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Optimization of Lead Bioremediation by Marine *Halomonas* sp. ES015 Using Statistical Experimental Methods

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***Abstract*—**Bioremediation technology is now used for treatment instead of traditional metal removal methods. A strain was isolated from Marsa Alam, Red sea, Egypt showed high resistance to high lead concentration and was identified by the 16S rRNA gene sequencing technique as *Halomonas* sp. ES015. Medium optimization was carried out using Plackett-Burman design, and the most significant factors were yeast extract, casamino acid and inoculums size. The optimized media obtained by the statistical design raised the removal efficiency from 84% to 99% from initial concentration 250 ppm of lead. Moreover, Box-Behnken experimental design was applied to study the relationship between yeast extract concentration, casamino acid concentration and inoculums size. The optimized medium increased removal efficiency to 97% from initial concentration 500 ppm of lead. Immobilized *Halomonas* sp. ES015 cells on sponge cubes, using optimized medium in loop bioremediation column, showed relatively constant lead removal efficiency when reused six successive cycles over the range of time interval. Also metal removal efficiency was not affected by flow rate changes. Finally, the results of this research refer to the possibility of lead bioremediation by free or immobilized cells of *Halomonas* sp. ES015. Also, bioremediation can be done in batch cultures and semicontinuous cultures using column technology.

***Keywords*—**Bioremediation, lead, Box–Behnken, *Halomonas* sp. ES015, loop bioremediation, Plackett-Burman.

I. Introduction

THE environment, and particularly the aquatic environment, has been focused within the last years due to the large amount of released chemicals. Thousands of synthetic chemical compounds are currently recorded for use in industry and agriculture, and thousands of tons of these are produced yearly. Regardless of the source or original intended use, fundamental amounts of these chemicals end up in the aquatic environment due to physicochemical, hydrologic and atmospheric processes [1], [2].

Lead (Pb) is ubiquitous and one of the earliest metals discovered by human. Pb's unique properties like softness, high malleability, ductility, low melting point and resistance to corrosion, led to an increase in its use in many industries as automobile, paint, ceramics, plastics, etc. which in turn has led to a massive increase in the presence of free Pb in biological systems and the inert environment [3]. The main reason of Pb prolonged persistence in the environment is the non-biodegradable nature of Pb ions. Human exposing to Pb occurs via various sources like leaded gasoline, industrial processes such as Pb smelting and coal combustion, Pb-based paints, Pb containing pipes or Pb-based solder in water supply systems, battery recycling, grids and bearings etc. [4].

The high Pb concentration in the effluent causes a direct danger to human and animals. Pb with no known biological function is highly toxic and it accumulates in humans [5]. Pb, mercury (Hg), cadmium (Cd) and chromium (Cr) (VI) show the highest toxicity in the toxicity list from among various metal ions, due to their major impact on the environment. Pb is a toxic heavy metal which forms complexes with oxo-groups in enzymes to affect virtually all steps in the hemoglobin synthesis process and porphyria metabolism. Toxic levels of Pb in human may cause encephalopathy, seizures and mental retardation [6]. Thus, removal of heavy metals, especially Pb metal from contaminated wastes is absolutely necessary. Traditional methods that have been used for heavy metals removal from wastewaters include chemical precipitation, ion exchange, membrane separation, reverse osmosis, evaporation and electrolysis [7].

Bioremediation processes are very attractive compared to physicochemical methods, because of the low cost and high efficiency at low metal concentrations [1]. In microbial bioremediation, microorganisms could be induced to degrade hazardous organic contaminants to environmentally safe levels in water, soils, sludge, subsurface materials, and residues [8]. The microbial ability to grow in the presence of heavy metals could aid in waste water treatment because microorganisms during waste water treatment are directly involved in the biological processes of organic matter degradation [9], because heavy metals' inhibitory effect is a common phenomenon which occurs in waste water and sewage biological treatment [10]. Heavy metals affect the microbial population by affecting their growth, morphology, biochemical activities and finally resulting in decreased biomass and diversity. Microbial survival depends on biochemical and structural properties, physiological, or genetic modifications which include changes in cell morphology, or environmental modifications in the metal speciation [11]. Microbial metal resistance mechanisms include metals precipitation as metal phosphates, metal

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carbonates and/or metal sulfides, volatilization by methylation or ethylation, membranes electronegative components and extracellular polymeric substances physical exclusion, metal efflux systems which is energy-dependent, and intracellular sequestration of low molecular weight cysteine-rich proteins [1]. Another investigation was suggested by Murthy et al. [5] that microbes are very small in size so they have a high surface area to volume ratio. They provide themselves with a large contact area which can interact with substances in the surrounding environment. The capability of the microorganisms to grow and survive in presence of high metal concentrations is attributed to stress induced selection of these microbes in specific environments. The aim of this study is to isolate a local strain capable of Pb bioremediation, and optimization of cultural conditions by statistical methods for maximum removal efficiency, and then applying optimum medium obtained for Pb removal by the loop bioremediation.

II. Materials and Methods

A. Bacterium and Cultivation

Bacterial strains capable of Pb bioaccumulation were isolated from different areas related to metal-based activities in Egypt: Aqaba Gulf, Red Sea, Max, Abukir and Airport. Samples were collected in sterile glass containers, and are used to inoculate ZoBell medium with the following composition (g/l): filtered sea water, 800 ml/L; distilled water, 200 ml/L; yeast extract, 1; peptone, 5; $FeSO_4.7H_2O$, traces, and final pH was adjusted to 7.5 [12]. Flasks were incubated at 120 rpm and 37 °C, overnight, 1 ml of culture broth from each flask was then transferred to ZoBell agar medium supplemented with 50 ppm of Pb, and incubated for 24, 48 and 72 hr. Bacterial colonies were purified by repeated subculturing on ZoBell agar medium. Bacterial sensitivity toward metal toxicity was further studied at higher concentrations of Pb (100, 150 and 250 ppm), for 72 hours of incubation at 37 ºC on ZoBell agar medium [12].

The growth of bacteria in presence of Pb was monitored by measuring the optical density at 600 nm using a spectrophotometer. Pb removal efficiency was calculated and the standard curve was constructed. Uninoculated flasks were used as negative controls in order to evaluate metal removal efficiency. The most potent strain, isolated from Marsa Alam and showing maximum tolerance at 250 ppm of Pb solution was used for further studies on bioremediation of the metal. The strain was identified by the 16S rRNA gene sequencing analysis by GACT Company-Germany using universal primers; 27F: FAGAGTTTGATCMTGGCTCAG and 1492R: CGGTTACCTTGTTACGACTT. NCBI tools including blast search, tree method (Fast Minimum Evolution) and pair wise alignment were applied for the providing sequence. PCR amplification program was set according to the following cycling parameters: 10 min of initial denaturation /enzyme activation at 95 °C, 5 min of denaturation at 95 °C, followed by 20 cycles of 30 s at 95 °C (denaturing), 1 min at 65 °C (annealing), and 90 s at 72 °C (extention), with a final extension at 72 °C for 10 min. The nucleotide sequences were analyzed with the BLAST database [13].

B. Optical Density Determination

The cultures broths were taken for growth determination photometrically at 600 nm using spectrophotometer (Pharmacia Biotech, Novaspec II).

C. Determination of Pb Removal Efficiency

Strains were grown in ZoBell medium supplemented with 250 or 500 ppm of Pb solution/ L of culture medium till 0.9 $O.D_{600}$, and then culture media were centrifuged at 5000 rpm for 15 min and residual metal concentration was determined in supernatant using atomic absorption spectrophotometry. Metal removal efficiency was expressed as a percentage of metal removed compared to initial metal concentration using:

$$(M^{++} \text{ removal efficiency } (\%) = (Ic - Fc) \times 100 / Ic) \quad (1)$$

where, Ic is the initial concentration (ppm) and Fc is the final concentration (ppm).

D. Plackett-Burman Design

The Plakett-Burman experiment design which is a fractional factorial design was used in this study to estimate the importance of many environmental factors relatively on the efficiency of Pb Pb^{++} removal. In this experiment, four independent variables were screened in twelve combinations organized according to the Plackett-Burman design matrix.

For each variable, high (+) and low (-) levels were tested (Tables I and II). All trials were done in duplicates and the average results were calculated as the responses. The following equation was used to calculate the main effect of each variable:

$$E\,xi = (\Sigma M\,i + - \Sigma M\,i-) / n \quad (2)$$

in which E_{xi} is considered as the variable main effect, while M_{i+} and M_{i-} are the Pb removal efficiency percentage in trails, in which the independent variable (xi) was existing in high and low levels, respectively, while n is considered as the number of trials divided by 2. The positive sign of the main effect means that the high concentration of this variable is near to the optimum and the negative sign means that the low concentration of this variable is near to the optimum. Using Microsoft Excel, statistical t-values for equal un-paired samples were calculated for determination of variable significance [14].

TABLE I
Different Levels of the Four Independent Variables Used in the Plackett-Burman Design

Factors (g/L)	Low level (-)	Level (0)	High Level (+)
Yeast extract (g/L)	0.5	1	2
Casamino acid (g/ L)	1.4	2.8	5.6
pH	6.5	7.5	8.5
Inoculums size (ml/ L)	0.5	1	2

TABLE II
THE PLACKETT-BURMAN EXPERIMENTAL DESIGN FOR FOUR FACTORS [15]

Trial	Yeast extract	Casamino acid	pH	Inoculums size
1	+	+	-	+
2	-	+	+	-
3	+	-	+	+
4	-	+	-	+
5	-	-	+	-
6	-	-	-	+
7	+	-	-	-
8	+	+	-	-
9	+	+	+	-
10	-	+	+	+
11	+	-	+	+
12	-	-	-	-

E. Box-Behnken Experimental Design for Three Factors

After evaluation of the most significant independent variables affecting the bioremediation process, the most significant variables were further chosen for estimation of their optimal level. For this reason Box-Behnken design, which is a response surface methodology (RSM), was applied. Three main steps were included in the optimization process: Performing the statistically designed experiments, the coefficients in a mathematical model were estimated, predicting the response and checking the adequacy of the model [16]. The three significant variables elucidated through Plackett-Burman experimental design for the tested *Halomonas* sp. ES015 isolate were yeast extract (X_1), casamino acids (X_2) and inoculum size (X_3). Low, middle and high levels of each of the independent variables were -1, 0 and +1, respectively (Table III). A design matrix for the 15 trials, along with the natural values for the three factors was constructed (Table IV). 15 trials were carried out in triplicate and mean values of Pb removal (ppm) were calculated. To predict the optimal point, a second order polynomial function was fitted to correlate the relationship between the independent variables and the response. The equation for the three factors was as:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (3)$$

where Y is the predicted response, β_0 is the model constant, X_1, X_2, and X_3 are the independent variables, β_1, β_2, and β_3 are the linear coefficients, β_{12}, β_{13}, and β_{23} are the cross-product coefficients, and β_{11}, β_{22}, and β_{33} are the quadratic coefficients. The experiment was conducted using medium supplemented with 500 ppm of Pb, incubated at 120 rpm and 37°C for 24 hours. Optical density was measured ($O.D_{600}$), and metal removal efficiency was determined using atomic absorption spectrophotometry.

TABLE III
DIFFERENT LEVELS OF THE INDEPENDENT VARIABLES USED IN THE BOX-BEHNKEN EXPERIMENT

Factor (g/L)	Low level (-)	Level (0)	High Level (+)
Yeast extract (g/L)	1	2	3
Casamino acid (g/L)	2.8	5.6	8.4
Inoculums size (ml/L)	1	2	3

TABLE IV
THE BOX-BEHNKEN EXPERIMENTAL DESIGN FOR THE INDEPENDENT VARIABLES

Trial	Yeast extract	Casamino acid	Inoculums size
1	0	1	-1
2	1	1	0
3	0	0	0
4	1	-1	0
5	-1	-1	0
6	1	0	1
7	0	-1	1
8	0	0	0
9	1	0	-1
10	-1	0	1
11	-1	0	-1
12	-1	1	0
13	0	-1	-1
14	0	0	0
15	0	1	1

F. Column Design (Loop Bioremediation)

A column bioremediation was used for continuous cultivation. The column was made of transparent cylindrical glass of 2.5 cm diameter and 25 cm height. The column was connected by a quick fit to a reservoir (1 L conical flask) and filled with sponge cubes for adsorption. 0.22% of cell solution was used to inoculate the support. After adsorption of cells on the packed support matrix, approximately 500 ml of the optimized medium supplemented with 500 ppm/L Pb nitrate solution was allowed to flow from top to bottom of the column in a closed circle using a peristaltic pump. Temperature was adjusted at 37 ºC using hot plate. Aeration was maintained by air pump and shaking of medium using magnetic stirrer. The pH of the solution was adjusted at 7.5. Flow rate was increased gradually from 0.3 to 7.5 ml/min.

The effluent from the bottom of the column was collected after the time intervals 18, 20, 22 and 24 hr, and the residual Pb concentration was measured after centrifugation by atomic absorption spectrophotometry. Fresh media supplemented with 500 ppm of Pb solution were used to replace culture media of the same composition under aseptic conditions for each run which is 24 hr of cultivation

The column experiment was continued for six successive cycles. Uninoculated column was used as a negative control in order to evaluate metal removal efficiency.

III. RESULTS AND DISCUSSION

A. Strain Identification

The partial sequence of 16S rRNA of the isolate was provided by GACT Company-Germany. According to the blast search of NCBI, the sequence similarity was found to be 86% similar to *Halomonas campaniensis* strain LS21 followed by 85% similarity to *Halomonas boliviensis* LC1 (Fig. 1) Consequently, the strain was identified to the genus level to be *Halomonas* sp. ES015. This genus belongs to phylum *Proteobacteria,* order *Oceanospirillales* and family *Halomonadaceae*. The sequence was given an accession number KT935521.

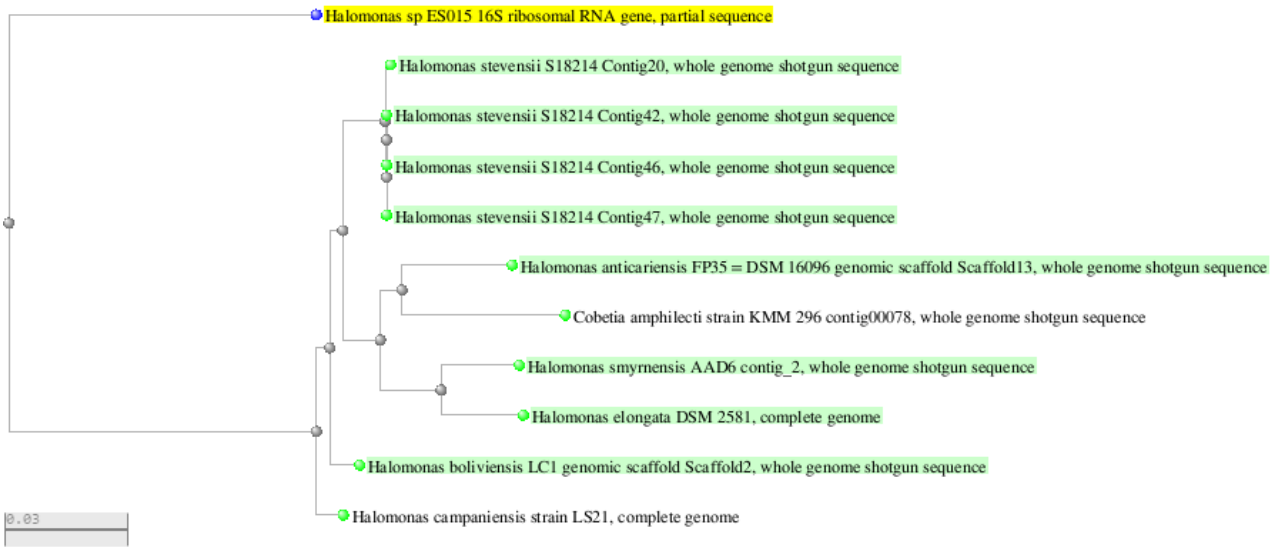

Fig. 1 Phylogenetic relationships between *Halomonas* sp. ES015 strain and closely related strains based on the 16S DNA partial sequence. The degree of relationship is represented as percentages according to NCBI blast database

B. Plackett-Burman Design

The Plackett-Burman design was applied to reflect the relative importance of various fermentation factors in combinations supplemented with 250 ppm/L Pb nitrate solution. The efficiency of Pb removal for each trial were determined, data represented in Table V showed that trial 4 followed by trial 8 yielded the highest Pb removal efficiency (99.59, 99.49%) respectively.

The principal statistical analysis of this experiment is shown in Table VI. Yeast extract, casamino acid, and inoculums size were the most significant factors at 90% tested degree of significance ($\alpha = 0.1$), in which the increase in yeast extract, casamino acid concentrations and the inoculums size level resulted in an increase in Pb removal efficiency. The main effect of each constituent on the Pb removal was estimated and represented graphically (Fig. 2). Main effects results showed that inoculum size, casamino acid, yeast extract and pH had high positive main effect which indicates that the high concentrations of these variables are near to the optimum and increase Pb bioremediation. According to these suggestions dictated by the applied Plackett-Burman experiment, with respect to the Pb removal, it can be predicted that the optimum medium for Pb removal from the culture of *Halomonas* sp. ES015 is (g/l): yeast extract, 2 g; casamino acid, 5.6 g; $FeSO_4.7H_2O$, traces; inoculum size, 2 ml; pH adjusted to 7.5 ~ 8.

TABLE V
EXPERIMENTAL RESULTS OF THE PLACKETT-BURMAN DESIGN OF *HALOMONAS* SP. ES015 PB REMOVAL

Trial	Optical density	Pb removal efficiency (%)
1	1.329	98.33
2	1.136	99.12
3	1.141	99.19
4	1.166	99.59
5	0.093	29.03
6	0.302	85.92
7	1.061	99.36
8	1.266	99.49
9	1.324	97.93
10	1.312	99.36
11	1.030	98.49
12	0.008	8.80

TABLE VI
STATISTICAL ANALYSIS OF THE PLACKETT-BURMAN EXPERIMENTAL DESIGN

Variable		Pb removal efficiency (%)						Mean	Main effect	T-value	Degree of significance (%)
Yeast extract	+	98.33	99.12	99.36	99.49	97.93	98.49	98.787	28.483	1.716113	90
	-	99.12	99.59	29.03	85.92	99.36	8.80	70.303			
Casamino-acid	+	98.33	99.12	99.59	99.49	97.93	99.36	98.970	28.83	1.743738	90
	-	99.19	29.03	85.92	99.36	98.49	8.8	70.132			
pH	+	99.12	99.19	29.03	97.93	99.36	98.49	87.187	5.270	0.280226	90
	-	98.33	99.59	85.92	99.36	99.49	8.80	81.915			
Inoculums size	+	98.33	99.19	99.59	85.92	99.36	98.49	96.813	24.520	1.424211	90
	-	99.12	29.03	99.36	99.49	97.93	8.80	72.288			

Degree of significance, $t\alpha 90 = 1.3722$

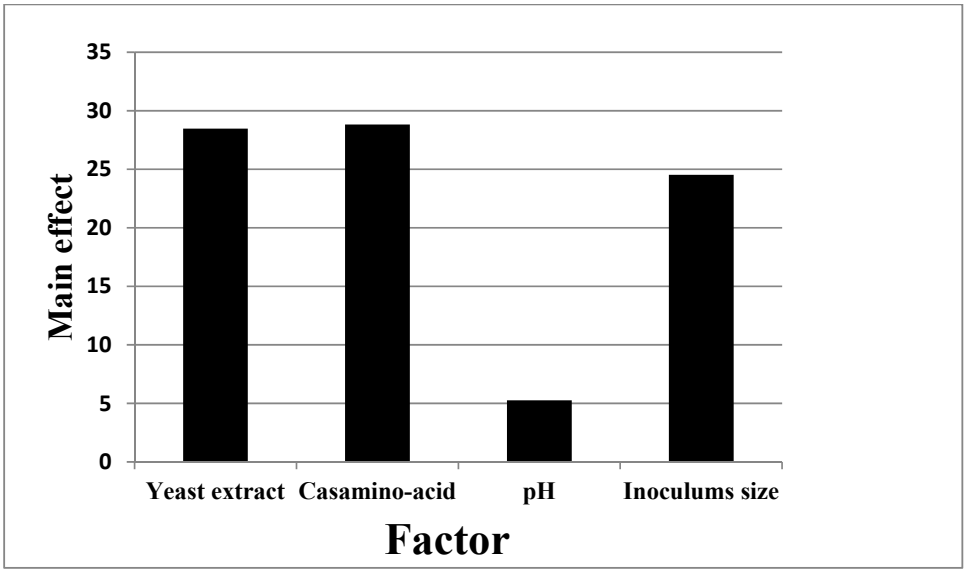

Fig. 2 The main effect of each variable on Pb removal efficiency by *Halomonas* sp. ES015

In order to evaluate the accuracy of the applied Plackett-`Burman experiment, a verification experiment was carried out. The predicted optimum levels of independent variables were examined and compared to the basal conditions setting and the anti- optimized levels. Data represented in Table VII, show that optimum culture medium led to high bioremediation efficiency compared to non- and anti-optimized medium settings, giving 99.3% bioremediation efficiency for optimized cultures while non-optimized and anti-optimized cultures gave 84%, 35% respectively from initial Pb concentration 250 ppm. And thus Pb removal efficiency was increased by optimized medium 2.83 times than that achieved by anti-optimized medium.

TABLE VII
A VERIFICATION EXPERIMENT FOR *PB* REMOVAL BY *HALOMONAS* SP. ES015 BY OPTIMIZED VERSUS NON-OPTIMIZED AND ANTI-OPTIMIZED MEDIA

Medium	Optical density	Pb removal efficiency (%)
Optimized	1.264	99.3
Non optimized	0.836	83.8
Anti-optimized	0.188	35.1

Bioremediation has an effect only when environmental conditions are suitable for microbial growth and activity. This could be achieved by optimizing environmental conditions to allow high rate of microbial growth and degradation [17], [18].

The results agreed with Mabrouk [19] who mentioned that high levels of yeast extract concentration and inoculum size had a significant effect on Cr reduction by halophilic *Streptomyces* sp. *MS-2* and also agreed with Abd-Elnaby et al. [20] who mentioned that high levels of yeast extract concentration and inoculum size had a highly positive main effect which positively affected the increase of Cd bioaccumulation by *Vibrio harveyi*. However, the results were different from that obtained by El-Ahwany [21] who mentioned that the three factors which showed high copper biosorption efficiency by *Oenococcus oeni PSU-1* are copper concentrations, immobilization and mixing speed.

C. Box-Behnken Experimental Design

To identify the optimum response region for Pb removal, the significant independent variables (yeast extract, X; casamino acids, Y; inoculums size, Z) were further explored at three levels based on the results of the two levels used in Plackett-Burman design illustrated in Fig. 2, Table VI to identify the optimum response region for Pb removal. Each of significant independent variables was further explored at three levels (1, 0, -1). The efficiency of Pb^{++} removal for each trial supplemented with 500 ppm/L Pb nitrate solution of box benken design was determined, and data was represented in Table VIII.

It was found that maximum bioremediation efficiency obtained at high values of yeast extract and casamino acid and middle value of inoculum size. On the model level, the correlation measures for estimating the regression equation are the multiple correlation coefficient R and the determination coefficient $R^{2.}$ In this Box-Behnken experiment, the value of R^2 was 0.925 and that of R was 0.961. Maximum values of R^2 and R, and also, the many significant effect, gave a proof that the model in a high accurate way qualifies the process, and shows an elevated degree of relationship between the experimental and the predicted values [22].

The optimal levels of the three factors, as obtained from the maximum point of the polynomial model, were estimated using the *solver* function of the Microsoft Excel tools, and found to be (3) 1% yeast extract, (8.4) 1% casamino acids and 0.15% inoculums size equal to 2.3 ml.

In addition, Figs. 3-5 show the simultaneous effects of the three most significant independent factors on each response using three-dimensional graphs generated by STATISTICA 5.0 software. Fig. 3 showed the effect of both yeast extract concentration and casamino acids on the reciprocal of Pb

removal. As shown, higher Pb removal (99.19%) was designated by the red color region and this region was achieved when yeast extract and casamino acids were at high concentrations. Moreover, Fig. 4 showed higher Pb removal (99.18%) which occurred around the middle value of inoculum size and higher concentration of yeast extract. For Fig. 5, it was shown that at the higher casamino acids concentration, the removal of Pb reached about 99.18% when met with the middle value of inoculums size. Accordingly, the more were the concentrations of both casamino acids and yeast extract, the more was the Pb removal, until reaching a region, deep red, where a maximum removal was achieved.

Also, the main effect of different variables was represented graphically in Fig. 6. According to these suggestions dictated by the applied Box-Behnken experiment, with respect to the Pb removal, it can be predicted that the optimum medium for Pb removal from the culture of *Halomonas* sp. ES015 is (g/l): yeast extract, 3g; casaminoacid, 8.4g; $FeSO_4.7H_2O$, traces; inoculum size, 2.3 ml; pH adjusted to 7.5 ~ 8.5.

In order to evaluate the accuracy of the applied Box–Behnken experiment, a verification experiment was carried out. The predicted optimum levels of independent variables were examined and compared to the non-optimized setting and the anti-optimized levels. The Pb removal results are shown in Table IX, where the Pb removal reached about 97% for optimized medium, 95% for basal medium and 79% for anti-optimized medium from initial Pb concentration 500 ppm/L.

Adinarayana and Ellaiah [23] have reported that three-dimensional (3D) response surface plots as a function of two factors, maintaining all other factors at fixed levels are more helpful in understanding both the main and the interaction effects of these two factors. In addition, 3D response surfaces and their corresponding contour plots can facilitate the straightforward examination of the effects of the experimental variables on the responses [24].

Kahkha et al. [25] indicated that there were considerable interactions between each of the independent variables (pH, dosage of adsorbent and initial concentration) and the Cd removal efficiency by Citrullus colocynthis. Also, Verma et al. [26] had studied the optimization of Pb(II) and COD sequestration by consortium of sulphate-reducing bacteria, using Box–Behnken design model of RSM to examine the effect of three independent operating variables, which were pH, temperature and time. The results were highly fitted to the quadratic model, and the interactive effectiveness of pH, temperature and time on Pb(II) and COD removal was highly significant.

TABLE VIII
EXPERIMENTAL RESULTS OF THE BOX-BEHNKEN DESIGN OF *HALOMONAS* SP. ES015 FOR *PB* REMOVAL

Trial	Optical density	Pb removal efficiency (%)
1	1.325	96.69
2	1.414	98.46
3	1.233	94.41
4	1.392	94.50
5	0.592	79.54
6	1.377	98.11
7	0.507	79.64
8	1.152	93.15
9	1.353	96.67
10	1.151	79.85
11	0.604	79.11
12	1.218	97.01
13	0.567	81.30
14	1.103	93.13
15	1.421	98.24

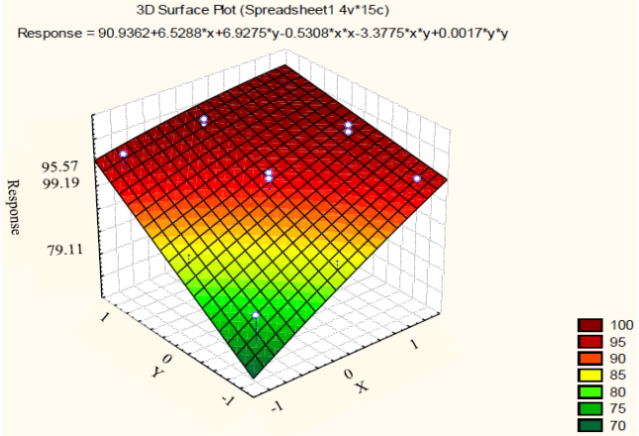

Fig. 3 Interaction effect between (X) yeast extract concentration (g/L) and (Y) casaminoacid concentration (g/L) on Pb bioremediation

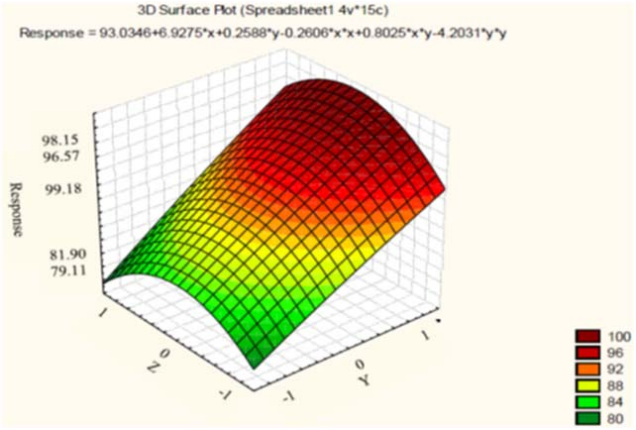

Fig. 4 Interaction effect between (X) yeast extract concentration (g/L) and (Z) inoculums size on Pb bioremediation

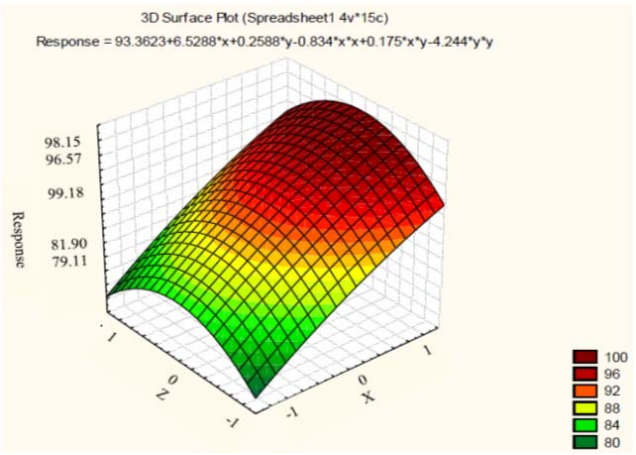

Fig. 5 Interaction effect between (Y) casaminoacid concentration (g/L) and (Z) inoculums size on Pb bioremediation

A. Loop Bioremediation

In column bioremediation experiment, Pb nitrate solution (Pb concentration 500 ppm) was allowed to flow from top to bottom of the column at different flow rates (0.3, 0.6, 1.2, 2.4, 4.8 and 7.5 ml /min) using a peristaltic pump. The results represented in Table X showed that, metal removal reached approximately 100% after 18 hours of incubation and then still relatively constant till 24 hours of incubation. Reusing cultures of *Halomonas* sp. ES015 adsorped on sponge cubes showed also relatively constant Pb removal efficiency till the 6th run over the range of time interval. Bioremediation processing continued for 6 successive cycles and metal removal efficiency not affected by flow rate changes. Processing time decreased from 24 hours to 18 hours with bioremediation efficiency reached approximately 100% from initial Pb concentration 500 ppm.

TABLE IX
A VERIFICATION EXPERIMENT FOR *PB* REMOVAL BY *HALOMONAS* SP. ES015 BY OPTIMIZED VERSUS NON-OPTIMIZED AND ANTI-OPTIMIZED MEDIA

Medium	Optical density	Pb removal efficiency (%)
Optimized	1.426	97.08
Non-optimized	1.350	95.62
Anti-optimized	0.530	79.74

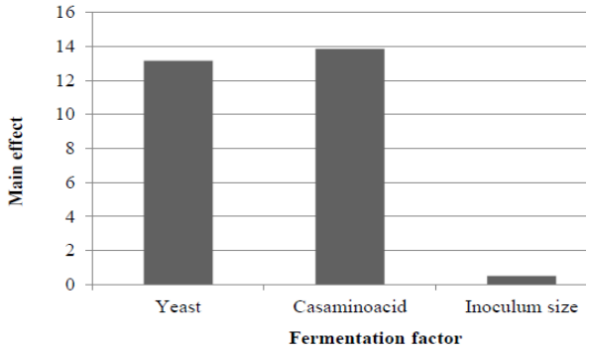

Fig. 6 Elucidation of fermentation factors affecting Pb removal in applied Box- Behnken experiment

For large scale systems, rigid supports are preferred. Microbial colonization and growth on the support surface produces a layer of self-immobilized cells held in contact with the perfuming flow [20]. Some investigators reported metal removal by column study using immobilized biomass as *Aeromonas hydrophila* [27], *Brevundimonas vesicularis* [28] and *Rhizopus nigricans* [29].

TABLE X
COLUMN EXPERIMENT (LOOP BIOREMEDIATION METHOD)

Run	Flow rate (ml/min)	Time(hour)	Pb Removal Efficiency (%)
1	0.3	18	99.94
		20	99.72
		22	99.78
		24	99.80
2	0.6	18	99.56
		20	99.25
		22	99.56
		24	99.72
3	1.2	18	98.54
		20	99.22
		22	99.01
		24	99.64
4	2.4	18	99.71
		20	99.29
		22	99.24
		24	99.70
5	4.8	18	99.56
		20	99.65
		22	99.57
		24	99.77
6	7.5	18	99.63
		20	99.60
		22	99.68

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