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Exploring multi-metal biosorption by indigenous metal-hyperresistant *Enterobacter* sp. J1 using experimental design methodologies

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Abstract

A novel experimental design, combining mixture design and response surface methodology (RSM), was developed to investigate the competitive adsorption behavior of lead, copper and cadmium by an indigenous isolate *Enterobacter* sp. J1 able to tolerate high concentrations of a variety of heavy metals. Using the proposed combinative experimental design, two different experiment designs in a ternary metal biosorption system can be integrated to a succinct experiment and the number of experimental trials was markedly reduced from 38 to 26 by reusing the mutual experimental data. Triangular contour diagrams and triangular three-dimensional surface plots were generated to describe the ternary metal biosorption equilibrium data in mixture design systems. The results show that the preference of metal sorption of *Enterobacter* sp. J1 decreased in the order of $Pb^{2+} > Cu^{2+} > Cd^{2+}$. The presence of other metals resulted in a competitive effect. The influence of the other two metals in ternary metal biosorption system can be easily determined by comparing the stray distance from the single metal biosorption. The behavior of competitive biosorption was successfully described and predicted using a combined Langmuir–Freundlich model along with new three-dimensional contour-surface plots.

Keywords: Multi-metal biosorption; *Enterobacter* sp.; Response surface methodology; Mixture design; Contour-surface plot; Triangular three-dimensional surfaces

1. Introduction

Heavy metals pollution comes from various industrial sources, such as metal plating, electroplating, mining, ceramic, batteries, pigment manufacturing, leather, photography, automobile, refrigeration industries [1,2]. Biomass of algae, fungi and bacteria has been known to readily adsorb or accumulate metal ions (known as biosorption or bioaccumulation) [3–9] and has been utilized to develop effective and economic means for the remediation of heavy-metal-polluted wastewater [6,10,11]. Accumulation of heavy metal by microorganisms is a promising approach because of its specificity and ability to function at low concentrations, or after conventional physico-chemical treatment. Therefore, it is an attractive prospective to use biosorption process for the removal of industrial heavy metal pollutants [12,13]. The research on metal biosorption has been performed with a variety of aspects as summarized in a review reported by Volesky and Holan [6].

Most of biosorption studies have been focused on single-metal systems [3,6,14–16]. In contrast, relatively less work has been contributed to elucidate the biosorption behavior in multi-metal systems, which are normally the composition of the industrial effluents [3,7,17–20]. Competitive biosorption of heavy metals has been investigated for binary and ternary-metal systems [21]. When more than one metal is present in a sorption system, evaluation, interpretation and representation of biosorption results become very complicated. This is the reason why much biosorption work was done with single-metal systems, though in practice wastewaters are polluted with multiple heavy metals [9]. Chen et al. [21] shows in their competitive sorption experiments that Pb^{2+} was sequestered preferentially over Cd^{2+} and Hg^{2+}, while the presence of the co-existing metals still decreased the equilibrium uptakes of preferably metal adsorbates [9]. Similar results were observed by Göksungur et al. [22] using ethanol treated yeast cells as the biosorbent. The

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decreased metal uptake in competitive conditions was thought to be a response to increased competition between like charged species for binding sites [22]. Pradhan et al. [23] also reported the biosorption preference of Fe > Ni > Cr by Microcystis in single, duplicate and trimetallic combination. They proposed that the preference of a metal sorbent may be closely related to electronegativity of the metal ions. [23]. For more effective applications of metal biosorbents, the competitive adsorption of the co-existing metal ions needs to be revealed. This information is of importance to predict the efficiency of the biosorption process, allowing optimization of the operation strategies. Since the interaction of metal biosorption from other metal ions may be synergistic, antagonistic or non-interactive, the sorption results cannot be predicted on the basis of single-metal studies [24]. The one-factor-at-a-time strategy frequently applied to single-metal system fails to consider possible interactions between the metal ions in aquatic systems [25]. Thus, there is a need to develop appropriate methodologies in the study of multi-metal biosorption [26–29]. This motivated us to design our experiments with statistical methods, which offer a valid basis for developing an empirical model for the multi-metal biosorption system.

In this study, lead, copper, and cadmium, which are frequently found in the industrial effluents in Taiwan, were the target metal pollutants. Two experimental design methodologies have been used; namely, response surface design (RSM) and mixture design to identify the characteristics of metal removal from multi-metal aqueous systems via biosorption. Most of RSM used in multi-variable systems was designed for optimization hunting. However, since in real industry wastewater, the concentration of heavy metals in the effluents is case- and time-dependent. Therefore, in this case, the RSM design was used to predict the biosorption results under different metal composition, instead of finding the metal composition leading to optimal biosorption capability. By using RSM l design and data collection, the parameters of the empirical model for multi-metal biosorption can be estimated more effectively. Furthermore, in practical situation, metal-containing wastewater may be diluted before treatment if the metal concentration is too high. Therefore, two mixture designs, standing for high and low overall metal concentrations with the same metal components, were presented in this study to investigate the effect of total metal concentration on biosorption ability. By using mixture design, the interaction of metal biosorption between a target metal ion and the other two co-existing metal ions can be conveniently compared through the contour plot on a trilinear coordinate scale and a triangular three-dimensional response surface plot. As all the experimental data were shown in a 3D space, the fitness of model prediction can be checked directly from the triangular 3D response surface plot. We also demonstrated the feasibility of predicting the behavior of competitive biosorption in practical wastewater treatment using a new 3D contour surface plot generated by a multi-metal biosorption model. Moreover, through sharing part of the experimental data in a RSM face-centered cube design and two augmented simplex-lattice mixture designs, the number of experimental trails can be markedly reduced.

2. Materials and methods

2.1. Bacterial strain and culture conditions

The biosorbent used in this study was *Enterobacter* sp. J1, which was screened from local industrial wastewater treatment plant according to the ability to tolerate elevated concentrations of heavy metals, including lead, copper, cadmium, mercury, zinc, cobalt, and nickel. There may be no direct correlation between high metal resistance and high metal biosorption capacity. However, microorganisms with metal resistance often express metal-binding proteins that may help increase metal binding affinity or capacity. [30–32] Also, microorganisms could have the advantage for application to the environment under metal toxicity stress. The J1 strain was able to grow on LB agar (Difco) amended with 1500 mg/l of Pb^{2+}, 500 mg/l of Cu^{2+}, 300 mg/l of Cd^{2+}, 750 mg/l of Zn^{2+}, and 300 mg/l of Ni^{2+}. The bacterial cultures were typically incubated in LB broth at 37 °C and 200 rpm agitation in shake-flask cultures.

2.2. Biosorbent preparation

The cells were harvested by centrifugation (9050 × g, 10 min) from early-stationary cultures with a cell density of approximately 1.5 ± 0.3 g/l. After rinsing twice with 0.05 M Tris-buffer (pH 5.0), the cells were re-suspended in designated heavy metal solutions for the biosorption experiments.

2.3. Measurement of heavy metals

The heavy metal adsorbates used in this study were lead ($PbCl_2$), copper ($CuCl_2$), and cadmium ($CdCl_2$), which were obtained from Riedel-de Haen, Inc. (Germany). Heavy metals in solutions were measured by a flame Atomic Absorption Spectrometer (AAS; Model 932 plus, GBC Scientific Equipment, Dandenong, VIC, Australia). Before measurement, the heavy metal solutions were appropriately diluted with 0.1 N HCl to ensure that the heavy-metal concentration in the sample was linearly dependent on the absorbance detected.

2.4. Metal adsorption experiments

The biosorbent was suspended in solutions containing heavy metals whose concentrations were set according to experimental design methodology. The cell concentration in the solution was in the range of 2.0 ± 0.3 g/l. The cell/metal suspension was gently agitated (75 rpm) at 25 °C. The pH of the solution was initially adjusted to 5.0 ± 0.2 to avoid precipitation of metals in the form of metal hydroxides [1,7,9,23,33]. After 24 h of incubation (25 °C, 75 rpm), samples were taken from the solutions, and the metal concentration in the supernatants was measured with atomic absorption spectrometer.

2.5. Experimental design

Heavy metals were prepared as aqueous solutions with different compositions according to the experimental design

Table 1
The list of experimental trials according to response surface methodology, mixture designs, and combinative design (no. 1–26) for multi-metal biosorption using *Enterobacter* sp. J1 as the biosorbent

Experiment	No.	RSM (Face-centered cube)			Mixture design (666 μM)			Mixture design (1333 μM)		
		Pb (μM)	Cu (μM)	Cd (μM)	Pb (μM)	Cu (μM)	Cd (μM)	Pb (μM)	Cu (μM)	Cd (μM)
	1	0	0	0						
	2	0	0	666.67	0	0	666.67			
	3	0	666.67	0	0	666.67	0			
	4	0	666.67	666.67				0	666.67	666.67
	5	666.67	0	0	666.67	0	0			
	6	666.67	0	666.67				666.67	0	666.67
Mutual experiments	7	666.67	666.67	0				666.67	666.67	0
	8	0	333.33	333.33	0	333.33	333.33			
	9	666.67	333.33	333.33				666.67	333.33	333.33
	10	333.33	0	333.33	333.33	0	333.33			
	11	333.33	66.667	333.33				333.33	666.67	333.33
	12	333.33	33.333	0	333.33	333.33	0			
	13	333.33	33.333	666.67				333.33	333.33	666.67
	14	333.33	333.33	333.33						
	15	333.33	333.33	333.33						
	16	333.33	333.33	333.33						
	17	333.33	333.33	333.33						
	18	333.33	333.33	333.33						
	19	333.33	333.33	333.33						
Different experiments	20	666.67	666.67	666.67						
	21				333.33	166.67	166.67			
	22				166.67	333.33	166.67			
	23				166.67	166.67	333.33			
	24							0	0	1333.3
	25							0	1333.3	0
	26							1333.3	0	0

methodologies (Table 1). The experimental design methodologies used (namely, response surface design and mixture design) are described in detail as follows

2.5.1. Response surface methodology (RSM)

RSM is usually used for optimization of experimental conditions. However, in this study, this methodology was used to find the interaction of co-existing metals during biosorption as well as to predict multi-metal biosorption results. In general, there are several design methodologies can be chosen, such as Box-Behnken design, central composite design and face-centered cube design. In this work, the face-centered cube design (FCCD), a modified version of the central composite design, was utilized to obtain the experimental data [34]. This design locates the star or axial points on the centers of the faces of the cube, representing the concentration ranges of the three heavy metals co-existing in the environment.

This design contained a total of 20 experiments, representing the 6 points on the six cubic surfaces and 8 points on the eight vertices, as well as the central point with 6 replications (Fig. 1). In geometry, three of six vertices and three of eight face-center points are on a plane (the big triangle), and another three edge points with other three face-center points are on the other plane (the small triangle). Two planes come with a normal vector (1,1,1) and the sum of the concentration values is the same for each point on the same plane. Therefore, the points on each triangle plane were not only used in RSM but also in the mixture design (introduced in Section 2.5.2). The composition of the three metals in each experiment is listed in Table 1, in which the first seven experiments and the last experiment were organized with a fractional factorial design. The experimental trials from 14 to 19 were the replications of central points. The last part of experimental trials (from 8 to 13) was organized on the center of the face of the design cube. Using RSM, the relationship between the variables (i.e., concentration of the three heavy metals) were expressed mathematically in the form of a multi-metal adsorption model [35], which gave the response as a function of relevant variables.

2.5.2. Mixture design methodology

In response surface designs described earlier, the concentration of each heavy metal is independent of that of the other two metals. While in the mixture design, the factors were the weight percentage of each heavy metal, and thereby their levels were dependent. Simplex lattice design commonly applied to study the effects of mixture components on the response variable is basically a boundary point design [34]. In order to cover the points in the interior of the simplex-lattice design, this study used augmented simplex-lattice design with axial points to increase the interior runs for the experimental design [34]. There were two groups of mixture designs, including the total metal ion concentrations of 667 and 1333 μM, respectively. Each design contained a total of 9 experiments as indicated in Fig. 1. The experimental design contained 6 points for the simplex lattice

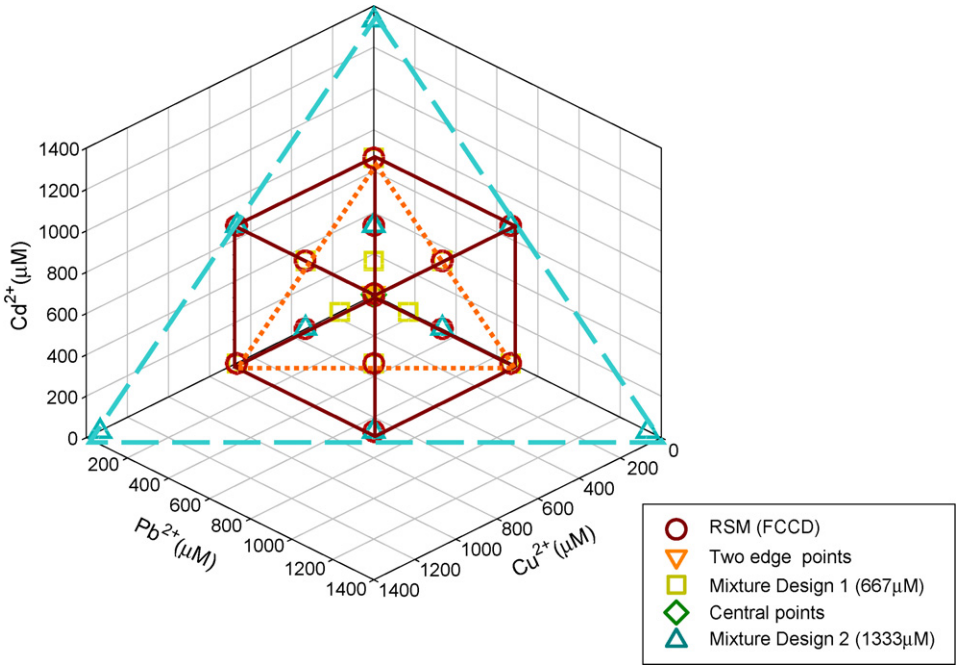

Fig. 1. The arrangement of metal compositions (experimental trials) in ternary-metal systems (Pb^{2+}, Cu^{2+} and Cd^{2+}) using the combinative design method. The cube with solid lines was FCCD; the triangle with long dash lines was one of mixture design (the total metal concentration was 1333 μM); and the triangle with dotted lines was another mixture design (the total metal concentration was 666 μM).

design and 3 points for axial runs. The composition of the three metals in each run was also listed in Table 1. The experimental points no. 21, 22 and 23 were the axial points for total concentration of 667 μM, while no. 9, 11 and 13 were the axial points for total concentration of 1333 μM. The experimental design software used for design table arrangement and data analysis was JMP V.3.2.2 (SAS Inc., 1989).

2.5.3. Combinative design

In order to increase the accuracy of analysis and reduce the number of experiments at the same time, a new design methodology was developed by combining the RSM and mixture design described above. As shown in Fig. 1 and Table 1, most of the experimental points, except for two edge points (0,0,0) and (666.67, 666.67, 666.67), were located on the two plates: $x+y+z=666.67$ and $x+y+z=1333.3$. The experimental points on each plate can be used to build a mixture design. Thus, we developed a combinative methodology by using part of experimental points in RSM to build up two mixture designs for the reduction of the number of total experimental runs (Fig. 1 and Table 1). The experimental runs can be separated into two groups; namely, "Mutual experiments" and "Different experiments" (Table 1). The experiments grouped in "Mutual experiments" were organized by sharing the data among the RSM and two mixture designs to reduce the experimental runs. The other experiments, which cannot be used in data sharing, were grouped in "Different experiments".

2.6. Model simulation

Langmuir model is valid for monolayer sorption onto a surface of a finite number of identical sites, while the empirical Freundlich equation is based on sorption on a heterogeneous surface. The combined Langmuir–Freundlich model (CLF) [36] could possess the characteristics of both models and more applicable to the real situation. Thus, in this study, CLF model (Eq. (1)) was used in this multi-metal biosorption system. Once the experimental results were obtained, the parameters of multi-metal models were calculated using the following equations:

$$q_{Pb}=\frac{a_{Pb}C1/n_{Pb}Pb}{1+b_{Pb}C1/n_{Pb}Pb+b_{Cu}C1/n_{Cu}Cu+b_{Cd}C1/n_{Cd}Cd} \quad (1)$$

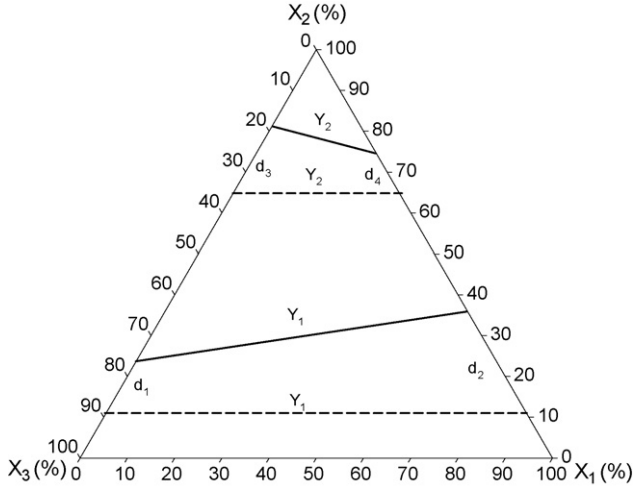

Fig. 2. Graphical representation for the information regarding the interaction of the target metal adsorbate with its co-existing metal ions during multi-metal biosorption in a triangular contour diagrams. The solid contour plot was generated for multi-metal biosorption system and the short dash contour plot was generated for single-metal biosorption system.

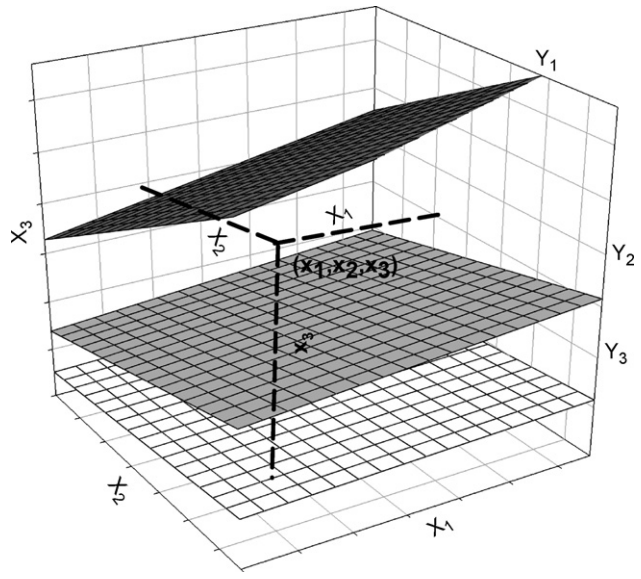

Fig. 3. Graphical representation for the methodology to predict the result of multi-metal biosorption with a three-dimensional contour-surface plots. A solution containing the three heavy metal ions can find a point in this three-dimensional contour-surface plots with the coordinates (x_1, x_2, x_3) representing the concentration of the three metal ions. The metal adsorption can be predicted by the location that is between two of contour surfaces (Y_1 and $Y2$).

where q_i (i = Pb, Cu and Cd) was the predicted response (heavy metal adsorption) used as the dependent variable; C_i (i = Pb, Cu and Cd) was the concentration of heavy metals used as the controlled variables; a_i, b_i and n_i (i = Pb, Cu and Cd) were the model parameters. The parameters of CLF model were estimated by multiple linear regression analysis using the least squares method (SigmaPlot V. 8.0, SPSS Inc.).

2.7. Graphical methodology for data validation

Three graphical methodologies were used to present the experimental results; namely, the contour plot on a trilinear coordinate scale, the triangular three-dimensional surface plot and the three-dimensional contour surface plot. The latter two are considered new graphical methods that developed from this work.

2.7.1. Triangular contour diagram (TCD)

The TCD plot has been widely used for three-component mixture design [34]. The contour plots were generated for single-metal and multi-metal biosorption systems and shown in Fig. 2. Three axes stood for the component ratio of three metals, X_1, X_2 and X_3. The solid lines were drawn for X_2 biosorption in multi-metal systems and the dot lines were in single-metal biosorption systems. Comparison between the two contour plots for the same biosorption system (Y_2 for high X_2 containing or Y_1 for low X_2 containing) provides information regarding the interaction of the target metal adsorbate with its co-existing metal ions during multi-metal biosorption. The distance (d_1 to d_4) between solid line and dash line on the axis shows the influence of co-existing metal ions (Fig. 2). For instance, if d_2 is larger than d_1, metal ion X_1 would play a more important role than X_3 in influencing biosorption ability when X_2 concentration is low. On the other hand, if d_3 is larger than d_4, metal ion X_3 has a

Table 2
Parameters estimated from simulations with combined Langmuir–Freundlich model for multi-metal biosorption isotherms of Pb, Cu, and Cd using *Enterobacter* sp. J1 as the biosorbent

Adsorbate	Combination Langmuir–Freundlich model[a]		
	n	a	b
Pb	0.813	1.34	3.01×10^{-2}
Cu	0.537	1.43×10^{-2}	7.05×10^{-4}
Cd	0.416	6.37×10^{-4}	3.27×10^{-5}

[a] $r^2 = 0.993$.

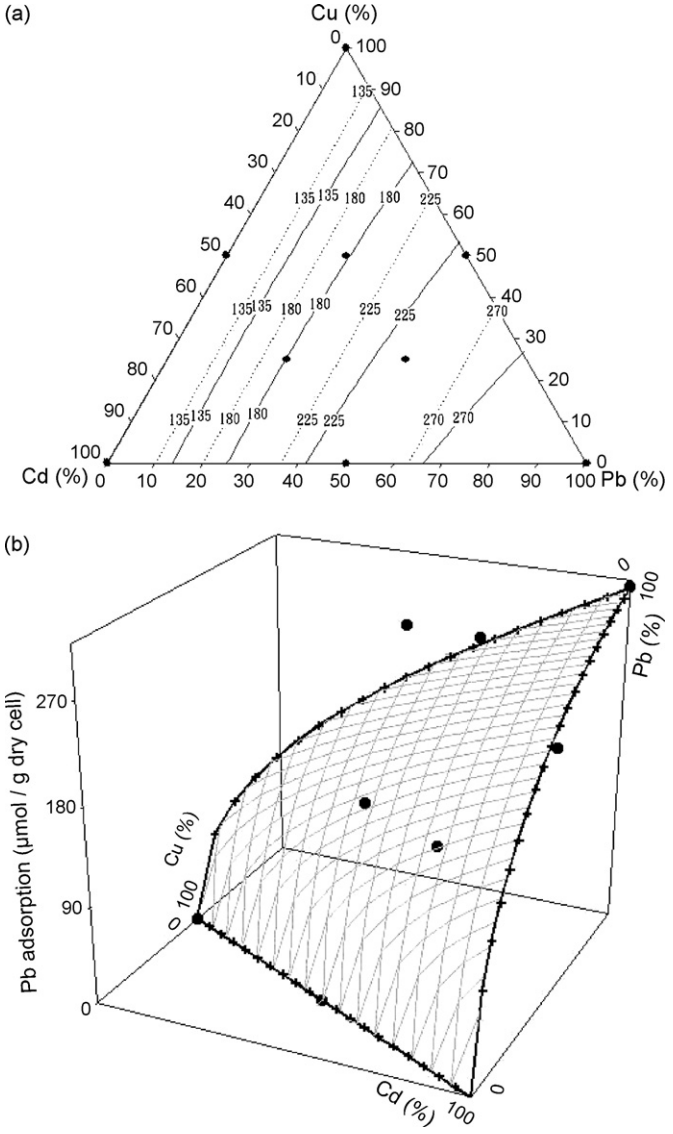

Fig. 4. Graphical representation for the biosorption of Pb^{2+} on *Enterobacter* sp. J1 in ternary metal mixtures of Pb^{2+}, Cu^{2+} and Cd^{2+} using the combined Langmuir–Freundlich model. (a) triangular contour diagrams, (b) triangular three-dimensional biosorption surfaces (the total metal concentration was 666 μM; the values indicated in (a) were biosorption capacity in a unit of μmol/g dry cell).

larger impact on biosorption than X_1 when X_2 concentration is high.

2.7.2. Triangular three-dimensional surface plot (T3DSP)

Although the TCD plot could be used to observe the interaction of three components in a mixture design system, it is difficult for TCD to show the experimental data on the plot, making it inconvenient for data analysis. Therefore, a triangular three-dimensional surface plot was developed for data validation in the mixture design systems. T3DSP was created based on the triangular contour diagram, as the response surface in T3DSP was generated from the model equation used in developing contour curves in TCD. Using the T3DSP plot method, the triangle prediction surfaces generated from both model simulation and the experimental data can be shown on the same plot. In this way, the deviation between experimental data and the model prediction values can be clearly observed.

2.7.3. Three-dimensional contour surface plot (3DCSP)

Three-dimensional contour surface plot was developed from two-dimensional contour plot that has been used to predict the experimental data in a two-variable system. In a 3D contour surfaces plot, the response surfaces were generated from the results of model simulation shown in the previous section. The biosorption is a function of the concentration of the three heavy metals as shown below.

$$Y = f(x_1, x_2, x_3) \quad (2)$$

where Y is biosorption capacity and x_1, x_2 and x_3 are the concentrations of heavy metals. Based on Eq. (2), the contour surfaces (shown in Fig. 2) can be generated with different Y value (i.e., Y_1, Y_2, Y_3). Since there are three independent vari-

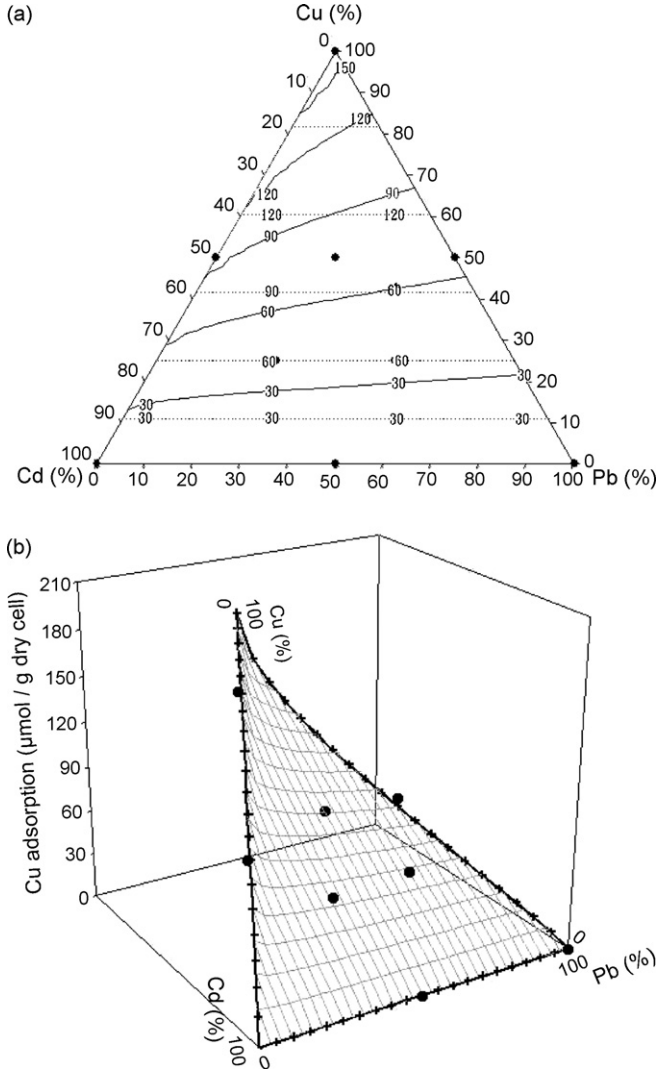

Fig. 5. Graphical representation for the biosorption of Cu^{2+} on *Enterobacter* sp. J1 in ternary metal mixtures of Pb^{2+}, Cu^{2+} and Cd^{2+} using the combined Langmuir–Freundlich model. (a) triangular contour diagrams, (b) triangular three-dimensional biosorption surfaces (the total metal concentration was 666 μM; the values indicated in (a) were biosorption capacity in a unit of μmol/g dry cell).

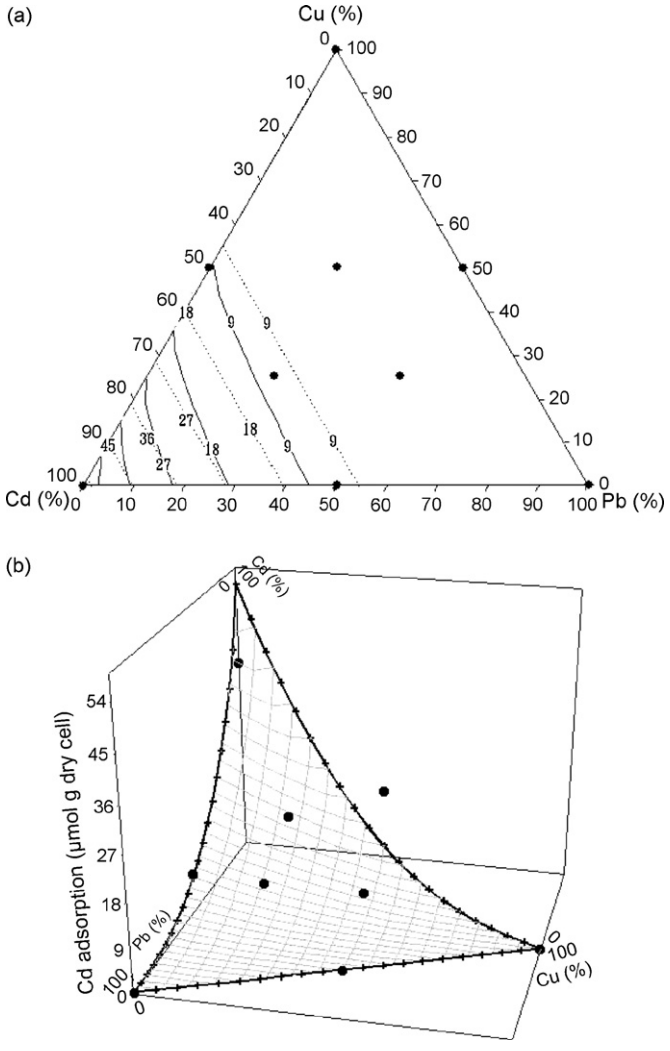

Fig. 6. Graphical representation for the biosorption of Pb^{2+} on *Enterobacter* sp. J1 in ternary metal mixtures of Pb^{2+}, Cu^{2+} and Cd^{2+} using the combined Langmuir–Freundlich model. (a) triangular contour diagrams, (b) triangular three-dimensional biosorption surfaces (the total metal concentration was 666 μM; the values indicated in (a) were biosorption capacity in a unit of μmol/g dry cell).

ables (lead, copper and cadmium) in our multi-metal biosorption system, each metal-bearing sample containing a known composition of three metal ions can find a relative point (x_1, x_2, x_3) in the three-dimensional contour surface plot as shown in Fig. 3. The multi-metal biosorption result of the relative point, located between two contour surfaces (Y_1 and Y_2), can also be estimated by interpolation methodology. Hence, if the wastewater contains a given composition of the three metal ions (Pb^{2+}, Cu^{2+} and Cd^{2+}), the relative point in this 3DCSP plot can be used to predict the result of multi-metal biosorption.

3. Results and discussion

3.1. Development of a new experimental design methodology – a combinative design

As shown in Fig. 1 and Table 1, the RSM face-centered cube design needed 20 experimental points and the two augmented simplex-lattice mixture designs needed 18 experimental points. Fig. 1 shows the distribution of the experimental points in the design space. The cube was for RSM and the two triangles were for the two mixture designs. It is observed that each mixture design shared six experimental points with the RSM design (Table 1). Thus, using combinative design successfully reduced the number of overall experimental trials from 38 to 26, as indicated in Table 1. The proposed experimental design methodology appeared to offer a more complete coverage of experimental trials with a reduction of total number of designed experiments.

3.2. Model simulation of biosorption isotherms

Results from multi-metal biosorption experiments listed in Table 1 were simulated with the combined Langmuir–Freundlich (CLF) model [36] (Eq. (1)). The estimated values of model parameters are listed in Table 2. The high r square value ($r^2 = 0.933$) suggests the feasibility of using CLF model to describe the experimental results obtained from the multi-metal biosorption experiments. Therefore, in RSM and mixture design analysis, the CLF model with the estimated parameters (Table 2) was used to generate the biosorption profiles.

3.3. Data analysis from mixture designs

Figs. 4–6 are graphical representations of the biosorption of Pb^{2+}, Cu^{2+} and Cd^{2+} ions, respectively, on *Enterobacter* sp. J1 from ternary mixtures of the three metals with a total metal concentration of 666 μM. The biosorption profiles were generated by using combined Langmuir–Freundlich model. In the triangular contour diagrams (Figs. 4a, 5a, and 6a), comparison of the single-metal (dashed lines) and multi-metal (solid lines) biosorption contour shows that the extent of influence on biosorption of a target metal by *Enterobacter* sp. J1 was in the order of $Cu^{2+} > Cd^{2+}$, $Pb^{2+} > Cd^{2+}$, and $Pb^{2+} > Cu^{2+}$ for Pb^{2+}, Cu^{2+} and Cd^{2+} biosorption, respectively. Hence, the biosorption preference of *Enterobacter* sp. J1 appeared to decrease in the order of $Pb^{2+} > Cu^{2+} > Cd^{2+}$. Meanwhile, in the triangular 3D biosorption surfaces (Figs. 4b, 5b, and 6b), the deviations between the 3D surfaces generated by CLF model and the real experimental data (solid dots) are clearly shown. The predicted surfaces essentially matched the experimental data, indicating that the CLF model could predict the behavior of multi-metal biosorption quite well.

To enhance the feasibility in practical applications, multi-metal biosorption experiments were also conducted at a higher total concentration (i.e., 1333 μM) using mixture experimental design. The results for Pb^{2+}, Cu^{2+} and Cd^{2+} biosorption are shown in Figs. 7–9, respectively. The trend of competitive biosorption (i.e., $Pb^{2+} > Cu^{2+} > Cd^{2+}$) was similar to that observed in the mixture design experiments with a total metal concentration of 666 μM. The predicted surface from model

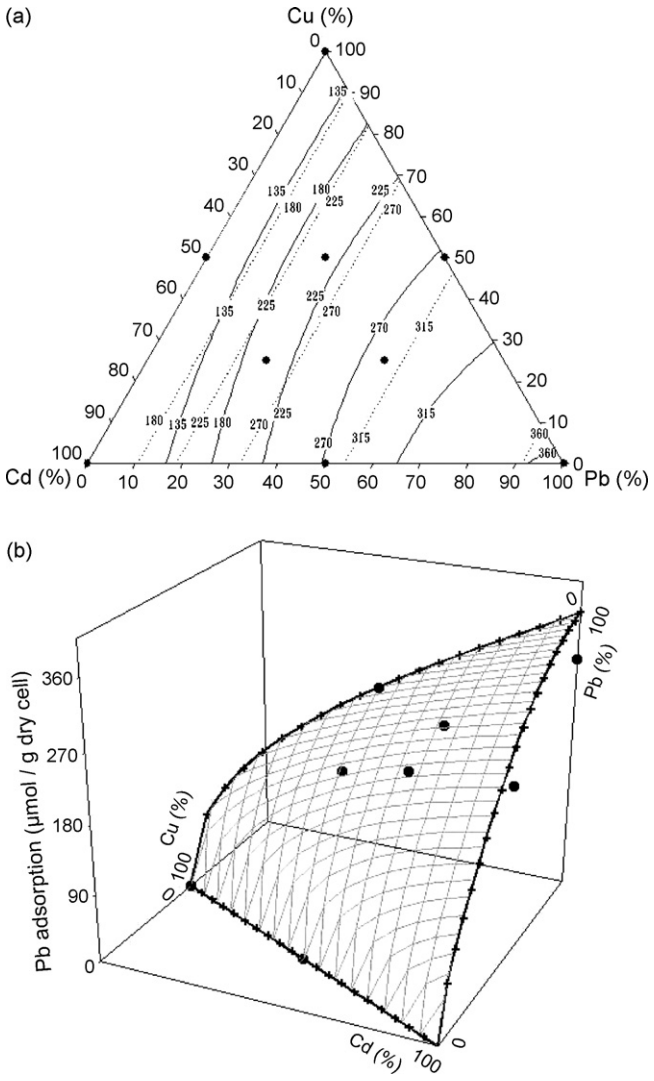

Fig. 7. Graphical representation for the biosorption of Cu^{2+} on *Enterobacter* sp. J1 in ternary metal mixtures of Pb^{2+}, Cu^{2+} and Cd^{2+} using the combined Langmuir–Freundlich model. (a) triangular contour diagrams, (b) triangular three-dimensional biosorption surfaces (the total metal concentration was 1333 μM; the values indicated in (a) were biosorption capacity in a unit of μmol/g dry cell).

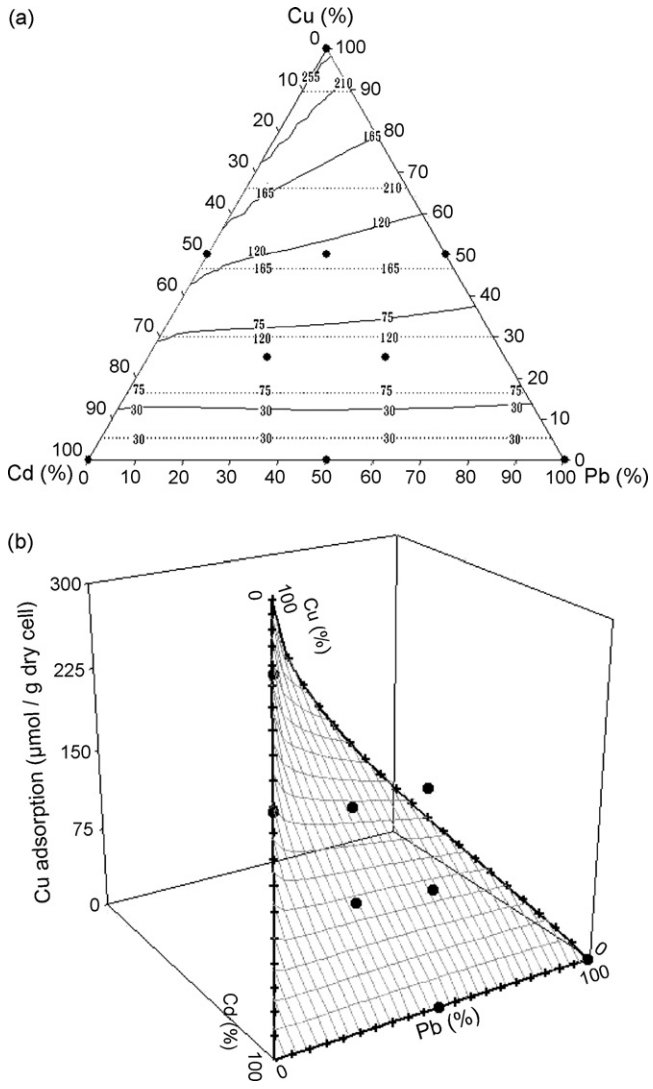

Fig. 8. Graphical representation for the biosorption of Cd^{2+} on *Enterobacter* sp. J1 in ternary metal mixtures of Pb^{2+}, Cu^{2+} and Cd^{2+} using the combined Langmuir–Freundlich model. (a) triangular contour diagrams, (b) triangular three-dimensional biosorption surfaces (the total metal concentration was 1333 μM; the values indicated in (a) were biosorption capacity in a unit of μmol/g dry cell).

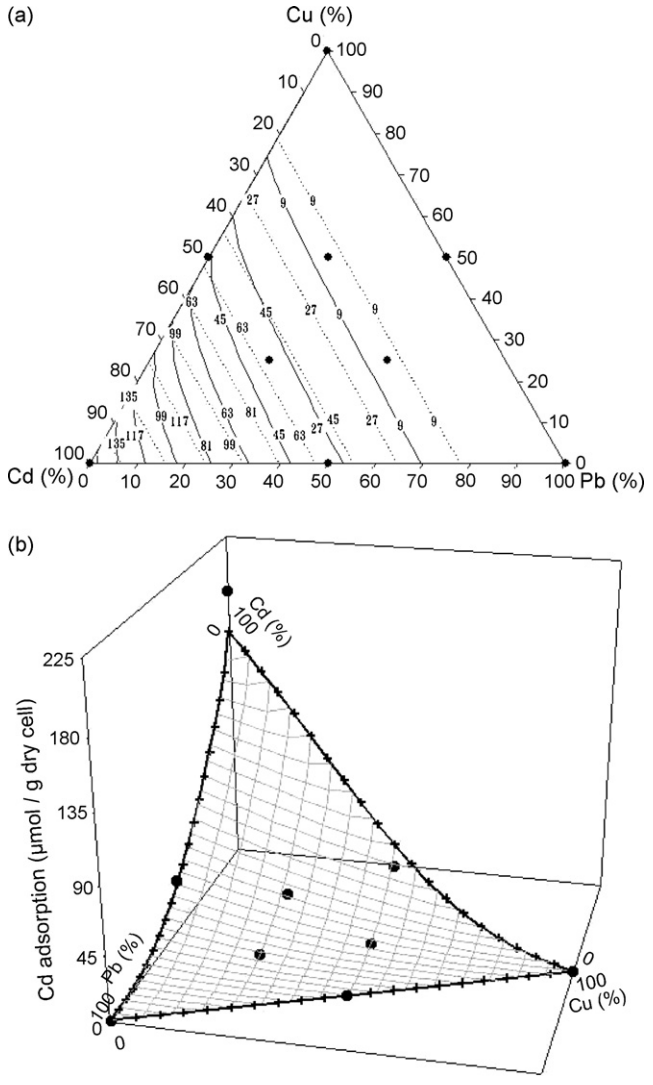

Fig. 9. Graphical representation for the biosorption of Cd^{2+} on *Enterobacter* sp. J1 in ternary metal mixtures of Pb^{2+}, Cu^{2+} and Cd^{2+} using the combined Langmuir–Freundlich model. (a) triangular contour diagrams, (b) triangular three-dimensional biosorption surfaces (the total metal concentration was 1333 μM; the values indicated in (a) were biosorption capacity in a unit of μmol/g dry cell).

simulation also matched the experimental data satisfactorily well. When comparing the results in Figs. 4–6 with those in Figs. 7–9, the shape of the triangular three-dimensional surface was quite similar for the runs with two different total metal concentrations (666 μM and of 1333 μM). This suggests that the interaction of the target metal adsorbate with its co-existing metal ions during multi-metal biosorption was similar regardless of total metal concentration. However, the total adsorption capacities in each run were in general higher for the run with a higher total metal concentration. This suggests that a total metal concentration of 666 μM had not completely saturate the adsorption sites on *Enterobacter* sp. J1. Yet, the metal sorption preference of the biosorbent was not alter regardless of the difference in total metal concentrations.

3.4. Data analysis from response surface methodology (RSM) design

Three-dimensional contour-surface plots were created by using CLF model for the prediction of ternary-metal biosorption by *Enterobacter* sp. J1. The predicted profiles for the selective biosorption of Pb^{2+}, Cu^{2+} and Cd^{2+} are illustrated in Figs. 10a–c, respectively. Each 3D surface in Fig. 10 indicates a contour with identical adsorption capacity of a target metal under a ternary metal environment. In other words, these surfaces act as scales (or markers) of biosorption capacity of each metal. Once a specific composition of a ternary metal system is identified, the adsorption capacity of each metal ion can be determined from these 3D contour surfaces using interpolation methodology. This implies that for each wastewater sample containing a known concentration of the three metal ions, the biosorption capacity

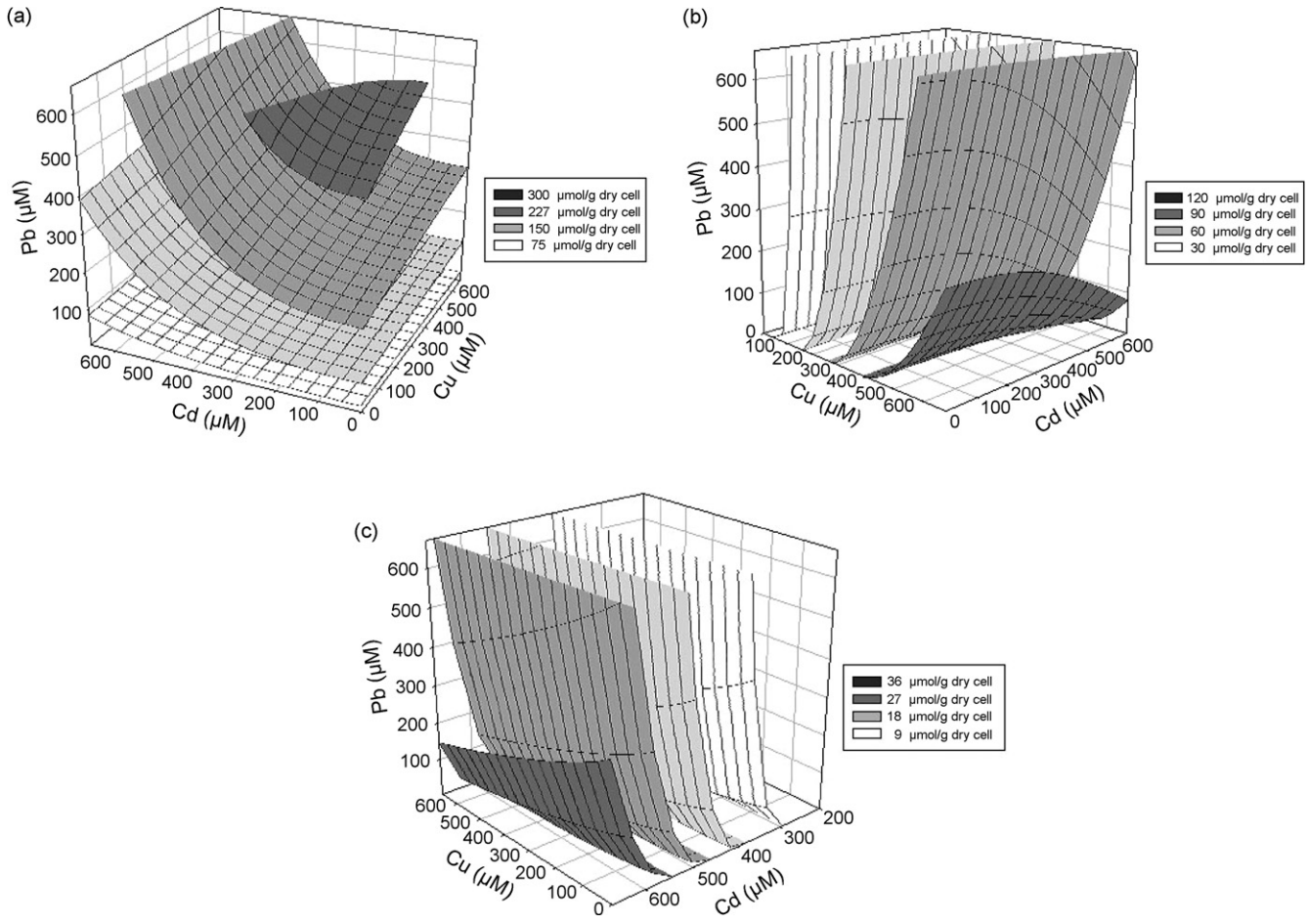

Fig. 10. Contour-surface plots created by combined Langmuir–Freundlich model for the competitive biosorption of (a) Pb, (b) Cu, and (c) Cd on *Enterobacter* sp. J1 in ternary-metal systems (total metal concentration was 666 μM).

of the three metal ions can be predicted by using the 3D contour-surface plot shown in Fig. 10. Therefore, this study appeared to develop a powerful and feasible tool for the prediction of the performance of a multi-metal biosorption process on the basis of the metal composition of the wastewater polluted with multiple metals.

4. Conclusions

This work demonstrated that combination of novel experimental design methodologies and graphical approaches provided an effective means to explore and predict the behavior of competitive biosorption in ternary-metal systems using an indigenous bacterium *Enterobacter* sp. J1 as the biosorbent. Using the combinative design could reduce the total number of experimental trials, while still maintaining high accuracy of analysis. The results show that the biosorption preference of *Enterobacter* sp. J1 decreased in the order of $Pb^{2+} > Cu^{2+} > Cd^{2+}$. The competitive metal biosorption could be described by combined Langmuir–Freundlich model. With the triangular three-dimensional surface plot developed in this work, the deviation between experimental data and model prediction can be visualized on a 3D diagram. Through the three-dimensional contour-surface plot, the results of multi-metal biosorption for a specific multi-metal solution can be predicted quantitatively via exploration of the contour surfaces. This provides a useful tool to estimate the removal efficiency of each metal ion during biotreatment of metal-contaminated wastewaters once the initial metal composition is known.

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