Response Surface Design for Removal of Lead by Different Lactic Acid Bacteria

Leila Goudarzi 1, Rouha Kasra Kermanshahi 1, 2, * and Gholamreza Jahed Khaniki 2

1Department of Microbiology, Faculty of Science, Alzahra University, Tehran, Iran
2Department of Environmental Health Engineering, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
*Corresponding author: Department of Microbiology, Faculty of Science, Alzahra University, Tehran, Iran. Tel: +98-9131150779, Email: rkasra@yahoo.com

Received 2020 January 15; Revised 2020 May 09; Accepted 2020 May 20.

Abstract

Background: Toxic heavy metals, such as lead, are widely used in industry and may cause serious health problems and ecological hazards for living organisms.

Objectives: The current study aimed to investigate the removal efficiency of lead by Lactobacillus strains using a methodological approach.

Methods: After selecting the bacteria with the maximum metals removal ability, experiments were conducted according to (i) the Plackett-Burman design (Minitab18 program) to screen several significant process factors and (ii) Central Composite Design (Design-Expert 11.1.2.0 program) to find out the optimum process conditions for the maximum capacity of metal removal efficiency.

Results: The optimum pH, metal, and bacterial concentration were 6.76, 391 mg.L⁻¹, and 4.60 g.L⁻¹ for lead removal ability of L. acidophilus ATCC4356. A quadratic model was developed to correlate the variables with removal efficiency. According to the results, this model was not statistically significant (P > 0.05).

Conclusions: The experimental removal efficiencies at the optimum condition for lead by L. acidophilus ATCC4356 (73.9%) were consistent with the predicted values. Consequently, due to their appreciate efficiency and the lower cost of the lead removal ability, these two bacteria may be a candidate as good biosorbents. The results also confirmed that the Response Surface Methodology is an appropriate methodology for modeling of removal efficiency.

Keywords: Lead, Response Surface Methodology (RSM), Lactic Acid Bacteria

1. Background

Lead (Pb) is one of the most abundant toxic heavy metals which tends to accumulate in living organisms and causes a variety of adverse health effects for humans (1-4). In humans, exposure to lead can cause renal, skeletal, hepatic, pulmonary, neurologic and hematological, cardiovascular, reproductive, and cardiovascular dysfunction (5-8).

In recent years, due to the expansion of industrial activities, contamination in the environment and the food chain has increased (9); Therefore, the digestive system of the body is prone to lead contamination (10). Since pollution caused by heavy metal has severe health consequences, their removal is of crucial importance. Recent studies were mainly focused on discovering new and cheap methods of metal ions removal (11). Among the conventional treatment methods, ‘adsorption’ has attracted considerable attention in recent years, mainly because of its metal binding capacities and cost-benefits (4). Since the dynamic characteristics of the adsorption process are complex, having the optimum working condition is essential to achieve optimum pollution removal efficiency. Adsorbents may be of mineral, organic, or biological origin by using live or dead microbial organisms (2). According to the literature, lactic acid bacteria (LAB) have efficient removal ability among the microbial organisms (3, 12-14). Recently, several studies have reported that LAB, and more particularly lactobacilli, which are generally recognized as safe probiotics, could identify heavy metals in food and water and perhaps gastrointestinal tract as well, due to their heavy metals binding ability (15, 16). Therefore, the applications of LAB in the food industry and probiotics products have expanded recently (6, 12). The ability to bind lead is reported for several probiotic and food-grade Lactobacillus strains (12, 17, 18).

2. Objectives

Several studies showed that culture conditions substantially affect the removal ability of heavy metals by mi-
croorganisms (2, 5, 7, 18). However, the effects of culture conditions on lactobacilli removal efficiency against lead are less investigated (8). Several optimization techniques are developed to achieve this goal (7). Classical and conventional methods cannot depict all factor combinations which can affect the experiments. Meanwhile, determining the optimal level using these methods requires a lot of time. The current study has used the Response Surface Method (RSM), which is a mathematical and statistical technique to investigate the association between a group of controlled experimental factors and measures’ responses according to one or more criteria (10, 19-21). The RSM is one of the most widely used methods as it develops, improves, and optimizes the processes, particularly in the presence of complex interactions (22). The greatest advantage of the RSM with CCD or Box-Behnken design is decreasing the number of experimental trials required to interpret multiple parameters (23). Therefore, the current study aimed to investigate how the cultural and bacterial conditions can affect lead removal ability of examined lactobacillus strains and to select the optimum conditions for removal activity.

3. Methods

3.1. Heavy Metals

Lead (2 mg.mL⁻¹) stock solutions were prepared from Pb (NO₃)₂ (Sigma-Aldrich), respectively, in Milli-Q water. The stock solutions were sterilized by passing through a 0.22 μm syringe filter and then stored at 4°C until use.

3.2. Bacterial Strains

In the current study, the five Lactobacillus strains were used, that all of which were purchased from the Iranian Research Organization for Science and Technology (IROST): Lactobacillus acidophilus ATCC4356, Lactobacillus Plantarum ATCC8014, Lactobacillus fermentum ATCC9338, Lactobacillus casei ATCC3932, and Lactobacillus rhamnosus ATCC7469. All strains were cultured in deMan, Rogosa, Sharpe (MRS) broth (Merck, Germany), under microaerophilic conditions at 37°C.

3.3. Determining Lead Removal Activities of Each Strain

The lead removal activity of the five strains was analyzed according to the methodology proposed by Zhai and colleagues (5). Briefly, cultured biomass was centrifuged at 8000 × g (Centrifuge Sorvall. RC-5B) for 20 min after incubation of the strains. Then, cell pellets were washed two times, weighed and resuspended in ultrapure water containing 50 mg.L⁻¹ lead to give a final bacterial concentration of 1 g.L⁻¹ (wet weight). The pH of the suspension was adjusted to 6 by dilute HNO₃ or NaOH. After 1 h incubation at 37°C, the suspension was centrifuged at 8000 × g for 20 min; a sample was taken from the supernatant for the analysis of residual lead concentration, which was measured by Inductively Coupled Plasma-optical emission spectrometry (ICP-MS). The lead removal activity (as a percentage) of the strains (bound by the bacteria) was expressed as follows:

\[ \text{Removal} \% = 100\% \times \left[ \frac{C_0 - C_1}{C_0} \right] \] (1)

where \( C_0 \) (mg.L⁻¹) and \( C_1 \) (mg.L⁻¹) are the initial and final metal ion concentrations in the solution, respectively (24). Strains with relatively high lead removal activity in the removal assay were selected for further optimization.

3.4. Optimization Experiment

The optimization experiment was performed in two steps. First, the important factors were selected, and then the optimum condition for lead removal by Lactobacillus strains was determined. The Plackett-Burman design (11) by the Minitab18 program was used for selecting the important factors. A 12 run Plackett-Burman design was applied to evaluate the important variables for six factors by two levels (the lower and higher levels of the factors were selected, respectively) (10). In the second step, the optimum condition for lead removal was done by RSM (9). The experiment was designed with 18 trials, according to the Central Composite Design (17). Each trial was performed in duplicates.

In the current study, different factors (live status, pH, temperature, bacterial and metal concentration) affecting the process of biosorption, were optimized and controlled by using boiling water (live/dead bacteria), acid/base addition (pH), cooling jackets (temperature), and baffles/agitators (same level of bacterial and metal concentration).

3.5. Statistical Analysis

Data were analyzed using Design-Expert 11.1.2.0 program. Analysis of variance (ANOVA), probability values (P value), and three-dimensional curves of the response surfaces were used to investigate the interaction between variables and the response.

4. Results

4.1. Lead Removal Activity of Lactobacillus strains

The percentage of lead removed (bound by the bacteria) by each of the five Lactobacillus strains is shown in Table 1. Metals removal efficiencies varied from 30.5 to 53.8%. Hence, L. acidophilus ATCC4356 was screened for further optimization, with the maximum percentage removal for lead (53.9 ± 0.01%).
4.2. Plackett-Burman Design Analysis to Find Critical Factors for Response Values of Lead Removal

Investigated factors, Plackett-Burman design, and the corresponding response measurements, which were randomized according to the run number, are shown in Table 2.

ANOVA and Pareto charts were used to analyze the significance of the effects. Pareto chart examines how the factors significantly affect the response and help in comparing the relative importance of the effects (4). The interpretation of this chart demonstrates that pH, biomass concentration (g.L\(^{-1}\)), and concentration of metals (mg.L\(^{-1}\)) were highly significant, and other factors were less significant (with a confidence interval of < 95% and P < 0.05) (Figures 1A and 2A). Normal probability plot (Figures 1B and 2B), residual versus order plot (Figures 1C and 2C), and residual versus fitted plot (Figures 1D and 2D) showed the same insinuations; thus, the model fits the data well and follows a normal distribution in both strains.

4.3. Optimum Factors for Response Values of Metal Removal and Analysis for the Central Composite Design

Biomass concentration, pH, and concentration of metals (lead) were left to be optimized after the Plackett-Burman design. The screening was planned according to the principle of the central composite design, such that the pH (A), metal concentration (B, mg.L\(^{-1}\)), and bacterial concentration (C, g.L\(^{-1}\)) were defined as independent values and lead removal (R, percentage) was defined as the response value in the mathematical modeling for L. acidophilus ATCC4356. The range of independent experimental variables, coded variables, and the results of the experiment are shown in Table 3.

The results of the ANOVA are shown in Table 4. Statistical parameters such as F value, R\(^2\), adj. R\(^2\), predicted R\(^2\), and lack of fit were evaluated and compared with the experimental results for the reliability of the model. The analysis showed that the lack of fit was not significant (P > 0.05) and two models were significant (P < 0.05). The coefficient of determination (R\(^2\)) for lead removal was 0.94 (that is, the fitted models were adequate). According to the analyses, a quadratic model equation was developed. The results of the ANOVA are shown in Table 4. The association between independent variables and response was drawn by second-order polynomial equations. Final equations were expressed as follows:

For coded factors:

\[
R (\text{Pb removal}) = +53.7 + 7.41 A - 0.36 B \\
+ 3.20 C - 9.28 AB + 9.22 AC \\
+ 9.76 BC - 2.28 A^2
\]

For actual factors:

\[
R (\text{Pb removal}) = +42.9 + 9.39 pH \\
+ 0.04 \text{ Pb concentration} \\
- 26.2 \text{ Bacterial Concentration} \\
- 0.03 pH * \text{ Pb concentration} \\
+ 3.03 pH * \text{ Bacterial Concentration} \\
+ 0.04 \text{ Pb concentration} \\
* \text{ Bacterial Concentration} - 0.52 pH^2
\]

4.4. Response Surface Modeling and Process Optimization

The 3D plots of the response surface for the highest removal efficiency in an optimized condition are presented in Figure 2A-C for R. Figure 2A represents the combined effect of pH (A) and lead concentration (B). Both parameters affect the lead removal synergistically. By increasing each of the two independent variables, the removal efficiency increases. The effect of pH (A) and bacterial concentration (C) on removal efficiency is shown in Figure 2B. The combined effect of pH (A) and bacterial concentration (C) affect the lead removal activity (Figure 2B) synergistically. The combined effect of lead concentration (B) and bacterial concentration (C) on metal removal efficiency is shown in Figure 2C. As shown, by moving toward the maximum bacterial concentration and lower lead concentration at a constant pH (6.8), the removal values decrease dramatically.

4.5. Verification

This step aimed to verify the optimization capability of the models generated according to the results of the CCD. The maximum metal removal efficiencies of Lactobacillus strains were investigated when the optimal process variable (A, B, and C) were equal to 6.76, 391 mg.L\(^{-1}\) and 4.65

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**Table 1. Removal of Lead from MRS Broth by Lactobacillus Strains**

| Lactobacillus Strains | % Pb Removed |
|-----------------------|-------------|
| L. acidophilus ATCC4356| 53.9 ± 0.013|
| L. plantarum ATCC8014 | 47.7 ± 0.025|
| L. rhamnosus ATCC7469 | 30.5 ± 0.054|
| L. fermentum ATCC9338 | 44.2 ± 0.046|
| L. casei ATCC9392    | 33.3 ± 0.028|

\(^{a}\)The bacteria were incubated 24 h in MRS broth supplemented with 5 mg.L\(^{-1}\) Pb. 
\(^{b}\)Values are mean ± SD of three replicates.

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Table 2. Experimental Design and the Results Derived from Each Run in Plackett-Burman Design

| Standard No. | Run | Factors | Removal of Lead (%) for *L. acidophilus* ATCC4356 |
|-------------|-----|---------|-----------------------------------------------|
|             | A   | B       | C     | D     | E     | F     |
| 7           | 1   | -       | +     | +     | +     | -     | 68.9  |
| 5           | 2   | +       | +     | -     | +     | +     | 55.6  |
| 1           | 3   | +       | -     | +     | -     | -     | 55.7  |
| 4           | 4   | -       | +     | +     | +     | -     | 71.1  |
| 3           | 5   | -       | +     | +     | -     | +     | 54.0  |
| 2           | 6   | +       | -     | -     | +     | -     | 53.6  |
| 12          | 7   | -       | -     | +     | -     | -     | 52.9  |
| 10          | 8   | +       | -     | -     | -     | +     | 53.9  |
| 8           | 9   | -       | -     | +     | +     | -     | 62.4  |
| 11          | 10  | -       | +     | -     | -     | +     | 54.3  |
| 9           | 11  | -       | -     | +     | +     | +     | 57.1  |
| 6           | 12  | +       | +     | +     | -     | +     | 59.7  |

Abbreviations: A, live status; B, time (hour); C, pH; D, bacterial concentration (g.L\(^{-1}\)); E, temperature (°C); F, metal (lead) concentration (mg.L\(^{-1}\)).

- and + represent the low and high levels, respectively.

Figure 1. A) Pareto chart, B) normal probability plot, C) residual versus order plot, and D) residual versus fitted plot for Plackett-Burman design (PBD) for lead removal of *L. acidophilus* ATCC4356.

For lead removal of *L. acidophilus* ATCC4356. The experimental and predicted removal efficiencies at the opti-
Figure 2. Fitted surface for the lead removal as: A) pH(A) and Pb concentration(B) (Bacterial concentration = 4.7), B) pH(A) and bacterial concentration(B) (Pb concentration = 391), C) Pb concentration(B) and bacterial concentration(C) (pH = 6.8).

5. Discussion

The results show that *L. acidophilus* ATCC4356 had the highest (53.9 ± 0.01%) lead removal efficiency. Therefore, this strain was selected for further investigation. Several maximum condition were found as 73.960 and 75.710% for *L. acidophilus* ATCC4356, respectively (95% PI low = 63.4 and 95% PI high = 79.4)
studies showed that physical and cultural conditions affect removal capacity (19, 20). Optimization helps us to find the condition with the best output for a system (7, 8, 17, 21). Few studies are conducted on the application of bacteria, particularly LAB, using a methodological approach. For example, Kumar and colleagues (25) used the Box-Behnken design matrix with RSM for removing Cr (VI), Ni (II), and Zn (II) ions with immobilized bacterial strain Bacillus Brevis. They investigated the effect of parameters such as pH, temperature, and initial concentration of metal ions on adsorption, and the maximum removal of Cr (VI) was observed as 77.24% at pH 2.0, temperature 40°C, and concentration 35 mg/L. Whereas, removal of Ni (II) ions was 75.2% at pH 6.0, temperature 40°C, and concentration 35 mg/L and 71.6% of Zn (II) ions at pH 4.0, temperature 32.5°C, and concentration 35 mg/L, respectively.

Zhai and colleagues (5) examined cadmium removal from rice using the Box-Behnken model with a combined RSM approach by L. plantarum. They investigated three effective factors for cadmium removal (i.e., temperatures, fermentation durations, and inoculation amounts of L. plantarum). In total, they performed 17 experiments using a quadratic model. Using this model, they calculated the regression equation coefficients, and the data were fit-

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| Table 3. Experimental Design for Lead Removal of L. acidophilus ATCC4356 (Response) with the Results |
|-----------------|-----------------|-----------------|-----------------|
| Run No. | A: pH (3.42, 7.58) | R: Lead Concentration (109.32, 400.68) | C: Bacterial Concentration (1.09, 4.01) | Response R Lead Removal (%) |
| 1 | 3.4 | 109 | | 50.4 |
| 2 | 5.5 | 255 | 2.5 | 49.0 |
| 3 | 5.5 | 10 | 2.5 | 56.0 |
| 4 | 5.5 | 255 | 2.5 | 50.8 |
| 5 | 2.0 | 255 | 2.5 | 34.7 |
| 6 | 3.4 | 401 | 4.0 | 56.5 |
| 7 | 5.5 | 255 | 0.1 | 48.6 |
| 8 | 5.5 | 255 | 2.5 | 54.0 |
| 9 | 7.6 | 109 | 4.0 | 70.2 |
| 10 | 9.0 | 255 | 2.5 | 60.0 |
| 11 | 5.5 | 255 | 5.0 | 60.3 |
| 12 | 7.6 | 109 | 1.3 | 66.1 |
| 13 | 1.4 | 401 | 1.1 | 50.3 |
| 14 | 5.5 | 500 | 2.5 | 53.2 |
| 15 | 7.6 | 401 | 4.0 | 72.0 |
| 16 | 5.5 | 255 | 2.5 | 53.8 |
| 17 | 5.5 | 255 | 2.5 | 52.5 |
| 18 | 5.5 | 255 | 2.5 | 59.6 |

| Table 4. ANOVA for Response Surface Quadratic Model of R (Lead Removal of L. acidophilus ATCC4356) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Source | Sum of Squares | df | Mean Square | F Value | P Value |
| Model | 1206 | 9 | 134 | 15.2 | 0.0004 |
| A: pH | 594 | 1 | 594 | 67.4 | 0.0003 |
| B: Pb concentration | 1.47 | 1 | 1.47 | 0.167 | 0.6935 |
| C: Bacterial Concentration | 110 | 1 | 110 | 12.6 | 0.0075 |
| AB | 173 | 1 | 173 | 19.7 | 0.0022 |
| AC | 171 | 1 | 171 | 19.4 | 0.0023 |
| BC | 193 | 1 | 193 | 21.9 | 0.0016 |
| A² | 53.0 | 1 | 53.0 | 6.02 | 0.0397 |
| B² | 2.50 | 1 | 2.50 | 0.283 | 0.6090 |
| C² | 2.06 | 1 | 2.06 | 0.233 | 0.6421 |
| Residual | 70.5 | 8 | 8.81 | | |
| Lack of Fit | 4.57 | 3 | 1.52 | 0.315 | 0.9473 |
| Pure Error | 65.9 | 5 | 13.2 | | |
| Cor Total | 1276 | 17 | | | |

aStd.Dev = 2.97; R-Squared = 0.944; Mean = 55.4; Adjusted R² = 0.882; C.V.% = 5.3; Predicted R² = 0.869
bSignificant
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Footnotes

Authors’ Contribution: Study concept and design: L. G., and R. K.; analysis and interpretation of data: L. G., and R. K.; drafting of the manuscript: L. G.; critical revision of the manuscript for important intellectual content: L. G., R. K., and GH. J.; statistical analysis: L. G.; Study supervision: R. K., and GH. J.

Conflict of Interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

Funding/Support: No grants were involved in supporting this work.
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