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Detoxification of heavy metals by probiotic bacteria: investigating the effect of temperature, time, and type of bacteria on the removal of lead and cadmium metals using the response surface methodology

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ABSTRACT

Pollution in industrial areas due to the release of heavy metals is one of the important environmental concerns. Heavy metals can have very adverse effects on human and animal health. In this regard, food products contaminated with heavy metals, even in low concentrations, can have harmful effects on human health. In this regard, the use of microorganisms is known as a new and low-cost method for the biological removal of metals. The purpose of this study was to investigate the effect of the type of microorganism (*Lactiplantibacillus paraplantarum*, *Lactobacillus paragasseri*, and *Limosilactobacillus reuteri*), temperature, and incubation time on the removal of lead and cadmium metals using the response surface methodology. The results showed that the removal rate of lead metal using microorganisms was significantly higher than that of cadmium. Increasing the time from 0 to 24 hours significantly increased the amount of metal removal. On the other side, increasing the temperature up to about 38 °C positively affected the removal of metals, but increasing the temperature further reduced the ability of microorganisms to remove metals. In general, the software determined the optimal conditions to achieve the maximum removal of lead and cadmium metals by 45.9% and 39.65%, respectively, at 24 hours incubation time and 33.98 °C temperature using *Lactobacillus paragasseri* bacteria. Therefore, according to the results of this research, the use of microorganisms such as *Lactobacillus paragasseri* is a useful solution for removing heavy metals from various sources, such as industrial wastewater.

1-Introduction

Rapid industrialization and urbanization, especially in developing countries, have significantly increased human exposure to heavy metals. Studies have shown that exposure to high levels of heavy metals causes severe damage to various organs and systems of the body, including the kidneys, liver, central nervous system, and reproductive system [1]. More than 20 types of heavy metals have been identified so far, among which cadmium (Cd), lead (Pb), and arsenic (As) have been identified as the most dangerous elements [2]. Based on the duration, exposure to heavy metals is mainly divided into three groups: acute (1-14 days), moderate (15-354 days), and chronic (≥ 365 days). In contrast to acute heavy metal poisoning, which is usually caused by skin contact, inhalation of large amounts of heavy metal vapors, or misuse of drugs in a short period of time, chronic heavy metal poisoning is caused by daily exposure to heavy metals in an insensible manner through food, water, air, or skin, which is a serious threat to public health [3]. In general, adverse effects of heavy metals include immunosuppression, carcinogenicity, disruption of the nervous system, especially in children, and inhibition of the activity of some vital enzymes involved in synthesizing biomolecules [4]. Various methods have been proposed for the removal of heavy metals, including chemical oxidation-reduction processes, adsorption processes, and electrolytic recovery, but the widespread application of these methods is limited due to high costs or environmental incompatibility [5]. In the 1990s, a new field of heavy metal recovery and removal using biological methods was introduced as

a low-cost method [6]. In this method, microorganisms or their biomass are used to absorb heavy metals [7]. Metal uptake by microorganisms is due to molecules containing hydroxyl and carboxyl functional groups on the bacterial surfaces that can form chemical bonds with heavy metals, which in turn causes metal uptake and deposition [8]. Studies have shown that heavy metals are absorbed through three mechanisms: 1) binding to peptidoglycans and teichoic acids by ion exchange reactions, 2) deposition through nuclear reactions, and 3) complex formation with nitrogen and oxygen ligands [9]. Studies have shown that the absorption of cadmium and lead from aqueous solutions is very rapid, so the mechanism of passive binding of metals to the bacterial surface compared to intracellular accumulation has been proposed as the main mechanism [10]. In many studies, the appropriate efficacy of some probiotics such as *Bifidobacterium* species, *Lactiplantibacillus paraplantarum*, *Limosilactobacillus reuteri*, *Lactobacillus paragasseri* and some yeasts for detoxification or removal of heavy metals has been reported [11-13]. In this regard, many studies have been conducted to evaluate the ability of lactic acid bacteria to remove heavy metals. For example, Kirillova et al. (2017) evaluated the effect of lactic acid bacteria on cadmium removal and reported that *L. plantarum* B-578 and *L. fermentum* 3-3 were able to remove 16 and 12% of cadmium, respectively, while *L. brevis* 20054, *L. buchneri* 20057 and *L. rhamnosus* I2L were unable to remove cadmium [13]. In this regard, it has been reported that the removal rate of heavy metals by microorganisms depends on various factors such as the species used,

temperature and pH [14]. Therefore, adjusting the optimal conditions to achieve maximum efficiency is of particular importance. In this regard, the response surface methodology (RSM) is a suitable method for optimizing complex processes that is successfully used to optimize food industry processes. RSM includes statistical and mathematical processes that can be used to examine one or more dependent and independent variables. This method shows the effect of independent variables alone or in combination in the process and interprets the process accurately by creating a mathematical model [15]. Therefore, the aim of this study was to investigate the effect of the type of microorganism used (*Lactiplantibacillus paraplantarum* PTCC 1965, *Lactobacillus paragasseri* PTCC 1897, and *Limosilabacillus reuteri* PTCC 1655 bacteria), temperature, and incubation time on the removal rate of cadmium and lead metals using the response surface methodology.

2-Materials and Methods

2-1- Microbial strains

Bacteria *Lactiplantibacillus paraplantarum* PTCC 1965, *Lactobacillus paragasseri* PTCC 1897 and *Limosilabacillus reuteri* PTCC 1655 were purchased from the Scientific and Industrial Research Organization in Tehran (Tehran Province, Iran). The microbial strains were activated in MRS culture medium according to the center's instructions. After activation, bacterial cells were separated from the culture medium using a centrifuge (Hettich, Germany) at $3000 \times g$ and 4°C for 10 minutes. The initial number of cells was diluted in a sterilized normal saline solution [16].

2-2- Biosorption Test

Stock solutions of lead and cadmium were prepared by dissolving $\text{Pb}(\text{NO}_3)_2$ and $\text{Cd}(\text{NO}_3)_2$ in distilled water to a concentration of 1000 mg/L and subsequently diluted to the target concentrations. The pH was adjusted to 4.5 by HCl (0.1 M) and NaOH (0.1 M). Bacterial suspension ($7 \log \text{CFU/mL}$) was added separately to Erlenmeyer flasks containing lead and cadmium, followed by incubation for 24 h at 30°C , 37°C and 44°C at 150 rpm. Sampling was performed every 4 h. Control samples were samples that did not contain bacteria. At the time of sampling, centrifugation was performed at $3000 g$ for 15 min and after separation of the supernatant, filtration was performed using Whatman filter paper No. 42. The residual concentration of metals was determined by atomic absorption (AA500; PG Instruments, UK) [17].

2-3-Statistical analysis

Optimization of the conditions for removing heavy metals, cadmium, and lead was carried out in the form of a central composite design using Design Expert software version 7 and the response surface methodology. The variables of temperature, time, and type of bacteria used were considered as independent variables, and the removal rate of cadmium and lead metals as dependent variables, and the effect of each of these independent variables on the dependent variables was investigated to determine the conditions for achieving the maximum removal rate of heavy metals.

3- Results and discussion

3-1- Lead removal content

According to Table 1, response surface analysis showed that the effects of the variables temperature (A), time (B), type of bacterial species (C), A^2 , B^2 , C^2 , AB, BC and AC were significant, and among them, the variables AB and BC showed a less significant effect than the other variables ($p < 0.05$). The regression coefficient of the equation is $R^2 = 0.97$, which indicates that the model was able to predict 97 % of the total changes in the range of values under study and is a suitable model for predicting the effect of process variables on the desired response. The relationship between the lead removal rate and the reaction

parameters is of the second order type, which follows equations 3-1, depending on the type of bacteria used.

Equation (1)

$$\text{Pb removal by } Lactiplantibacillus \text{ paraplantarum} = (-9.82 + 0.62 B + 0.68 A - 0.0034 AB - 0.0112 B^2 - 0.0106 A^2)^2 - 0.06$$

Equation (2)

$$\text{Pb removal by } Lactobacillus \text{ paragasseri} = (-14.87 + 0.65 B + 0.81 A - 0.0034 AB - 0.011 B^2 - 0.0106 A^2)^2 - 0.06$$

Equation (3)

$$\text{Pb removal by } Limosilatobacillus \text{ reuteri} = (-14.73 + 0.66 B + 0.81 A - 0.0034 AB - 0.011 B^2 - 0.0106 A^2)^2 - 0.06$$

Table 1- Analysis of variance for Pb removal

	Sum of squares	df	F-value	p-value
Model	292.66	11	188.74	< 0.0001
A-Time	240.37	1	1705.16	< 0.0001
B-Temperature	7.63	1	54.12	< 0.0001
C-Strain	4.82	2	17.08	< 0.0001
AB	1.58	1	11.21	0.0015
AC	1.51	2	5.35	0.0078
BC	8.43	2	29.92	< 0.0001
A^2	24.53	1	174.04	< 0.0001
B^2	3.79	1	26.89	< 0.0001

3-2-Evaluation the effect of time, temperature, and type of bacteria on the rate of lead removal

As shown in Figure 1, using *Lactiplantibacillus paraplantarum* bacteria, at constant temperatures, increasing the incubation time from 0 to 24 hours significantly increased the rate of lead removal. On the other hand, at constant

times, increasing the incubation temperature from 30 to about 35 ° C increased the rate of lead removal, but further increase in temperature to 44 ° C had a negative effect. In general, the highest rate of lead removal (44.9%) was achieved at a temperature of 30.14 ° C and a time of 23.71 hours.

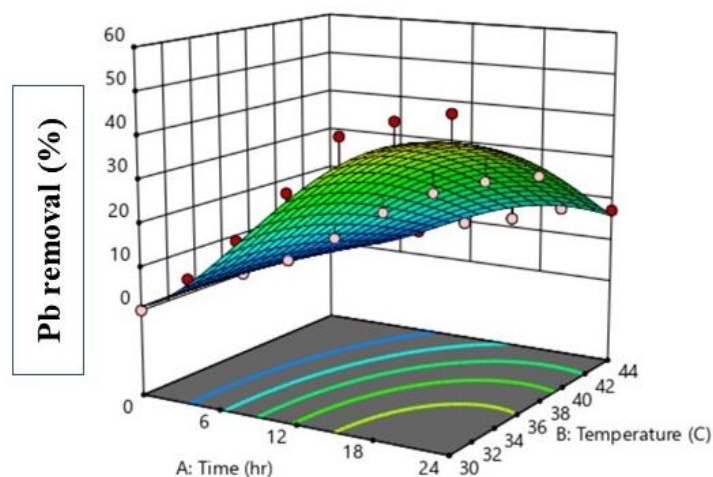


Figure 1- The effect of time and temperature on the Pb removal by *Lactiplantibacillus paraplantarum*

Figure 2 shows the effect of incubation time and temperature with *Lactobacillus paragasseri* on the lead removal rate. In general, at constant incubation temperatures, increasing the time from 0 to 24 hours significantly increased the lead removal rate. For example, at a constant temperature of 37°C, increasing the time from 0 to 24 hours increased the lead

removal rate from 0 to 45%. On the other hand, at constant times, increasing the incubation time to 38°C increased the lead removal rate, but further increases in temperature had a negative effect. In general, the highest lead removal rate (45.92%) was achieved at a temperature of 34.61°C and a time of 23.5 hours.

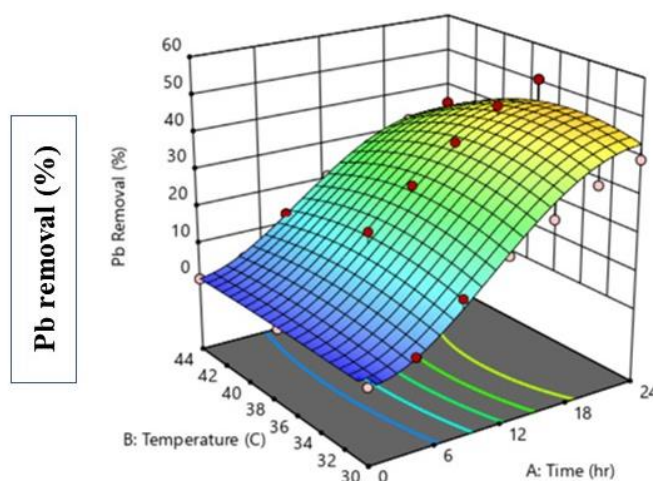


Figure 2- The effect of time and temperature on the Pb removal by *Lactobacillus paragasseri*

Figure 3 shows the effect of incubation time and temperature with *Limosilatobacillus reuteri* on the lead removal rate. According to the figure, at fixed incubation times, increasing the temperature to 37.6°C increased the lead removal rate, but further

increase in temperature had a negative effect. On the other hand, at fixed incubation temperatures, increasing the time significantly increased the lead removal rate. The highest lead removal rate

(51.1%) was achieved at 23.95 hours and 34.58°C.

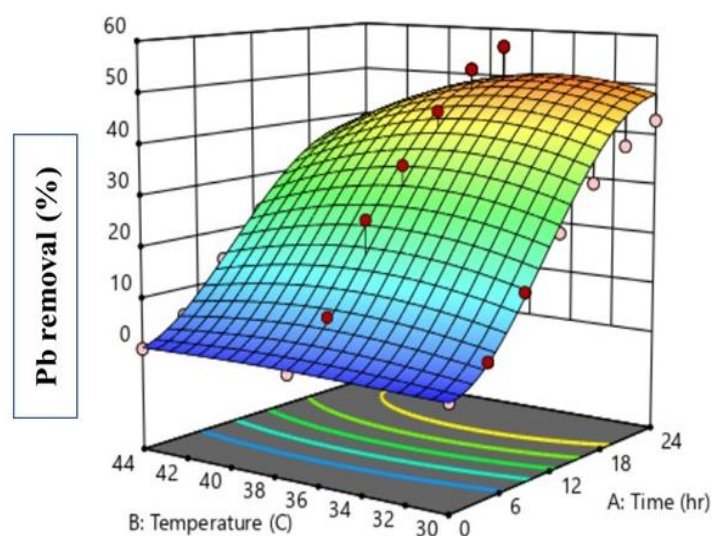


Figure 3- The effect of time and temperature on the Pb removal by *Limosilatobacillus reuteri*

3-3- Cadmium removal content

According to Table 2, response surface analysis showed that the effects of the variables temperature (A), time (B), type of bacterial species (C), A^2 , B^2 , C^2 , AB and BC were significant, and among them, variables AB and C showed a less significant effect than other variables ($p < 0.05$). The effect of variable AC on the rate of cadmium removal was not significant ($p < 0.05$). The regression coefficient of the equation was $R^2 = 0.97$, which indicates that the model was able to predict 97 percent of the total changes in the range of variables under study and is a suitable model for predicting the effect of process variables on the desired response

(cadmium removal). The relationship between the rate of cadmium removal and reaction parameters is of the second order type, which follows equations 6-4, depending on the type of bacteria used.

Equation (4)

$$\text{Cd removal by } Lactiplantibacillus \text{ paraplantarum} = (-7.99 + 0.5 B + 0.54 A - 0.002 AB - 0.008 B^2 - 0.008 A^2)^2 - 0.06$$

Equation (5)

$$\text{Cd removal by } Lactobacillus \text{ paragasseri} = (-11.5 + 0.53 B + 0.63 A - 0.0028 AB - 0.008 B^2 - 0.008 A^2)^2 - 0.06$$

Equation (6)

$$\text{Cd removal by } Limosilatobacillus \text{ reuteri} = (-11.84 + 0.51 B + 0.65 A - 0.0027 AB - 0.008 B^2 - 0.008 A^2)^2 - 0.06$$

Table 2- Analysis of variance for Cd removal

	Sum of squares	df	F-value	p-value
Model	228.32	11	155.54	< 0.0001
A-Time	200.02	1	1498.86	< 0.0001
B-Temperature	5.21	1	39.02	< 0.0001
C-Strain	1.79	2	6.72	0.0026
AB	1.01	1	7.57	0.0082
AC	0.79	2	2.96	0.0606

BC	4.76	2	17.85	< 0.0001
A ²	12.34	1	92.43	< 0.0001
B ²	2.40	1	17.97	< 0.0001

3-4- Evaluation the effect of time, temperature, and type of bacteria on the rate of cadmium removal

Figure 4 shows the simultaneous effect of temperature and incubation time with *Lactiplantibacillus paraplantarum* bacteria on the rate of cadmium removal. In times of less than 6 hours, increasing the temperature to 34°C increased the rate of cadmium removal, but a further increase in temperature had a negative effect. In times

of more than 6 hours, increasing the temperature to about 37°C increased the rate of cadmium removal, and a further increase in temperature decreased the percentage of cadmium removal by the bacteria. On the other hand, at constant incubation temperatures, increasing the time significantly increased the rate of cadmium removal. The highest rate of cadmium removal (36.51%) was achieved at a time of 23.89 hours and a temperature of 30.14°C.

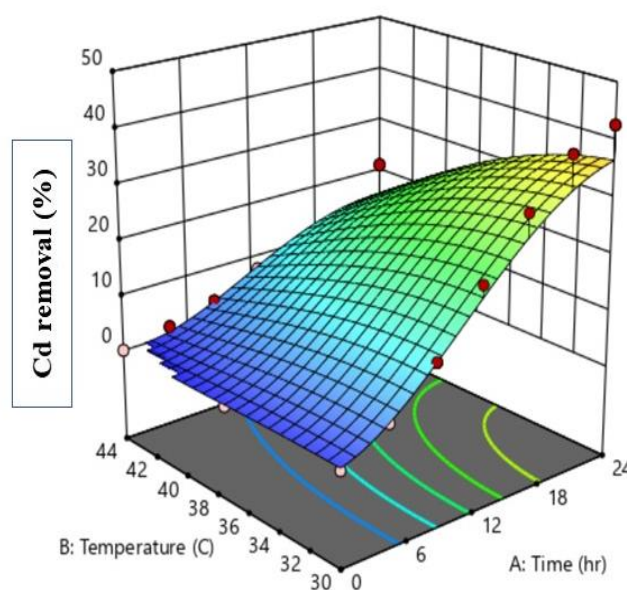


Figure 4- The effect of time and temperature on the Cd removal by *Lactiplantibacillus paraplantarum*

Figure 5 shows the simultaneous effect of temperature and incubation time with *Lactobacillus paragasseri* on the rate of cadmium removal. At fixed incubation times, increasing the temperature to 39°C increased the rate of cadmium removal, but

further increase in temperature had a negative effect and decreased the rate of cadmium removal. On the other hand, at fixed incubation temperatures, increasing the time significantly increased the rate of cadmium removal. The highest rate of cadmium removal (39.43%) was achieved at 23.71 hours and 33.34°C.

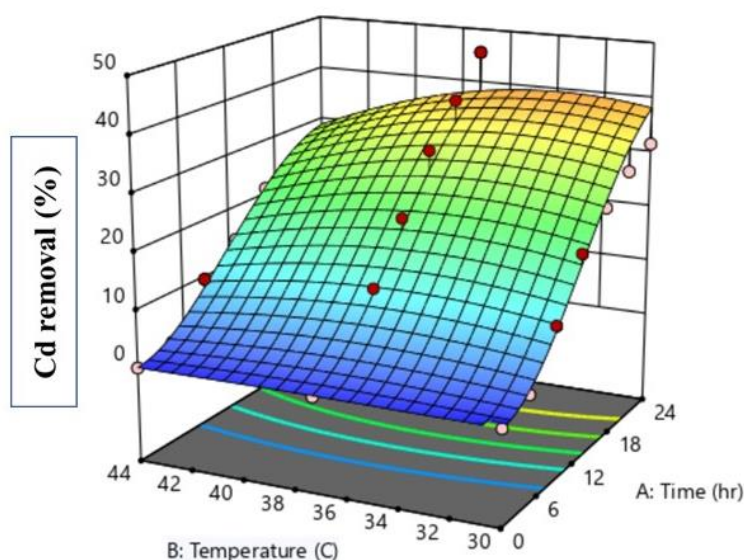


Figure 5- The effect of time and temperature on the Cd removal by *Lactobacillus paragasseri*

Figure 6 shows the simultaneous effect of temperature and incubation time with *Limosilatobacillus reuteri* on the rate of cadmium removal. At fixed incubation times, increasing the temperature to about 40°C increased the cadmium removal rate, but further temperature increase showed a

negative effect and the rate of cadmium removal decreased. On the other hand, at fixed incubation temperatures, increasing the time significantly increased the rate of cadmium removal. The highest rate of cadmium removal (35.35%) was achieved at 23.77 hours and 35.44°C.

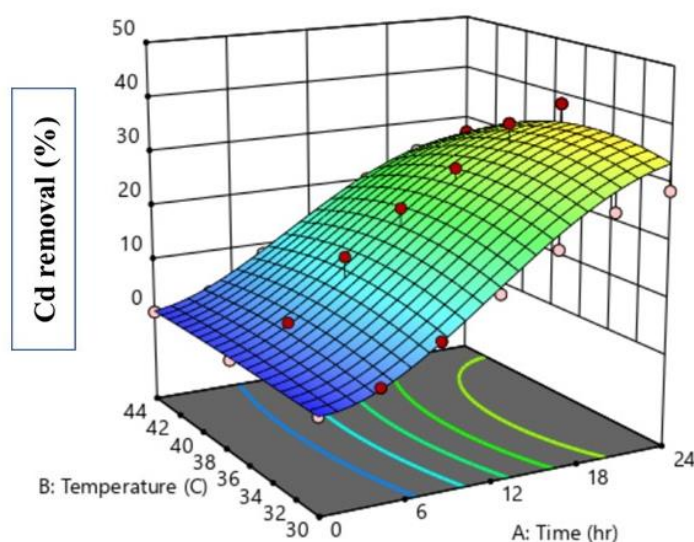


Figure 6- the effect of time and temperature on the Cd removal by *Limosilatobacillus reuteri*

In general, two general mechanisms have been proposed for the removal of heavy metals by microorganisms: 1)

bioaccumulation, which is a metabolism-dependent process in which metal ions penetrate the plasma membrane and

accumulate inside the cell, and 2) biosorption, which is a metabolism-independent process in which metal ions bind to the cell surface [17]. In this regard, it has been reported that mechanisms such as adsorption, ion exchange, complexation, and chelation can play a role in the biological absorption of metals [13].

As previously stated, in all the bacteria evaluated, increasing the incubation time significantly increased the removal of lead and cadmium metals. This could be due to the increase in the contact time of the metals with microorganisms, which had sufficient time to absorb and remove the metals. Similar to these results, Elsanhoty et al. (2016) reported that increasing the incubation time from 30 to 300 minutes significantly increased the removal of cadmium metal by *Lactobacillus acidophilus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Streptococcus thermophiles*, *Lactobacillus plantarum* and *Bifidobacterium angulatum* [18]. Also, Bhakta et al. (2012) reported that increasing the incubation time increased the removal of lead metal through adsorption on the surface of microorganisms but had no effect on the removal of cadmium [19]. On the other hand, Teemu et al. (2008) reported that incubation time did not have a significant effect on the removal rate of cadmium metal by microorganisms. This difference in results could be due to differences in the concentration of metals in the medium, the type of microorganism used, and the incubation conditions [20].

On the other hand, increasing the incubation temperature to more than 40°C significantly reduced the removal of heavy metals. In general, the highest removal of metals was achieved at a temperature of 34-

40°C. This finding could be due to the destructive effect of temperature on the microorganisms under evaluation, as the increase in temperature caused their inactivation or decreased activity, which consequently reduced their ability to remove metals from the environment. Cho and Kim (2003) reported that increasing the temperature increased the removal of cadmium metal by [21]. In general, it has been reported that removing heavy metals by microorganisms depends on various factors, including incubation temperature and time, pH, and metal concentration [22].

On the other hand, a comparison of the removal rates of lead and cadmium metals showed that, in general, the microorganisms evaluated had a more remarkable ability to remove lead than cadmium; in other words, the removal rate of lead by microorganisms was significantly higher than that of cadmium. This can be attributed to the larger ionic size and heavier atomic weight of lead compared to cadmium, which causes it to interact more with biological components (microorganisms) [23].

4- Optimization and evaluation of model validity

After analyzing the data, as shown in Figure 7, the software determined the optimal conditions to achieve the highest removal rates of lead and cadmium metals, 45.9 and 39.65 percent, respectively, with an incubation time of 24 hours and a temperature of 33.98 degrees Celsius using *Lactobacillus paragasseri* bacteria. In order to evaluate the validity of the model, the removal rates of lead and cadmium metals were measured under the mentioned conditions. The results showed that the removal rates of lead and cadmium metals

were 47.1 and 38.54 percent, respectively. These results indicate the appropriate ability of the model to predict the effect of

temperature, time, and type of bacteria on the responses under study (removal rates of lead and cadmium metals).

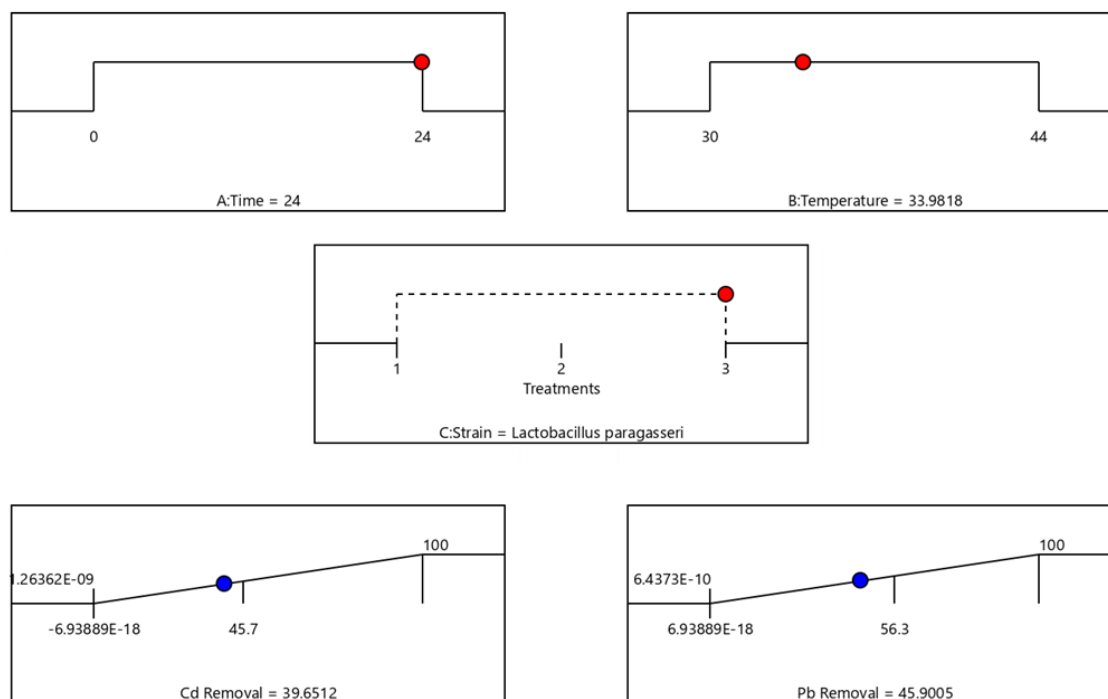


Figure 7 - Optimal conditions for achieving maximum removal of lead and cadmium metals

5-Conclusion

The results of this study showed that *Lactiplantibacillus paraplantarum*, *Lactobacillus paragasseri* and *Limosilatobacillus reuteri* bacteria have a significant ability to remove lead and cadmium metals. Using the response surface methodology as a suitable technique for optimizing multivariate processes showed that among the bacteria evaluated, *Lactobacillus paragasseri* bacteria was able to remove lead and cadmium heavy metals at a temperature of 33.98 °C and a time of 24 hours by 45.9 and 39.65 percent, respectively. Therefore, it can be concluded that the use of the *Lactobacillus paragasseri* is a cheap and environmentally friendly technique that is significantly capable of removing heavy metals. Therefore, it can be used as a highly

efficient method for removing heavy metals from industrial wastewater.

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سم زدایی فلزات سنگین توسط باکتری‌های پروبیوتیک: بررسی تاثیر دما، زمان و نوع باکتری بر میزان حذف فلزات سرب و کادمیوم با استفاده از روش سطح پاسخ

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آلودگی در مناطق صنعتی به دلیل رهائش فلزات سنگین یکی از نگرانی‌های مهم زیست محیطی است. فلزات سنگین می‌توانند تأثیرات بسیار نامطلوبی بر سلامت انسان و حیوانات داشته باشند. در این راستا همچنین محصولات غذایی آلوده به فلزات سنگین حتی در غلظت های کم، می‌توانند اثرات مضر را بر سلامتی انسان داشته باشند. در این راستا استفاده از میکروارگانیسم‌ها به عنوان روشی جدید و کم هزینه جهت حذف بیولوژیکی فلزات شناخته شده است. هدف از این پژوهش بررسی تاثیر نوع میکروارگانیسم (باکتری‌های *Lactobacillus*، *Lactiplantibacillus paraplantarum* و *Limosilactobacillus reuteri* و *paragasseri*) مورد استفاده، دما و زمان انکوباسیون بر میزان حذف فلزات سرب و کادمیوم با استفاده از روش سطح پاسخ بود. نتایج نشان داد که میزان حذف فلز سرب با استفاده از میکروارگانیسم‌ها به‌طور معنی‌داری بیشتر از کادمیوم بود. و افزایش زمان از ۰ تا ۲۴ ساعت به طور معنی داری باعث افزایش میزان حذف فلزات گشت. افزایش دما تا حدود ۳۸ درجه تأثیری مثبت بر میزان حذف فلزات داشت اما افزایش بیشتر دما از توانایی میکروارگانیسم‌ها جهت حذف فلزات کاست. به‌طور کلی نرم افزار شرایط بهینه جهت دستیابی به بیشترین میزان حذف فلزات سرب و کادمیوم به‌ترتیب به میزان ۴۵/۹ و ۳۹/۶۵ درصد را زمان انکوباسیون ۲۴ ساعت و دمای ۳۳/۹۸ درجه سانتی گراد با استفاده از باکتری *Lactobacillus paragasseri* تعیین کرد. بنابراین با توجه به نتایج این پژوهش استفاده از میکروارگانیسم‌ها همچون *Lactobacillus paragasseri* راهکاری مفید جهت حذف فلزات سنگین از منابع مختلفی همچون پساب‌های صنعتی می‌باشد.