Pb and pH gradually increased because they have positive effects on biosorption. The factors that no significant effect on biosorption were not included in the PSA.

As shown in Table 4, the maximum amount of Pb adsorption was 115.33 mg/g with an initial concentration of Pb of 80 mg/g, pH 5 and biosorbent dosage of 0.2 g/L. This point was chosen for further optimization by response surface optimization using Box-Behnken design.

The Results of Box-Behnken Design and the Response Surface Analysis

Optimal conditions and interactions between different factors were found by response surface optimization using Box-Behnken design. Three significant independent variables (pH, the initial concentration of Pb and biosorbent dosage) at three

Table 4. Experimental design and response values of path of steepest ascent for the biosorption of Pb by genetically engineered *S. cerevisiae*.

Run no.	pH	Concentration of Pb (mg/g)	Adsorbent dosage (g/L)	Pb (mg/g)
1	2	20	0.5	13.22
2	3	40	0.4	35.61
3	4	60	0.3	63.27
4	5	80	0.2	115.33
5	6	100	0.1	103.45

levels (-1, 0, +1) were used in BBD, and the amount of Pb adsorption was determined as the response value. The experimental and predicted results are displayed in Table 6. A total of 17 trials were performed in this study.

The relationship between variables and response was assessed with a second order polynomial equation and the regression equation coefficients were calculated. The data were fitted to the second polynomial equation as follows:

$$Y = 126.18 + 1.53*X_1 + 2.15*X_2 - 7.38X_3 - 2.39*X_1*X_2 - 0.72*X_1*X_3 + 1.46*X_2*X_3 - 8.84*X_1^2 - 1.83X_2^2 - 3.21X_3^2$$

Biosorption of Pb with genetically engineered *S. cerevisiae* was evaluated with ANOVA. The results of the ANOVA and analysis of regression model are shown in Table 7.

As shown in Table 7, the model was significant ($P<0.001$and $F = 38.58$). The lack-of-fit analysis for the model was found to be not significant ($P = 0.3164$), which shows that the quadratic model is valid for biosorption of Pb.

Table 5. Concentrations of variables at different levels in Box-Behnken Design for biosorption of Pb by genetically engineered *S. cerevisiae*.

Symbol	Variable	Range and level		
		-1	0	1
X_1	pH	4.50	5.00	5.50
X_2	Concentration of Pb (mg/L)	75.00	80.00	85.00
X_3	Adsorbent dosage (g/L)	0.15	0.2	0.25

Table 6. Box-Behnken Design matrix with experimental and predicted values for the biosorption of Pb by genetically engineered *S. cerevisiae*.

Run no.	pH	Concentration of Pb (mg/L)	Adsorbent dosage (g/L)	Pb (mg/g)	
				Experimental	Predicted
1	5	80	0.20	128.27	126.18
2	5	85	0.15	127.96	129.57
3	5.5	80	0.25	106.55	107.56
4	5.5	80	0.15	124.54	123.76
5	5	80	0.20	124.27	126.18
6	5	85	0.25	117.91	117.73
7	5.5	75	0.20	116.31	116.91
8	4.5	80	0.25	105.16	105.94
9	4.5	80	0.15	120.26	119.25
10	5	75	0.15	127.29	127.47
11	5	80	0.20	126.68	126.18
12	4.5	75	0.20	108.25	109.08
13	5	80	0.20	125.59	126.18
14	5	75	0.25	111.39	109.78
15	4.5	85	0.20	119.47	118.87
16	5	80	0.20	126.09	126.18
17	5.5	85	0.20	117.99	117.16

Table 7. ANOVA for Box-Behnken Design.

Source	Sum of squares (SS)	Degrees of freedom	Mean squares (MS)	F-value	P-value	Significance
Model	951.09	9	105.68	38.58	<0.0001	Significant
X_1	18.76	1	18.76	6.85	0.0346	
X_2	50.45	1	50.45	18.42	0.0036	
X_3	435.72	1	435.72	159.07	<0.0001	
X_1X_2	22.75	1	22.75	8.31	0.0236	
X_1X_3	2.09	1	2.09	0.76	0.4116	
X_2X_3	8.56	1	8.56	3.12	0.1205	
X_1^2	329.22	1	329.22	120.19	<0.0001	
X_2^2	14.14	1	14.14	5.16	0.0573	
X_3^2	43.39	1	43.39	15.84	0.0053	
Residual	19.17	7	2.74			
Lack-of-fit	10.55	3	3.52	1.63	0.3164	Not significant
Pure error	8.62	4	2.16			
Total	970.27	16				

$R^2 = 0.9802$, $R^2_{Adj} = 0.9548$, adequate precision=18.614.

The value of R^2 is 0.9802, and the value of Adj-R^2 is 0.9548, which shows that the model values are highly correlated. The correlation coefficient of 1.38% shows that the BBD experiment exhibits good reliability and accuracy. The value of Adeq precision in this study is 18.61 and shows that the model reflects the test results reasonably well. The study shows that there is a close relationship between the predicted values and the experimental values. Therefore, the model is suitable for predicting the amount of Pb adsorption by genetically engineered *S. cerevisiae*.

Response Surface Plots

The Effect of pH and the Concentrations of Pb on the Pb Uptake

It is generally known that the appropriate pH is a particularly important condition for biosorption [34]. However, Pb in wastewater exists in the form of $Pb(OH)_2$ when pH is greater than 7 [35-36]. Therefore, we chose pH values ranging from 3 to 6 in this study.

Fig. 1 shows the effect of pH and the initial concentration of Pb on the adsorption process, with the adsorption dosage at zero level. The interaction between the initial concentration of Pb and the pH was significant. The adsorption capacity of Pb increases as pH values increase from 4.5 to 5 and the initial concentration of Pb increases from 75 to 85 mg/L. However, the adsorption capacity of Pb gradually decreases when pH is greater than 5.

A lower pH can decrease the adsorption capacity of Pb, due primarily to competition between Pb^{2+} and H^+ in aqueous solution [37]. The degree of protonation of a biosorbent surface weakens with increasing pH, thereby promoting binding capacity between Pb and functional groups of active sites of the biosorbent. In other words, the higher the value of pH, the higher the amount of adsorbed Pb. However, the adsorption capacity of Pb gradually decreases when pH is greater than 5, due primarily to formation of soluble hydroxyl complexes [38]. Therefore, a higher sorption capacity of Pb can be obtained when pH = 5.

On the other hand, the adsorption capacity of Pb increases with a higher initial concentration of Pb. This is mainly due to an increased driving force resulting from the concentration gradient produced by the higher initial concentration of Pb [39]. All things being equal, if the concentration of Pb^{2+} is higher in solution, the collision times between Pb^{2+} and active site of biosorbent will increase, which increases efficiency of the adsorption process [40].

The Effect of pH and Biosorbent Dosage on the Pb Uptake

Fig. 2 shows the effect of pH and the biosorbent dosage on the adsorption process, with the initial concentration of Pb at zero level. The interaction between biosorbent dosage and pH was not significant. As the biosorbent dosage rises, the adsorption capacity of Pb declines. The amount of Pb adsorption gradually declined as the biosorbent dosage increased from 0.15 to 0.25 g/L. This is mainly due to existing, unsaturated adsorption sites on the biosorbent surface during the adsorption process [41]. The mechanisms involved with a reduction in adsorptive capacity with increasing concentration of adsorbent are complex,and include primarily electrostatic interactions, interference between binding sites, overlapping adsorption sites and others [42-43].

The Effect of Biosorbent Dosage and the Initial Concentration of Pb on the Pb Uptake

Fig. 3 shows the effect of biosorbent dosage and the initial concentration of Pb on the adsorption process,

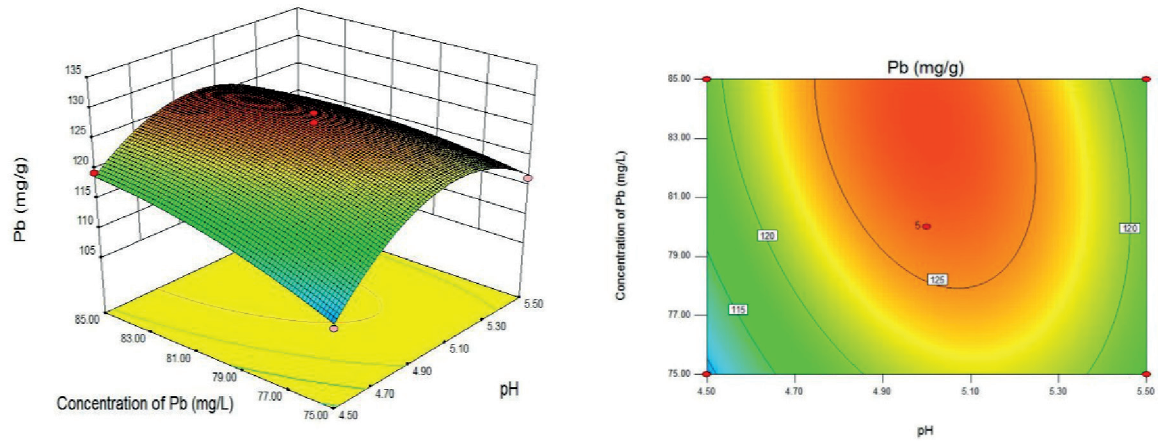

Fig. 1. Surface and contour plots for the effect of pH and initial concentration of Pb on Pb removal.

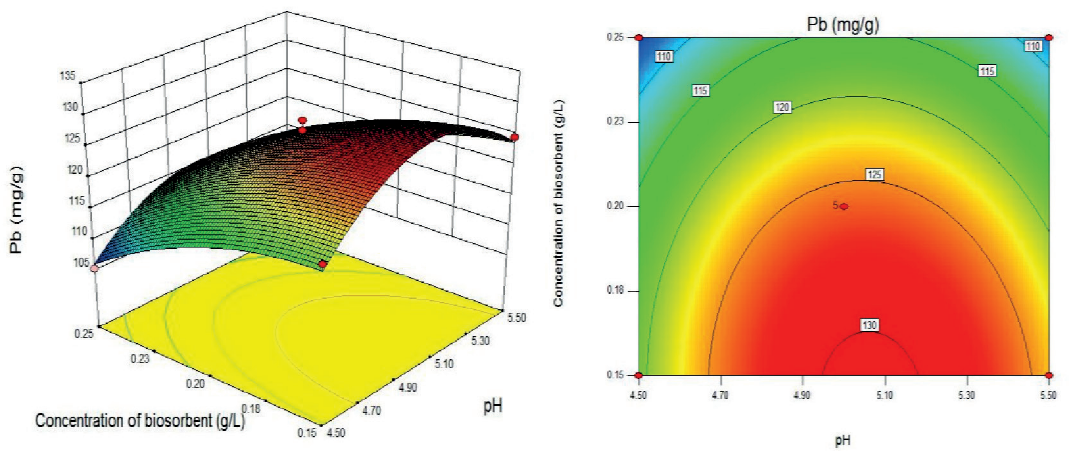

Fig. 2. Surface and contour plots for the effect of pH and adsorbent dosage on Pb removal.

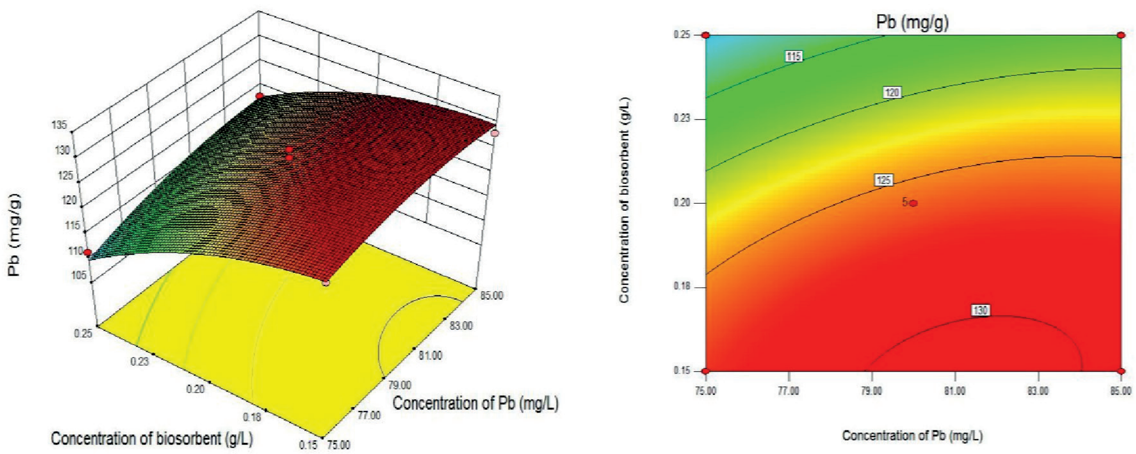

Fig. 3. Surface and contour plots for the effect of adsorbent dosage and initial concentration of Pb on Pb removal.

Table 8. Comparison of maximum Pb binding capacities of reported biomass.

Biomass	q (mg/g)	References
Simplicillium chinense QD10	57.80	Jin et al. [44]
Stenotrophomonas maltophilia	80.23	Li et al. [45]
Modified tea factory waste	22.11	Nuhoğlu et al. [46]
Absidia cylindrospora	12.30	Albert et al. [47]
Moringa oleifera	45.83	Imran et al. [48]
Recombinant saccharomyces cerevisiae expressing metallothionein	129.60	This study

with the pH at zero level. As shown in the contour map, we can see that the interaction between adsorption dosage and the concentration of Pb was not significant. This study shows that the amount of Pb that was adsorbed increased as the ratio of the concentration of Pb to biosorbent dosage increased.

The optimal conditions for adsorption were pH 5.05, an initial concentration of Pb of 81.12 mg/L, and a biosorbent dosage of 0.15 g/L. The maximum predicted value of adsorbed Pb was 130.57 mg/g.

The Model Validation Experiment

The validation experiment was performed under optimum adsorption conditions (pH = 5.05, an initial concentration of Pb = 81.12 mg/L, a biosorbent dosage = 0.15 g/L, temperature = 30ºC, time = 20 min and speed of agitation = 100 rpm). The results show that the predicted values of Pb adsorption (130.57 mg/g) are closer to the measured values (129.60 mg/g). The prediction accuracy reached 99.26%. The experiment demonstrated good precision reliability and validity of this method for removal of Pb from wastewater.

Table 8 shows a profiles from various organisms was used to remove Pb from wastewater in recent studies [44-48]. Although it is difficult to directly compare adsorption ability of Pb between various organisms because different experimental conditions existed, genetically engineered *S. cerevisiae* seem to exhibit far more potential for removing Pb from wastewater.

Conclusion

Because the binding capacity of wild type microorganisms for heavy metal is not sufficiently high, recent research has focused on the development of new biological material to increase the binding capacity for metal ions. In this study, genetically engineered *S. cerevisiae* were used for adsorption of Pb from wastewater. The PBD design and our statistical analysis indicate that the initial concentration of Pb, pH values and the concentration of biosorbent were three critical factors influencing Pb biosorption by genetically engineered *S. cerevisiae*. The optimal biosorption conditions were as follows: the initial concentration of Pb = 81.12 mg/L, pH = 5.05 and the biosorbent dosage = 0.15 g/L. Under optimal conditions, the maximum adsorption quantity of Pb was 129.60 mg/g. The model validation experiments showed good correlation between the predicted and experimental values. The performance of adsorption of Pb by genetically engineered *S. cerevisiae* is improved by response surface parameter optimization. In future research, genetically engineered *S. cerevisiae* was immobilized to carry out biosorption of Pb. Summarizing, genetically engineered *S. cerevisiae* show higher adsorption capacity of Pb than other reported adsorbents and will be an effective biosorbent for industrial use.

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Conflict of Interest

The authors have declared no conflict of interest. All two authors have contributed in this study.

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