

Lead bioremoval from milk by *Saccharomyces cerevisiae*

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ABSTRACT

The present study refers to the application of *Saccharomyces cerevisiae* for bioremoval of lead from milk, the most important dairy product. First, the impact of process variables on lead bioremoval by *S. cerevisiae* has been studied by Plackett-Burman Design. Then, the optimization of bioremoval process was carried out by three main factors; contact time, biomass and initial heavy metal concentration by using a central composite design. The analysis of variance showed that the models were highly significant. The model also described that lead bioremoval from milk is affected by all three factors. The optimized bioremoval was achieved after 4 days with 22×10^8 CFU inoculation of yeast in milk containing 70 µg/l of lead concentration. The reconfirmation test indicated that in the above mentioned optimum condition the highest yield of lead bioremoval was achieved at 70%. 3D plots analysis represented dual interaction effects on metal bioremoval. This study showed that *S. cerevisiae* is a natural potential biosorbent for lead bioremoval from milk. This approach could be also considered as a green technology useful in eliminating the heavy metal contaminations of drinking water and foodstuff.

1. Introduction

Milk contamination with different pollutants such as heavy metals, dioxins and mycotoxins considered as the main concern to public health all around the world due to their toxic effects on humans (Fischer et al., 2015). (Derakhshesh and Rahimi, 2012). So the presence of toxic metals in the food chain, which could be the result of environmental pollution, needs to be continuously controlled (Semaghiul et al., 2008). The negative effects of heavy metals on human health have been reported by many researches (Kanwal et al., 2018; Mahurpawar, 2015; Arif et al., 2015). (Hadiani et al., 2018). However heavy metals naturally exist in the environment, but different industries increase their amounts in human and animals' food chains directly or indirectly by using many types of these metals (Mishra et al., 2018; Yadanaparathi et al., 2009).

Lead is one of the most toxic pollutants in the environment (Phillips et al., 2003) resulted in human and animal poisoning (Ozmen and Mor, 2004). Lead is reported to induce intellectual problems in children and increase blood pressure and cardiovascular disease in adolescents (Commission of the European Communities,

2001). Lead may enter the food chain through various ways like the animals' contaminated feed and water, automobile emissions, lead batteries, textile, petrochemical industries, mining, smelting, paint industries and phosphate fertilizers (Chunjuan et al., 2018; Patra et al., 2008).

Various methods have been applied to remove these pollutants from the environment such as ion exchange, chemical precipitation, membrane technologies and activated carbon, electrochemical treatment and Polymer-based composites but most of them are very expensive and not applicable in food industry (Kobielsk et al., 2018; Zhao et al., 2018; Zabochnicka-Åšwi and Krzywonos, 2014). Nowadays there is a growing interest in using biological materials to improve the chemical and functional properties of dairy products especially milk (Ayar et al., 2009).

Biosorbents are the low-cost biologic originated sorbents like yeasts, bacteria, algae and fungi, which absorb the pollutants fast and efficient (Wang et al., 2018; Tran et al., 2015). Hadiani et al. (2018). Researches have shown that the biosorption method would be a desirable candidate for bioremoval of the heavy metals in food even in very low levels (Massoud et al., 2019). *Saccharomyces cerevisiae* is one

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of these biosorbents that widely use in food and beverage industries (Azhar and Abdulla, 2018; Amirnia et al., 2015). It is an ideal microorganism for research on the bioremediation studies (Wang and Chen, 2006). There are some benefits in working with *S. cerevisiae* such as easy application and cultivation on large scale and also its' health safety (Jianlong and Can, 2006; Soares and Soares, 2012).

The absorption of metal ions to the functional groups of the yeast cell wall depends on the nature of microorganisms and some chemical properties of the absorption media (Ozer and Ozer, 2003). Different factors are effective in biosorption of heavy metals such as the initial concentration of heavy metal, biomass dosage, contact time and presence of other compounds (Parvathi and Nagendran, 2007; Mapolelo and Torto, 2004; Ozer and Ozer, 2003). For optimization of the study, it is needful to examine the independent variables simultaneously. By increasing the number of variables, monitoring all the combined effects will be difficult and also time and cost-consuming, so there is a need to use the fractional factorial design method (Jahadi et al., 2012; Khosravi-Darani et al., 2008).

According to "Codex Standard for Contaminants and Toxins in Food", the maximum permissible limits for Pb concentration in milk should be less than 0.02 mg/L. Some studies have reported lead contamination, more than the maximum standard level, of milk in some countries around the world such as Hungary, 0.033 mg/L (Póti et al., 2012); China, 0.07 mg/L (Qin et al., 2009); South Africa, 0.096 mg/L (Ataro et al., 2008); Croatia, 0.047 mg/L (Sikirić et al., 2003) and also some cities in Iran; Hamadan, 0.045 mg/L (Pajohi-Alamoti et al., 2017); Arak, 0.040 mg/L (Rezaei et al., 2014); Isfahan, 0.048 mg/L and Mashhad, 0.33 mg/L and Ahvaz, 0.38 mg/L (Rahimi, 2013); Shahrekord, 0.39 mg/L (Derakhshesh and Rahimi, 2012).

Lead has major toxic effects and its residues in milk is a great concern, the determination of Pb level in milk is particularly notes by researchers but the practical ways of its removal is still a significant issue. Applying microorganisms as effective biosorbents in foodstuffs are so valuable.

This study aimed to evaluate the potential of *S. cerevisiae* for bioremoval of low concentrations of Pb (at ppb levels) in milk for the first time. To optimize the study, the effect of some independent variables suggested in other studies like Pb concentration, biomass dosage, contact time, inoculation temperature, shaking rate and viability of biomass, was first studied with Design Expert software (version, 7.1.5, STAT-EASE Inc., Minneapolis, USA). The 3 independent variables (biomass dosage, contact time and metal concentration) showed the highest coefficient estimate among all. Then, the interactions of these significant variables were investigated at five levels via central composite design (CCD) under response surface methodology.

There is no published document about the capability of *S. cerevisiae* in bioremoval of lead in milk and this is the first time that the valuable yeast, *S. cerevisiae*, is applied to remove the low level (ppb) of Pb concentration in milk successfully, so the findings of this study would open a new window in using this green technology for heavy metals removal in food industry.

2. Materials and methods

2.1. Instrumentation

The quadrupole inductively coupled plasma mass spectrometer (ICP-MS) applied in this project was an Agilent 7500a (Agilent Technologies, CA, USA) equipped with a Babington nebuliser, a Peltier-cooled quartz spray chamber and a standard torch (2.5 mm). Before each test run, the instrument was adjusted by an aqueous multi-element standard solution (Agilent, Madrid, Spain) of 10 ng/ml Pb. The treatments were digested using the following system for the microwave digestion: Milestone Ethos Micro SYNTH oven with programmable power control (10W increments, maximum power 1000 W)

with segmented rotor MPR-600 (operating pressure up to 35 bar maximum; operating temperature 260 °C maximum) with 10 reaction vessels (Khan et al., 2014).

2.2. Biomass

S. cerevisiae PTCC 5020 was prepared from Research and Technology Department of Ministry of Sciences (Persian Type Culture collection) in form of freeze-dried, and then cultured in a special sterilized media. The liquid medium applied for the yeast culture included (gram per liter): yeast extract, 1; glucose, 30; KH₂PO₄, 1; (NH₄)₂SO₄, 9; K₂HPO₄, 0.2 and MgSO₄, 2.5. The medium was then autoclaved at a temperature of 121 °C and pressure of 1.4 atm for 20 min for sterilization. After cooling, the yeast cells were cultured for 16 h at 27 °C and 50 rpm and then stored in refrigerator for biosorption studies.

2.3. Biomass seed culture

For each treatment combination, seed culture of *S. cerevisiae* was activated daily after inoculation of 5% v/v from master culture (2.2) in the same liquid medium and shaking for 16 h at 27 °C and 50 rpm. Then, activated seed culture was counted by serial dilution method, 1 ml of the seed culture, was diluted at a ratio of 1:10, and repeated 10 times serially. Then, from the dilution, 1 ml was added into a Nutrient Agar medium by pour plate method in duplicate. The plates incubated in 30 °C for 3 days (Sieuwerts et al., 2008). Finally, yeast colonies were counted of and showed the mean of 10×10^8 CFU/ml.

2.4. Reagents

All analytical reagent chemicals were from Merck (Darmstadt, Germany). Standard solutions of Pb (1000 mg/l in 0.1 M HNO₃) was provided by Panreac (Panreac Quimica SA, Barcelona, Spain). In order to prevent the metal contamination, all glass containers were soaked in HNO₃15% (v/v) overnight and rinsed with deionized water (Elga Labwater, Wycombe, Bucks, UK) before analysis. Also, Then, they were sterilized by autoclaving at a temperature of 121 °C and pressure of 1.4 atm for 20 min to prevent microbial contamination.

2.5. Optimization of Pb bioremoval

The various factors that may influence the bioremoval yield are chosen due to the literature reviews; Hadiania et al. (2018); Farhan and Khadom (2015); Galedar and Younesi (2013) and Ghorbani et al. (2008). Due to the pre-experience, it is required to examine the variables' interaction effects in a bioremoval study. The common approach in experimental planning begins with a screening design that all possible factors are considered and then the significant factors are selected, and after all the project continues with an experimental optimization design, such as central composite design (Coruh et al., 2012; Hanrahan and Lu, 2006). So, in the present study, central composite design was used.

2.6. Plackett-Burman Design

According to the factors that have been studied in pre-experienced trials and the literature reports for bioremoval of heavy metal by *S. cerevisiae* (Hadiania et al., 2018; Farhan and Khadom, 2015; Galedar and Younesi, 2013; Ghorbani et al., 2008), 6 independent variables involved in Pb bioremoval by this microorganism including: *S. cerevisiae* biomass dosage, contact time, Pb concentration, shaking rate, inoculation temperature and biomass viability. The variables and their experimental levels are shown in Table 1. The results were also based on the pre-experience and literature reviews. However, Pb concentration was selected in very low levels due to the previous study of our

Table 1
Investigated variables and their levels in Pb bioremoval by *S. cerevisiae*.

Variable	Low level	High level
<i>S. cerevisiae</i> biomass dosage ($\times 10^8$ CFU)	5	40
Inoculation temperature ($^{\circ}$ C)	4	40
Contact time (day)	0	4
Metal concentration (μ g/l)	40	100
Shaking rate (rpm)	0	50
Biomass viability	Live	Non-living

team on the drinking water; so the target of this study is to determine the ability of *S. cerevisiae* to absorb the low levels of Pb ions in milk, whereas no information has been available up to now. So, a plan was designed by Design Expert software for investigation of the optimum conditions for the maximum Pb removal.

To perform the bioremoval study, 100 ml sterile growth media as mentioned in section 2.2, with added Pb ions in concentration levels of 40 and 80 μ g/l were prepared in 250 ml flasks. Then the media was inoculated with activated seed culture (3 ml in 100 ml growth media equal to 22×10^8 CFU) as described in Section 2.3. Then, each flask was incubated at 25 $^{\circ}$ C on the shaker for 24. Also, to carry out the study on non-living cells, they were added to the milk at 4 or 40 $^{\circ}$ C after pasteurization. At the end of contact time, yeast cells were centrifuged at $2000 \times g$ for 15 min. The supernatant solution was then separated to measure the residual Pb using ICP- MS. All experiments were done in triplicates. The Pb ions removed by *S. cerevisiae* are calculated by Eq. (1):

$$R = \frac{C_0 - C_e}{C_0} \times 100 \quad (1)$$

while R is the % Pb removal by yeast biomass, C_0 is the initial concentration of Pb in milk in μ g/l, and C_e is the concentration of Pb in μ g/l in milk after biosorption (Goksungur et al., 2005). Then the data were processed by using the Minitab (version 14) statistical software and 3 variables were identified as the significant ones; *S. cerevisiae* biomass dosage, contact time and Pb concentration. The optimization trial was then distinguished by fractional factorial design. The designed experimental runs are shown in Table 2.

2.7. Response surface methodology design (RSM)

Response surface methodology (RSM) is the combination of mathematical and statistical techniques for analyzing the effect of several independent variables on the responses (Aslan and Cebeci, 2007). RSM is an important application in process design and optimizing as well as improving the existing design. This method has become more practical with using interactive computer programs among the variables based on experimenter's prior knowledge and finally it illustrates the parameters overall effects on the process (Bas and Boyac, 2007).

Table 2
Test runs designed by Plackett-Burman Design in Pb bioremoval by *S. cerevisiae*.

Run	Biomass dosage ($\times 10^8$ CFU)	Inoculation Temperature ($^{\circ}$ C)	Contact time (day)	Metal concentration (μ g/l)	Shaking rate (rpm)	Biomass viability
1	5	40	4	80	0	Live
2	22	4	1	80	50	Live
3	5	4	4	80	50	Non-living
4	22	40	1	80	0	Non-living
5	5	4	1	40	0	Live
6	22	4	4	40	0	Non-living
7	5	40	1	40	50	Non-living
8	22	40	4	40	50	Live

The results of Plackett-Burman Design revealed that three variables (*S. cerevisiae* biomass dosage, contact time and heavy metal concentration) were significant in bioremoval of Pb. For more accurate predictions on optimum conditions of Pb bioremoval by *S. cerevisiae* and also minimize the number of test runs, the central composite design (CCD) under RSM was designed. The RSM includes a group of experimental methods designed to evaluate the correlation between controlled experimental factors and responses in one or more selected criteria (Kunamneni and Singh, 2005).

The experimental design was performed using Design Expert software. The biomass viability showed a better effect on its' live status on Pb bioremoval, so all experiments were done by using the live yeast and the biomass inoculated at 4 $^{\circ}$ C to the milk after pasteurization. On the other hand, by the fact that the shaking rate was not a priority of this study, it was considered at 50 rpm. So, the 3 factors of inoculation temperature, shaking rate and yeast state were held constant at 4 $^{\circ}$ C, 50 rpm using live *S. cerevisiae* respectively. According to the Plackett-Burman Design results, the 3 significant factors (*S. cerevisiae* biomass dosage, contact time and Pb concentration) were designed at five levels in CCD under RSM. Table 3, shows the experimental ranges of the 3 significant variables for CCD trials.

2.8. Statistical analysis

The statistical analysis of results was done with MINITAB statistical software (version 14) and response surface plots were drawn. The data were statistically treated by analysis of variance (ANOVA). All data are presented as the mean value \pm standard deviation ($M \pm SD$) of independent experiments on different days. P-values ≤ 0.05 was considered statistically significant.

3. Results and discussion

The analysis of variance (ANOVA) showed the effect of 6 variables designed by Plackett-Burman Design. Then by applying the RSM, it was revealed the level of Pb biosorption as the function of three variables, as given in Table 4. The P-values less than 0.05 indicate that the model terms are significant. In this case Pb concentration, biomass dosage and contact time are significant model terms due to having the higher Coefficient values.

Table 3
The levels of experimental variables for the central composite experimental design.

Independent process variable	Range and level				
<i>S. cerevisiae</i> biomass dosage ($\times 10^8$ CFU)	$-\alpha$ (-1.68)	-1	0	+1	$+\alpha$ (+1.68)
Initial Pb concentration) μ g/l)	5	12	22	31	40
Contact time (day)	40	50	70	90	100
	0	1	2	3	4

Table 4
ANOVA results for Pb biosorption parameters.

Variables	Sum of Squares	Mean Square	F-value	p-value	Coefficient Estimate
Shaking rate	104.90	104.90	1003.53	0.0201	1.46
Biomass viability	134.13	134.13	1283.17	0.0178	1.95
Pb concentration	2000.02	2000.02	19132.82	0.0046	12.91
Biomass dosage	2620.20	2620.20	25065.67	0.0040	14.78
Contact time	4497.72	4497.72	43026.61	0.0031	19.36
Inoculation temperature	228.12	228.12	2182.22	0.0136	0.83

3.1. Effect of *S. cerevisiae* biomass dosage and contact time on the Pb removal

The contact time is one of the main factors that greatly influences the bioremoval (Ammini et al., 2012). The impact of contact time and biomass dosage on biosorption yield in the present study, can be predicted from the three-dimensional (3D) surface plot in Fig. 1. The 3D surface plot is a graphical diagram showing 2 factors, whereas all other factors are held in fixed levels. These plots would help to understand both main effects of variable and their interactions, better. As shown in Fig. 1, by increasing the contact time from 1st to 4th day, the Pb biosorption increased from 0 to 80% as well as increasing the biomass dosage from 5 to 40 × 10⁸ CFU. The optimum level of *S. cerevisiae* dosage for bioremoval was 22 × 10⁸ CFU. The highest efficiency was 70% in the 4th day. It is reasonable that by increasing the contact time, more Pb ions would connect to *S. cerevisiae* receptors, phosphate, amine and carboxyl groups in the cell wall, and the biosorption process would be more sufficiently by time (Han et al., 2006).

Some previous studies have reported that contact time in the biosorption medium is an important parameter which affects the biosorption by various biosorbents (hadiani et al., 2018; Farhan and Khadom, 2015; Khakpour et al., 2014; Samandari and Gazi, 2013). The findings showed that *S. cerevisiae* has high affinity for Pb in low level concentrations (Wang and Chen, 2006). The maximum Pb biosorption of 70% was obtained for Pb at 22 × 10⁸ CFU of *S. cerevisiae* dosage.

3.2. Effect of *S. cerevisiae* biomass dosage and Pb concentration on the Pb removal

The interaction effect of biomass dosage and Pb concentration is shown in Fig. 1. By increasing the Pb concentration from 4 to 100 µg/l, the Pb removal developed up to 70%. The bioremoval increased up was 22 × 10⁸ CFU and then decreased in 40 × 10⁸ CFU. The optimum *S. cerevisiae* biomass dosage for the maximum removal of Pb was 22 × 10⁸ CFU. However, the yeast surface area increases with enhancing its' dosage and therefore more absorption sites would be available, but practically, the obtained results showed that the metal removal decreases by increasing biomass dosage. This could be explained that by raising the biosorbent dosage, the number of available sites for biosorption increases, but these sites may have stayed unsaturated within the biosorption process (Vasudevan et al., 2002). It is shown in Fig. 2 that an optimum Pb biosorption yield of 70% was found in the Pb concentration of 70 µg/l, and biomass dosage of 22 × 10⁸ CFU.

Hadiani et al. (2018) reported the same results as the Pb and Cd bioremoval were reduced with enhancing *S. cerevisiae* biomass dose.

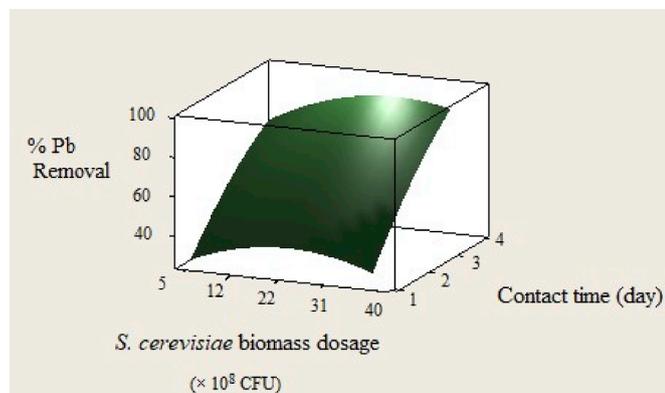


Fig. 1. 3D plot of interactive effect of *S. cerevisiae* biomass dosage and contact time on the Pb removal.

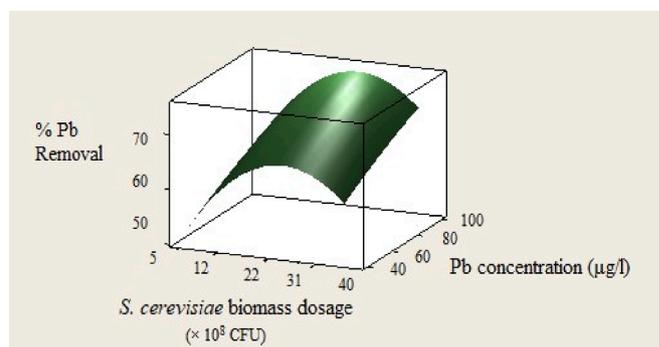


Fig. 2. 3D plot of interactive effect of *S. cerevisiae* biomass dosage and Pb concentration on the Pb removal.

Also Fadel et al. (2017) observed that the biosorption rate raised by increasing the Mn²⁺ concentration and fall by increasing *S. cerevisiae* biomass dosage. The results of our study also supported by the literature references, demonstrate that higher levels of biomass dose could cause interference between binding sites (Park and Choi, 2002). The increase in electrostatic interaction at high levels of biomass dosage could prevent metal bioremoval. As the biomass dosage is low, metal ions in the solution would be able to adsorb to the surface of the biomass and also enter to the intracellular parts via the concentration gradient of metal ions (Wang, 2002). Also, at lower biosorbent doses, the inter-cellular distance is more which improves the metal biosorption process and these conditions certifies the optimal electrostatic interaction between cells (Park and Choi, 2002).

In recent years, researches on removal of heavy metals by using microorganisms have received massive attention. *S. cerevisiae* is a unique biomaterial in metal biosorption. There are some studies on Pb bioremoval by *S. cerevisiae* and Table 5 shows a comparison of these studies.

Hadiani et al. (2018) observed that biomass dose, solution pH, and initial Pb ion concentration were the significant factors in Pb removal by *S. cerevisiae* in drinking water and the removal was 70.3% in the optimum conditions. Farhan and Khadom (2015) reported that the significant variables were temperature, initial concentration and contact time in uptake capacity of metal ions by *S. cerevisiae* in aqueous solutions and the removal efficiency was 79.2%. El-sayed (2013) also mentioned the initial metal ion concentration, biomass dosage and contact time as the main factors in biosorption by *S. cerevisiae* in aqueous solutions and the maximum biosorption capacities was 75%. Wang and Chen (2006) also reported that the main variables in biosorption of Pb by *S. cerevisiae* in effluent were initial metal ion, initial biomass concentration and contact time with the highest capacity of 80%.

In this project, also the significant variables are contact time, biomass and initial heavy metal concentration in Pb bioremoval by this valuable yeast in milk with the efficiency of 70%. The biosorption mechanism is the same in all of the mentioned studies as *S. cerevisiae* is a potent microorganism for uptaking the heavy metals. Phosphate, amine and carboxyl groups in the yeasts' cell wall were the main biosorption sites for metal ions. The highest biosorption in Wang and Chen study is probably due to the higher amount of heavy metals in

Table 5
Comparison of Pb bioremoval by *S. cerevisiae* in different studies.

<i>S. cerevisiae</i> Strain	Media	% Removal	Ref.
PTCC 5010	Drinking water	70.3	Hadiani et al. (2018)
PTCC 5010	Aqueous solutions	79.2	Farhan and Khadom (2015)
-	Aqueous solutions	75	El-sayed (2013)
-	Effluent	80	Wang and Chen (2006)
PTCC 5020	Milk	70	This study, 2019

effluent, but totally the removal capacities were nearly the same, in the range of 70–80%, which could be another confirmation of this yeasts' capacity in heavy metals biosorption.

4. Conclusion

The Lead residues in milk make particular concerns due to its' consumption largely by a majority of people. So finding a useful and safe solution to this issue would be so important. Most of the microorganisms can be useful biosorbents for metals removal, but the higher affinity of *S. cerevisiae* biomass for lead absorption is so valuable. The present project has reported the application of RSM to determine the conditions of maximum Pb biosorption by this desirable yeast. To confirm the accuracy of yeast bioremoval results, the analytical quality assurance was performed before the instrumental analysis. The results showed that bioremoval rate of Pb by *S. cerevisiae* increases by increasing the biomass dosage and contact time. The optimum condition for Pb uptaking of 70% was obtained by Design-Expert software at the initial Pb concentration of 70 µg/l and *S. cerevisiae* biomass dosage of 22×10^8 CFU. It was observed that Pb biosorption raised with increasing Pb concentrations and the optimum Pb concentrations was 70 µg/l. On the other hand, with increasing biomass dosage over the optimum level, the Pb biosorption reduced.

The results of this study revealed that by design of experiments based on the significant factors containing Pb concentration, contact time and biomass dosage, the lead bioremoval in very low concentration levels by *S. cerevisiae* from milk would be applicable. Other previous researches also confirmed the same results, but in much higher metals concentrations (ppm). Therefore, our findings based on this study demonstrate that using *S. cerevisiae* as a novel green technology, can be applied in heavy metals bioremoval and decontamination from water, food and drinks in the food processing industry.

Declaration of competing interest

There is no conflict of interests to declare.

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