Application of Biosurfactant Producing Microorganisms to Remediate Heavy Metal Pollution in El-Gabal El-Asfar Area

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ABSTRACT

In the present study, the effects of biosurfactant producing cells of different bacterial species namely *Pseudochrobactrum lubricantisa*, *Lysobacter novalis*, and crude biosurfactant extracted from *Aspergillus niger* were investigated as a cheap source to alleviate the availability of Pb^{2+} for plants grown in El-Gabal El-Asfar area, Egypt. This is owing to these areas are basically depend on the reused wastewater for irrigation. Biosurfactant producing microorganisms were isolated from three different farms aged for 20, 40, and 60 years. The obtained results showed that, the biosurfactant producing bacteria decreased the availability of Pb^{2+} in the outlet leachate during the leaching columns experiments. In contrast, Sr^{2+} leachate showed the reverse trend that was observed with Pb^{2+}. Taguchi approach indicated that, the mechanisms of biosurfactant producing bacteria are very complicated and interfused with each other. The most factors affected the available form of Pb^{2+} and Pb^{2+} existed in organic form was Pb^{2+} concentrations, however the exchangeable Pb^{2+}, Pb^{2+} bond to carbonate, Pb^{2+} bond to sulfate, and plant content the most influential factors was biosurfactant producing bacteria. The most influential factors affected NPK uptake by watercress were found to take the following sequence biosurfactant producing bacteria > Sr^{2+} doses > Pb^{2+} doses.

Keywords: Biosurfactant; Heavy metals; Remediation; Pb^{2+}; Sr^{2+}; Taguchi approach

INTRODUCTION

Water scarcity is in arid and semi-arid countries persuaded farmers for reusing drainage wastewater as alternative source for irrigation. The Egyptian government has established El-Sallam canal project that depends on blending of drainage wastewater (El-Serw and Hadous drainages) and Nile River water at constant ratio 1:1 to overcome water scarcity(Abou-Shady, 2017). Also, soils close to industrial areas such as Burg El Arab have been contaminated with rare inorganic contaminates such as vanadium (Eissa et al., 2017). Accordingly, industrial and rural areas have been contaminated with inorganic and organic contaminates that is represent a threat to human being and animals. The main pollution source in industrial areas is the untreated wastewater. So that, the treatment of industrial wastewater should be carried out before the outlet discharge (Peng *et al*., 2011; Abou-Shady, 2016a; Abou-Shady, 2016b; Abou-Shady, 2017; Eissa *et al*., 2017).

Several approaches have been introduced to treat heavy metal polluted soils such as electrokinetic remediation, chemical washing, precipitation, etc (Abou-Shady, 2012; Almeria *et al*., 2012a; Abou-Shady *et al*., 2018). Heavy metals interact with humic substances (humic and fulvic acids) containing soils and comply with its distribution in soil profile (Abou-Shady, 2008; Khalil *et al*., 2009). Biosurfactant producing microorganisms is an emerging technique that may be produced by different sources such as microorganisms, plants, and animals. Biosurfactant producing microorganisms has a direct influence on several factors that control the behavior of heavy metals such as sorption, ion exchange, electrostatic interaction, resistance to aqueous phase transport, dissolution, rate limited mass transfer, and precipitation. The biosurfactant producing microorganisms possesses a biodegradable behavior compared with synthetic components that is considered friendly to environmental. Another advantage relieves from biosurfactant producing microorganisms that is considered a cost effective material produces in situ (Milleer, 1995; Franzetti *et al*. 2014).

Several attempts have been carried out to restore heavy metal (Zn, Cu, Cr, Hg, Cd and Pb) containing wastewater and polluted soil using the biosurfactant producing microorganisms (Zouboulis *et al*. 2003; Yuan *et al*., 2008; Chen *et al*. 2011; Elouzi1*et al*., 2102; Huang and Liu, 2013). The efficiency of the biosurfactant producing microorganisms has been improved using ethylene diamine tetra acetic acid (EDTA) and citric acid. On the other hand, adding the biosurfactant producing microorganisms to soils

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contaminated with heavy metals is considered an effective factor to improve phytoremediation endeavor (Slizovskiy *et al*., 2011; Rajkumar *et al*., 2012).

The objectives of this study were to study the impact of the biosurfactant producing microorganisms on heavy metals containing polluted soil using soil collected from El-Gabal El-Asfar area, Egypt as representative of polluted soils in Egypt. To identify the influence of biosurfactant producing bacteria; the changes of dominant species of Pb^{2+} in the studied soils were studied. Also, the effects of bacteria species, Sr^{2+}, and Pb^{2+} on nitrogen, phosphorous, and potassium (NPK) uptake by plant were investigated using Taguchi approach.

MATERIALS AND METHODS

Isolation of biosurfactant producing microorganisms

Soil samples were collected from three different farms aged for 20, 40, and 60 years at El-Gabal El-Asfar area, Egypt as it listed in Table (1). Serial dilutions were carried out from rhizospheric zone and plated on nutrient agar media. After incubation period, the bacterial colonies were picked according to its dominance and variations. A total of fifteen colonies were isolated from nutrient agar and purified using successive streaking on nutrient agar media (Sanders, 2012).

Screening of biosurfactant producing microorganisms

The ability of isolates to produce biosurfactant was investigated according to oil spreading assay (Morikawa *et al*., 1993); blood hemolysis test (Sarvanan and Vijayakumar, 2012; Anandaraj and Thivakaran, 2010); blue agar plate (BAP) method (Sarvanan and Vijayakumar, 2012; Satpute *et al*., 2008); and emulsification test (Bodour *et al*., 2004).

Separation of Biosurfactant

Fungal isolate was grown on growth medium containing: NH_4NO_3 (1g/L), KH_2PO_4 (0.2g/L), $MgSO_4.7H_2O$ (0.2g/L), glutamic acid (10g/L), and olive oil refinery residue (60g/L). After seven days of incubation period the culture broth was filtered through a Whatman filter paper (No. 1) and centrifuged at 5000 rpm for 20 min. The cell free broth was concentrated by dry freezing then three volumes of chilled acetone were added and allowed to stand for 10 h at 4 ?C. The precipitate was collected by centrifuging and evaporating to dryness to remove the residual acetone and re-dissolved in the sterile water (Pruthi and Cameotra 1997).

Identification of selected isolates

Morphological and microscopic identification

The selected bacterial isolates were examined by using different morphological and biochemical tests, includes Gram staining, Motility test, Indole test, Methyl red test, Starch hydrolysis, Gelatin liquefaction test, Oxidase test, and Catalase test. The selected fungal isolates were identified based on the colony morphology, colony growth rate and microscopy features using standard taxonomic keys and monographs (Pitt and Hocking, 2009).

Molecular characterization of selected bacterial isolates

Genomic DNA of isolates was extracted directly from the colonies grown on solid medium and was used as template for PCR according to modified method of Ishikawa et al., (2000). The purified product was sequenced using (DYEynamic ET Terminator Cycle Sequencing Kit, Amersham Pharmacia Biotech. (ABI 3130) at Animal Health Institute, Agricultural Research Center, Cairo, Egypt.

Phylogenetic analysis

The 16S rRNA sequences of the isolates were assembled in BioEdit software (Hall, 1999). The nucleotide sequence was compared with similar sequences found in the NCBI database through BLAST program (NCBI website). Multiple nucleotide sequences alignment was performed using BioEdit and Clustal W. Neighbour joining phylogenetic tree was constructed using Phylip 3.65 (Felsenstein, 1993). The phylogenetic tree was constructed using the neighbor joining NJ method (Saitou and Nei 1987). The clustering stability of the tree was evaluated by bootstrap analysis of 100 data sets.

Leaching experiments

Leaching experiments were carried out using polyethylene tube of 6 cm in diameter and 20 cm length. The effective length of soil inside polyethylene columns was 10 cm. Soil collected from El-Gabal El-Asfar area was placed inside polyethylene columns and artificially polluted with Pb^{2+} and Sr^{2+} at constant concentrations 150 mg kg^{-1} and 10 mg kg^{-1}, respectively. The sources of Pb^{2+} and Sr^{2+} were $Pb(NO_3)_2$ and $SrCl_2.6H_2O$, respectively with analytical grade. The broth culture of the selected isolates and crude biosurfactant extract and their mixture were added separately to the polyethylene tube. The control experiment was treated with 10 ml distilled water to keep soil wet during experimental period. Distilled water (10 ml) was added to the soil column to keep soil wet during experiment period (15 days). The total concentration of Pb^{2+} and Sr^{2+} in outlet leachate was detected using Inductivity Coupled Argon

Table 1. The locations of the different soils samples collected from El-Gabal El-Asfar

No	Locations		Cultivation period
	Latitude	Longitude	
1	30 ′13 ″11.6 N^o	31 ′23 ″14.9 E^o	60 years
2	30 ′11 ″37.9 N^o	31 ′23 ″ 2.6 E^o	40 Years
3	30 ′11 ″29.6 N^o	31 ′23 ″33.9 E^o	20 Years

Table 2. Physical and chemical properties of the main soil (aged for 60 years)

Soil Properties		El-Gabal El-Asfer area
Soil Texture		Loamy sand
Particle size distribution	Sand %	81.1
	Silt%	7.6
	Clay%	11.3
Total $CaCO_3$ (%)		8.3
OM (%)		4.31
CEC(meq 100g^{-1})		23.9
pH (1:2.5)		7.72
EC (dS m^{-1})		3.98 (Saline Soil)

Table 3. Orthogonal array (L_{16}OA) shows the studied factors and their levels

Trials	Biosurfactant producing microorganisms	Sr^{2+} (mg kg^{-1})	Pb^{2+} (mg kg^{-1})
1	Without	0	0
2	A*	3	50
3	A	5	100
4	A	10	150
5	Without	0	50
6	B**	3	0
7	B	5	150
8	B	10	100
9	Without	0	100
10	C***	3	150
11	C	5	0
12	C	10	50
13	Without	0	150
14	A+B+C	3	100
15	A+B+C	5	50
16	A+B+C	10	0

* *P. lubricantisa*
** *L. novalis*
*** Crude biosurfactant extracted from *A. niger*

Plasma (ICAP 6500 Duo, Thermo Scientific England). The physical and chemical properties of the studied soils are listed in Table (2) according to (Jackson, M. L. 1973).

Taguchi approach experiments

In this experimental work, the experiential design was carried out using Taguchi approach (L_{16}OA), in which three factors at five and four levels were studied. Watercress (Eruca vesicaria subsp. Sativa) was grown for 45day in pots, in which each pot was filled with I kg soil. The first factor was biosurfactant producing microorganisms (without additions, *Pseudochrobactrum lubricantisa* (A), *Lysobacter novalis* (B), and crude biosurfactant extracted from *Aspergillus niger* (C), and mixed addation (A+B+C). The second factor was Sr^{2+} doses (0, 3, 5, and 10 mg kg^{-1}). The third factor was

Pb^{2+} doses (0, 50, 100, and 150 mg kg^{-1}). More details about Taguchi approach may be found in the following studies (Abou-Shady and Peng, 2012; Jin *et al*., 2012; Abou-Shady *et al*., 2012a, Abou-Shady *et al*., 2016b). The sixteen trials and the studied factors and there levels, are presented in Table (3). The sequential extraction of Pb^{2+} was determined according to (Tessier *et al*., 1979). The calculation of S/N (signal-to-noise) ratio was carried out based on the optimized values derived from (the larger–the better) according to the following equation:

$$\text{The larger–the better (MSD)} = -10 \log_{10}\left(\frac{1}{n}\sum_{j=1}^{n}\frac{1}{SP_j^2}\right)$$

where MSD is the mean square deviation, n is the number of observations, and SP_j^2 is the experimental response (Abou-Shady *et al*., 2012a).

The experimental responses that illustrated the effects of biosurfactant producing bacteria, Sr^{2+} doses, and Pb^{2+} doses on different forms of Pb^{2+} and the NPK uptake by plant were calculated according to the following equations:

$$\text{The variation of } Pb^{2+}(\%) = \frac{\text{The detected value of different form of } Pb^{2+}}{\text{Total concentration of } Pb^{2+} \text{ in each trial}} * 100$$

$$\text{The variation of NPK (\%) uptake by plant (watercress)} = \frac{(\text{Total concentration of (NPK) in blank experiment} - \text{The detected value of (NPK) in each trial})}{\text{Total concentration of (NPK) in blank experiment}} * 100$$

RESULTS AND DISCUSSION

Screening for biosurfactant substances

Fifteen morphologically distinct microbial colonies were isolated from three different farms aged for 20, 40, and 60 years at El-Gabal El-Asfar area, Egypt, and screened for the most effective biosurfactant producing isolate. The activity of microbial colonies to produce biosurfactant were determined using different methods namely oil spreading assay, blood hemolysis test, blue agar test, and emulsification activity as it listed in Table 4. Oil spreading method represent an easy and sensitive method that can be used to test the production of biosurfactant. This technique measures the diameter of displacement caused when a drop of a biosurfactant containing solution is placed on an oil water surface (Morikawa *et al*., 2000). The ability of isolates to lyses the erythrocytes when it grown on blood agar media indicated the production of biosurfactant. The hemolytic activity of biosurfactant was first discovered by (Bernheimer and Avigad, 1970). Blood agar lysis has been used to quantify surfactant (Moran *et al*., 200°) and rhamnolipids (Johnson and Boese-Marrazzo, 1980). Dark blue halo zone appeared around the isolated colonies in the methylene blue agar plate confirmed the presence of an anionic biosurfactant. This colorimetric assay based on the formation of insoluble ion pair between anionic surfactants, cationic cetyl trimethyl ammonium bromide (CTAB), and methylene blue (Siegmund and Wagner, 1991). Emulsification test represents a quantitative method to assay the potency of isolates to produce biosurfactant. The isolated bacteria were tested for their abilities to emulsify crude oil by adding 2 ml of olive oil to 2 ml of culture supernatant and kept overnight. The emulsification activities were calculated in terms of percentage as it shown in Table (4).

Table 4. Screening of biosurfactant producing microorganisms

Isolate No.	Blood hemolysis	Displacement zone (mm)	Methylene blue agar plate	E24
GA1	β hemolysis	3.0	positive	57.1
GA2	α-hemolysis	2.0	negative	42.9
GA3	β hemolysis	3.0	positive	66.7
GA4	β hemolysis	3.0	positive	60.0
GA5	α-hemolysis	3.0	negative	66.7
GA6	α-hemolysis	3.0	negative	66.7
GA7	β hemolysis	4.0	positive	66.7
GA8	β hemolysis	3.0	positive	50.0
GA9	β hemolysis	9.0	positive	85.0
GA10	β hemolysis	7.0	positive	80.0
GA11	β hemolysis	3.0	positive	56.7
GA12	α-hemolysis	2.0	positive	52.0
GA13	α-hemolysis	3.0	negative	60.0
GA14	β hemolysis	5.0	negative	72.0
GA15	β hemolysis	7.0	positive	84.0

The results showed that the isolates namely GA9, GA10 and GA15 gave the maximum emulsification activity (85, 80, and 84 %) and the highest displacement zone of oil (9, 7, and 7 mm), respectively. Thus they were chosen for further studies.

Identification and phylogenetic analysis

The morphological and biochemical characters of selected bacterial isolates are presented in Table (5). Isolate GA15 is a Gram negative, aerobic, rod-shaped, motile and had peige color and the 16S rRNA nucleotide sequence revealed that it belongs to *L. novalis* with 96% blast identity, while isolate GA9 is Gram negative bacillus or short rods, the colonies were creamy. The 16S rRNA nucleotide sequence revealed that it belonged to *P. lubricantisa* with 95% blast identity. Phylogenetic tree of the two bacterial strains and closely related strains indicated the grouping of isolate GA9 with *P. lubricantisa* with bootstrap value of 53% and GA15 was grouped with *L. novalis* with 100% bootstrap value as it illustrated in Figs. (1 and 2).

Table. 5. The morphological and biochemical characters of selected bacterial isolates

Characteristics	Bacterial isolates	
	GA9	GA15
Gram staining	-ve	-ve
shape	Rod	Rod
Motility test	Non-motile	motile
Spore forming	Non-spore forming	Non-spore forming
Indole test	+ve	+ve
Methyl red test	-ve	-ve
Starch hydrolysis	+ve	+ve
Gelatin liquefaction test	+ve	+ve
Oxidase test	+ve	+ve
Catalase test	+ve	+ve

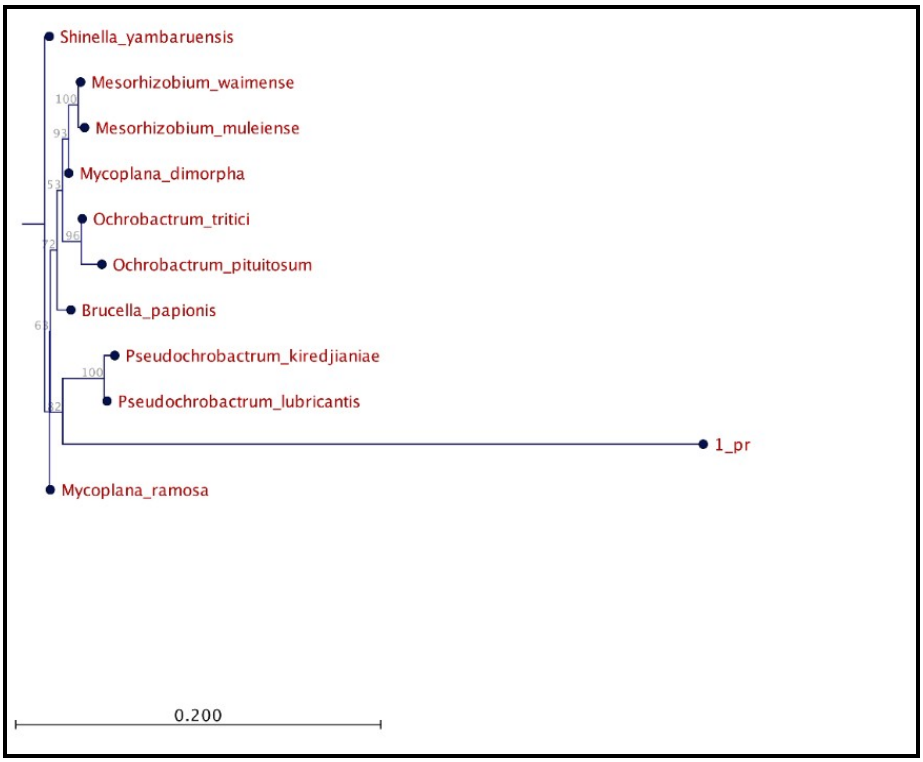

Fig. 1. Neighbor joining phylogenetic tree of partial 16S rRNA sequence of isolates GA9. The scale bar represents 10% nucleotide substitutions. Percentages of bootstrap values recovered from 100 trees are presented on the nodes

Fig. 2. Neighbor joining phylogenetic tree of partial 16S rRNA sequence of isolates GA15. The scale bar represents 10% nucleotide substitutions. Percentages of bootstrap values recovered from 100 trees are presented on the nodes

The fungal isolate GA10 was found to belong to *A. niger*. It showed fast growing black colonies on Czapek Dox agar medium. Surface is charcoal black, granular, flat and reverse side is colorless to white. Microscopic characters of fungal isolate were investigated under light microscope. The conidiophores are long, smooth, colorless or brown and vesicle is large and round.

Leaching experiments

The main goal of this part was to choose the appropriate pollutant in which biosurfactant producing bacteria may decrease it availability in soil. Afterward, Taguchi approach will be used to preciously identify the effects of different parameters. The effects of isolated bacteria on the leaching of artificially polluted soils with Pb^{2+} and Sr^{2+} at constant concentrations of 150 mg kg^{-1} and 10 mg kg^{-1} were studied as it shown in Figs. (3 and 4). Fig. (3) shows the increased and decreased percent of Pb^{2+} and Sr^{2+} compared with the out let leachate of the untreated soils.

The application of biosurfactant producing bacteria significantly decreased the mobility of Pb^{2+} in the polluted soil and in contrast increased Sr^{2+} concentrations in the outlet leachate. The most effective bacteria that increased the precipitation/ adsorption of Pb^{2+} was *L. novalis* bacteria in which the reduction in Pb^{2+} concentration reached to > 69 % compared with untreated soil. The order of biosurfactant producing bacteria that decreased the availability of Pb^{2+} in polluted soils took the following sequence *P. lubricantis* bacteria (B) > the mixture addition of (A+B+C) > *L. novalis* bacteria (A) > *A. niger* (crude biosurfactant) (C). When three species of bio-treatment mixed (A+B+C) together the response of Pb^{2+} in the treaded soil took the same tendency observed with *L. novalis* bacteria (B). Accordingly, Biosurfactant producing bacteria may be used as immobilize material to decrease plant uptake of Pb^{2+} in El-Gabal El-Asfar area. Hogan *et al*., reported that Pb^{2+} strongly bonded with biosurfactant compared with moderate bond that was obtained with other rare elements such as Ca^{2+} and Cd^{2+}, and weak bond with Sr^{2+}, Ni^{2+}, and Mn^{2+}. The biosurfactant may be adsorbed strongly with Pb^{2+} simultaneously with soil particles. So, that in our experiment Pb^{2+} did not leached out compared with Sr^{2+}. Hong *et al*., 1997 studied the removal percent of Cd^{2+} and Pb^{2+} using aescin as a biosurfactant and reported that the carboxylic and saccharide moieties had the main role to isolate these elements during soil washing.

Fig. (4) presents the effect of biosurfactant producing bacteria on Sr^{2+} mobility in the treated soils. The results indicated that, Sr^{2+} levels significantly increased in the outlet leachate with very high percent reached to 803% (8 fold from Sr^{2+} concentration obtained with the untreated leachate) when *P. lubricantis* bacteria (A) was added to the treated soil.

The impacts of either *L. novalis* bacteria (B) or *A. niger* (crude biosurfactant) (C) were almost the same

about 580% (5.8 fold from Sr^{2+} concentration obtained with the untreated leachate) as it depicted in Fig. (4). Blending the three *P. lubricantis* bacteria (A), *L. novalis* bacteria (B), and *A. niger* (crude biosurfactant) (C) together increased the concentrations of Sr^{2+} in the outlet leachate higher than *L. novalis* bacteria (B) and *A.*

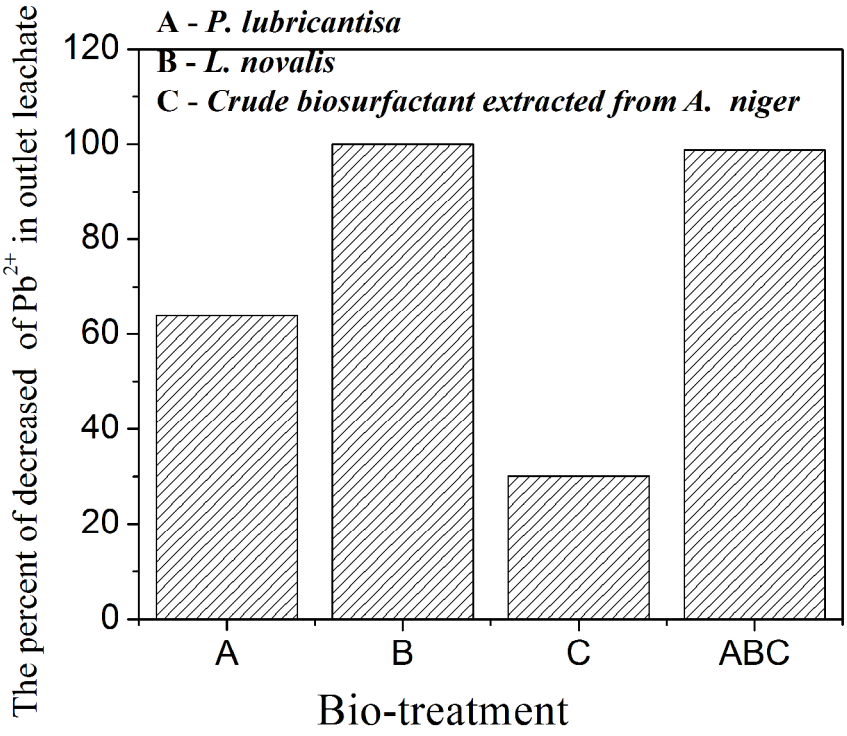

Fig. 3. The variation of outlet Pb^{2+} containing leachate compared with blank experiments

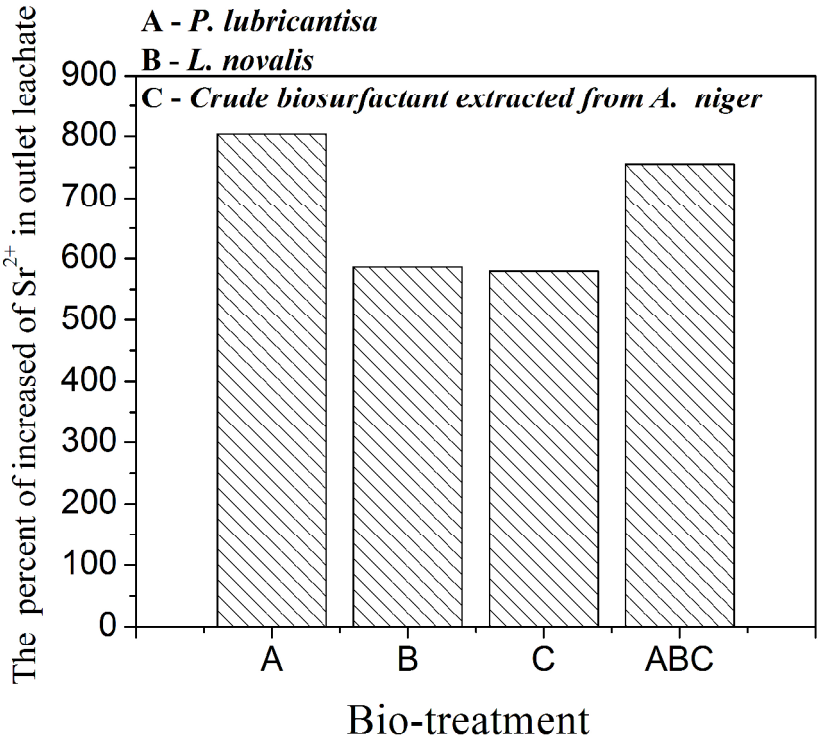

Fig. 4. The variation of outlet Sr^{2+} containing leachate compared with blank experiments

niger (crude biosurfactant) (C), but less than *P. lubricantis* bacteria (A). According to data observed from column leaching experiments, Pb^{2+} was chosen as the most alternative polluted element to study the effect of biosurfactant producing bacteria of Pb^{2+} uptake by plant using Taguchi approach.

Taguchi approach experiments

Taguchi approach (L_{16}OA) was utilized in which 16 trials were carried out in lab pots. The experiment response and it signal to noise ratio (S/N) are presented in Table (6). Figure. (5) shows the effect of biosurfactant producing bacteria on the variation of Pb^{2+} species in the treated soils, and the results are opposite with that obtained in the leaching experiments Fig. (3). The contradictory of results observed in the leaching experiment and Taguchi approach may be owing to that in Taguchi approach Pb^{2+} behaviors were studied based on different Pb^{2+} forms derived from sequential extraction.

L. novalis bacteria (B) and *A. niger* (crude biosurfactant) (C), and mixed addition (A+B+C) did not show any noticeable effect on the reduction of available Pb^{2+}. *L. novalis* bacteria (B) showed the best results to decrease the exchangeable form of Pb^{2+} compared with other species of bacteria. The addition of mixture addition (A+B+C) presented an increment of sulfate form of Pb^{2+} compared with the sole addition of bacteria. *A. niger* (crude biosurfactant) (C) increased the carbonate bonded to Pb^{2+}. Pb^{2+} existed in organic forms was influenced with the addition of mixture addition (A+B+C). The addition of *A. niger* (crude biosurfactant) (C) and blank experiments showed a stable concentrations of Pb^{2+} content in plant.

The addition of *P. lubricantis* (A) and *L. novalis* (B) resulted in an increment of Pb^{2+} content in plant as it presented in Fig. (5).

Fig. (6) shows the effects of Sr^{2+} doses on different forms of Pb^{2+} simultaneously with the addition of biosurfactant producing bacteria. The available Pb^{2+} oscillated with the addition of Sr^{2+}. In the blank experiment, the available Pb^{2+} was found with comparatively high concentrations more that found when Sr^{2+} doses were added. On the other hand, increasing Sr^{2+} concentrations decreased the content of exchangeable Pb^{2+}, however at high concentrations of 150 mg kg^{-1} the exchangeable content was increased again to be close to that found in the blank experiment. The carbonated content of Pb^{2+} did not influence with either low or high concentrations of Sr^{2+} (0, 3, and 10 mg kg^{-1}). It's clear seeing that, the medium concentration of Sr^{2+} increased Pb^{2+} carbonated content of. Pb^{2+} bonded to sulfate did not influence with the low concentration of Sr^{2+}, however at high concentrations it was found that, Pb^{2+} bonded to sulfate was increased noticeably. Pb^{2+} bonded to organic compounds was greatly affected with the low concentrations of Sr^{2+} (3 mg kg^{-1}), the rest of concentration did not show any noticeable effect. Total concentration of Pb^{2+} in plant increased with increasing Sr^{2+} doses.

Table 6. The variations of different forms of Pb^{2+} (%) and its calculated (S/N) in the treated soil

Available		Exchangeable		Carbonate bond		Sulfate bond		Organic bond		Plant	
Pb^{2+} (%)	S/N ratio	Pb^{2+} (%)	S/N ratio	Pb^{2+} (%)	S/N ratio	Pb^{2+} (%)	S/N ratio	Pb^{2+} (%)	S/N ratio	Pb^{2+} (%)	S/N ratio
257.98	48.23	1.98	5.95	4.70	13.45	4.12	12.30	21.89	26.80	15.16	23.61
24.99	27.95	0.0006	-64.30	0.64	-3.89	1.41	2.97	0.03	-30.99	73.52	37.33
31.38	29.93	0.32	-9.78	3.17	10.03	3.38	10.57	3.35	10.49	47.87	33.60
0.53	-5.54	0.59	-4.54	4.67	13.39	3.86	11.73	3.36	10.52	76.47	37.67
39.57	31.95	0.0012	-58.06	0.53	-5.49	2.65	8.46	3.96	11.94	60.31	35.61
30.00	29.54	0.0015	-56.76	0.45	-6.92	1.86	5.40	2.44	7.74	82.64	38.34
23.75	27.51	0.0012	-58.65	0.47	-6.50	1.96	5.84	0.84	-1.55	52.38	34.38
39.28	31.88	0.21	-13.59	0.00	-63.45	1.11	0.92	1.33	2.45	47.57	33.55
57.19	35.15	0.20	-14.11	0.0009	-61.21	2.67	8.52	2.54	8.10	11.36	21.11
38.19	31.64	0.12	-18.61	0.0014	-57.38	1.85	5.35	1.49	3.44	13.26	22.45
53.13	34.51	0.0042	-47.59	0.74	-2.62	7.29	17.25	6.76	16.59	84.83	38.57
44.56	32.98	0.29	-10.62	0.00	-54.88	2.17	6.73	2.69	8.58	28.87	29.21
29.06	29.26	0.23	-12.66	2.12	6.52	1.42	3.05	1.51	3.58	33.91	30.61
8.05	18.12	0.28	-11.10	1.65	4.34	2.56	8.16	0.00	-58.07	53.21	34.52
24.43	27.76	0.01	-43.87	1.49	3.48	3.29	10.34	2.00	6.03	59.31	35.46
269.70	48.62	0.03	-31.84	6.87	16.74	47.35	33.51	32.60	30.26	59.75	35.53

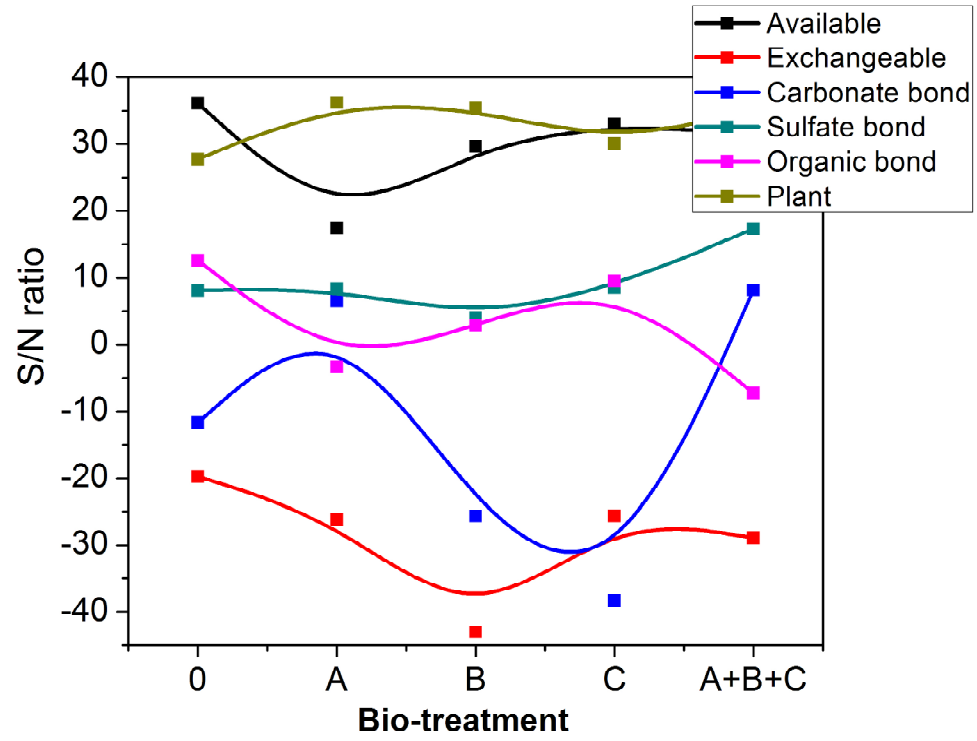

Fig. 5. Effect of biosurfactant producing bacteria on the variation of Pb^{2+} species in the treated soils (S/N is signal to noise ratio)

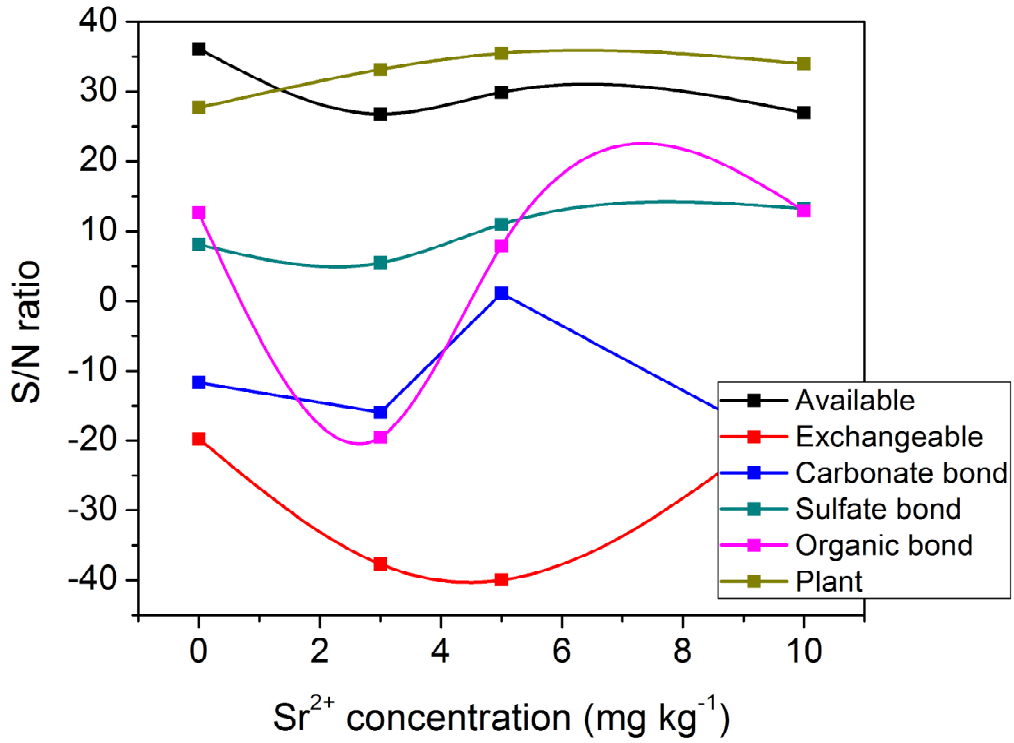

Fig. 6. Effect of Sr^{2+} doses on the variation of Pb^{2+} species in the treated soils (S/N is signal to noise ratio)

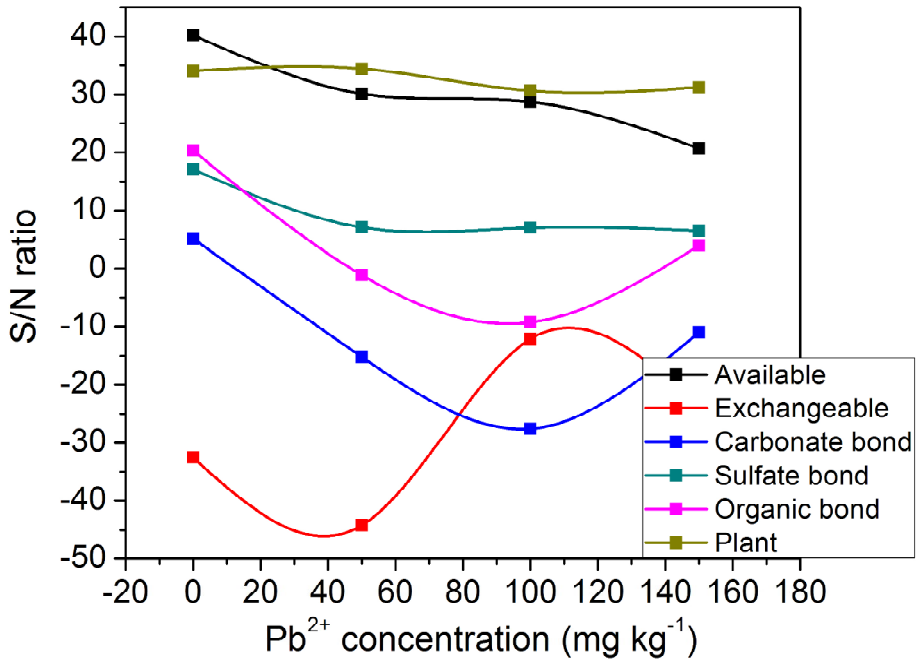

Fig. 7. Effect of Pb^{2+} doses on the variation of Pb^{2+} species in the treated soils (S/N is signal to noise ratio)

The effects of Pb^{2+} concentrations on variations of different forms are presented in Fig. (7). Increasing the addition of Pb^{2+} decreased the percentage of available form in the treated soil. This is may be due to great immobilization impact of biosurfactant producing bacteria. However, the exchangeable forms of Pb^{2+} were quite high at Pb^{2+} concentrations (100) mg kg^{-1}. Addition of Pb^{2+} with low concentration of 50 mg kg^{-1} or high concentrations of 150 mg kg^{-1} did not increase the concentrations of Pb^{2+} in exchangeable form. Pb^{2+} bonded to carbonate was decreased when Pb^{2+} doses were added at concentration of 50 and 100 mg kg^{-1}. On the other hand, the higher concentrations of Pb^{2+} (150 mg kg^{-1}) increased again Pb^{2+} carbonate content.

Pb^{2+} bonded to sulfate was quite high with the blank experiment. Pb^{2+} bonded to sulfate did not influence with different concentration varied from 50 to 150 mg kg^{-1}. Pb^{2+} existed in organic forms were almost the same at concentrations of 50 and 150 mg kg^{-1}, however at concentration equal to 100 mg kg^{-1} the formation of Pb^{2+}

bonded to organic was noticeability decreased. The highest values of Pb^{2+} bonded to organic form was observed with the naturally exited concentration. Finally, plant uptake of Pb^{2+} did not influence with the wide range of Pb^{2+} concentrations. Pb^{2+} concentrations in the outlet leachate in the 16 trials did not detect using Atomic Absorption Spectrophotometer.

Figure. (8) shows that, the most affected factors influenced the available form of Pb^{2+} in studied trials were Pb^{2+} doses > biosurfactant producing bacteria > Sr^{2+} doses, however for exchangeable form the most influenced factors took the following order biosurfactant producing bacteria > Sr^{2+} doses > Pb^{2+} doses. Pb^{2+} bonded to carbonate was influenced with biosurfactant producing bacteria more than Pb^{2+} and Sr^{2+} concentrations. For Pb^{2+} bonded to sulfate the most influential factor was biosurfactant producing bacteria followed with Pb^{2+} doses and Sr^{2+} doses. The most factors affected the content of Pb^{2+} in organic form was Pb^{2+} doses > biosurfactant producing bacteria > Sr^{2+} doses. Finally, the total concentrations of Pb^{2+} in plant were affected with the associated of biosurfactant producing bacteria followed with Sr^{2+} doses and Pb^{2+} doses. In general, our results are not in harmony with Herman *et al*., 1995 in which (a rhamnolipid) biosurfactant was isolated from *Pesudomonas aeruginosa*, and investigated to remove Cd^{2+}, Pb^{2+}, and Zn^{2+} containing polluted soils. The addition of a rhamnolipid improved the desorption of Pb^{2+} by 43% compared with ion exchange treatments. In general, the effect of associated elements on the behavior of Pb^{2+} containing soil during the treatment with biosurfactant was unclear (Hogan *et al*., 2017).

The analysis of variance derived from Taguchi approach are listed in Tables (7-12). The obtained F-factors values from the recommended tables were 3.15 and 4.78 at α (risk) levels of 0.05 and 0.01, respectively. The obtained results showd that, the effect of Pb^{2+} doses were significant at α (risk) 0.01 towards the available

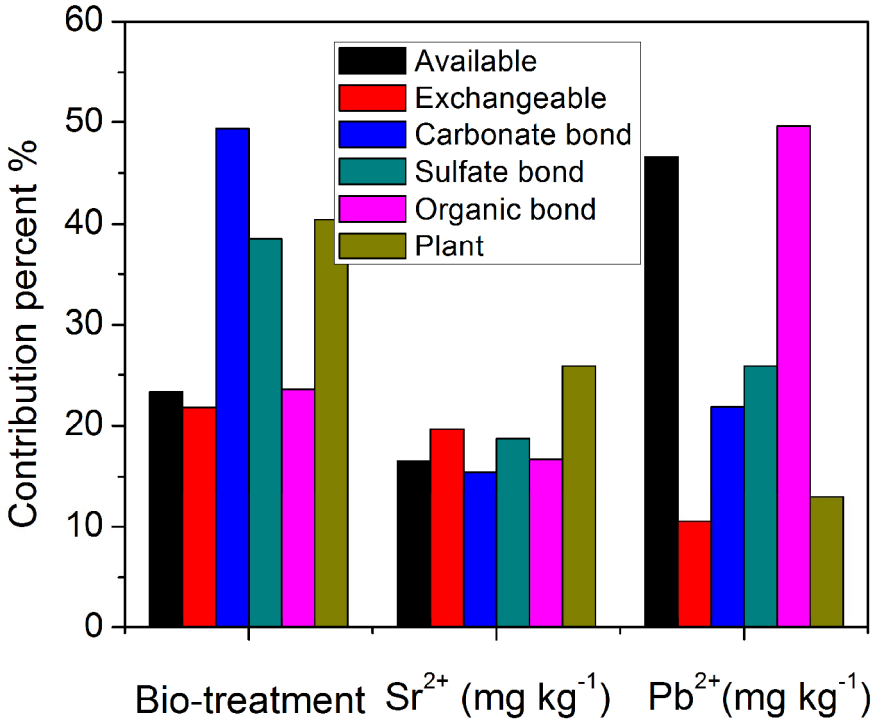

Fig. 8. The contribution percent of biosurfactant producing bacteria, Sr^{2+} doses, and Pb^{2+} doses towards different Pb^{2+} forms

Table 7. ANOVA analysis shows the significant effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses on the available form of Pb^{2+}

Factors	DOF	SS	V	F	P
Bio-treatment	4	22714.98	5678.745	2.142372	23.29902
Sr^{2+}	3	16105.86	5368.619	2.025374	16.51997
Pb^{2+}	3	45419.03	15139.68	5.711619	46.58684
Error	5	13253.4	2650.681		
Total	15	97493.27			

Table 8. ANOVA analysis shows the significant effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses on the exchangeable form of Pb^{2+}

Factors	DOF	SS	V	F	P
Bio-treatment	4	0.776294	0.194073	0.566078	21.77217
Sr^{2+}	3	0.699999	0.233333	0.680591	19.63238
Pb^{2+}	3	0.375047	0.125016	0.364649	10.51868
Error	5	1.714193	0.342839		
Total	15	3.565532			

Table 9. ANOVA analysis shows the significant effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses on Pb^{2+} bond to carbonate

Factors	DOF	SS	V	F	P
Bio-treatment	4	32.05967	8.014917	4.610017	49.34581
Sr^{2+}	3	10.03208	3.344028	1.923417	15.44125
Pb^{2+}	3	14.18469	4.72823	2.719581	21.83288
Error	5	8.692937	1.738587		
Total	15	64.96938			

Table 10. ANOVA analysis shows the significant effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses on Pb^{2+} bond to sulfate

Factors	DOF	SS	V	F	P
Bio-treatment	4	731.4075	182.8519	2.873391	38.56897
Sr^{2+}	3	355.4714	118.4905	1.861996	18.74491
Pb^{2+}	3	491.3024	163.7675	2.573492	25.90762
Error	5	318.1814	63.63628		
Total	15	1896.363			

Table 11.ANOVA analysis shows the significant effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses on Pb^{2+} bond to organic materials

Factors	DOF	SS	V	F	P
Bio-treatment	4	279.4234	69.85586	2.911508	23.56911
Sr^{2+}	3	198.1329	66.04429	2.752647	16.71233
Pb^{2+}	3	588.0279	196.0093	8.169431	49.59961
Error	5	119.9651	23.99302		
Total	15	1185.549			

Table 12. ANOVA analysis shows the significant effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses on Pb^{2+} uptake by plant

Factors	DOF	SS	V	F	P
Bio-treatment	4	3480.978	870.2445	2.440584	40.43463
Sr^{2+}	3	2231.518	743.8392	2.086083	25.92104
Pb^{2+}	3	1113.546	371.1821	1.040973	12.93482
Error	5	1782.861	356.5723		
Total	15	8608.903			

form of Pb^{2+}. The effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses on the exchangeable form of Pb^{2+} are not significant towards the exchangeable form of Pb^{2+}. The effect of bacteria species was significant at α (risk) 0.05 towards Pb^{2+} bond to carbonate. The effects of Sr^{2+} doses, and Pb^{2+} doses were insignificant towards Pb^{2+} uptake by plant and Pb^{2+} bonded to sulfate. The effects of Pb^{2+} concentrations were significant at α (risk) 0.01 towards Pb^{2+} bond to organic materials.

Effect of biosurfactant producing bacteria on NPK uptake by watercress determined by Taguchi approach

The effects of biosurfactant producing bacteria, Pb^{2+} doses and Sr^{2+} doses on nitrogen (N), phosphorous (P), and potassium (K) uptake by watercress is presented in Fig. (9). Table (13) lists the variations of NPK (%) and its calculated (S/N) in the treated soil. Data presented in Fig. (9) shows the effect of surfactant producing bacteria on NPK uptake by watercress . The effect of biosurfactant producing bacteria, Pb^{2+} doses and Sr^{2+} doses did not show any noticeable effect on nitrogen uptake by watercress and the obtained result were almost the same with that obtained in the blank experiments.

The *P. lubricantis* bacteria did not affect the phosphorous uptake by watercress and showed the same tendency observed with the blank experiments. However, *L. novalis* bacteria and *A. niger* (crude biosurfactant) decreased phosphorous uptake by watercress. The mixture addition (A+B+C) improved the phosphorous uptake by watercress again.

Fig. (9) illustrates that potassium uptake by watercress decreased compared with the blank experiment. However, the mixture addition showed the same tendency observed with phosphorous in which the mixture addition enhanced potassium uptake by watercress. Hogan *et al*., 2017 reported that K^{+} and Mg^{2+} were weakly bonded with biosurfactant (rhamnolipid) compared with Ca^{2+} and Fe^{3+}.

Data illustrated in Fig. (10) presents the effects of Sr^{2+} doses on the NPK uptake by watercress determined by Taguchi approach. Different doses of Sr^{2+} did not markedly show any impact on the nitrogen uptake by watercress. Increasing Sr^{2+} doses at 3 mg kg^{-1} and 5 mg kg^{-1} decreased phosphorous uptake by watercress, however at higher concentrations of 10 mg kg^{-1} the phosphorous uptake by watercress increased again. Potassium uptake by watercress was significantly decreased with deferent doses of Sr^{2+} varied from 0-10 mg kg^{-1}. The effects of Pb^{2+} doses on NPK uptake by watercress are presented in Fig. 11. The obtained results indicated that the same tendency obtained with biosurfactant producing bacteria and Sr^{2+} doses for nitrogen uptake by watercress was observed, in which there was no substantial variation in nitrogen uptake with different Pb^{2+} doses. The phosphorous uptake by

watercress was increased when Pb^{2+} doses were increased from 50 mg kg^{-1} to 100 mg kg^{-1}.

Table 13.The variations of NPK uptake by watercress (%) and its calculated (S/N) in the treated soil

Nitrogen		Phosphorous		Potassium	
N (%)	S/N ratio	P (%)	S/N ratio	K (%)	S/N ratio
65.63	36.34	15.47	23.79	33.27	30.44
68.75	36.75	36.21	31.18	22.48	27.04
68.75	36.75	44.50	32.97	26.61	28.50
75.00	37.5	34.65	30.79	31.25	29.90
78.13	37.86	44.66	33.00	43.95	32.86
68.75	36.75	34.13	30.66	23.49	27.42
68.75	36.75	18.65	25.41	37.80	31.55
78.13	37.86	46.01	33.26	23.49	27.42
75.00	37.50	49.61	33.91	49.19	33.84
78.13	37.86	35.90	31.10	38.21	31.64
75.00	37.50	30.59	29.71	25.00	27.96
71.88	37.13	22.77	27.15	19.15	25.64
68.75	36.75	50.02	33.98	59.07	35.43
68.75	36.75	21.15	26.51	25.71	28.20
81.25	38.20	35.80	31.08	26.11	28.34
75.00	37.50	62.27	35.89	63.10	36.00

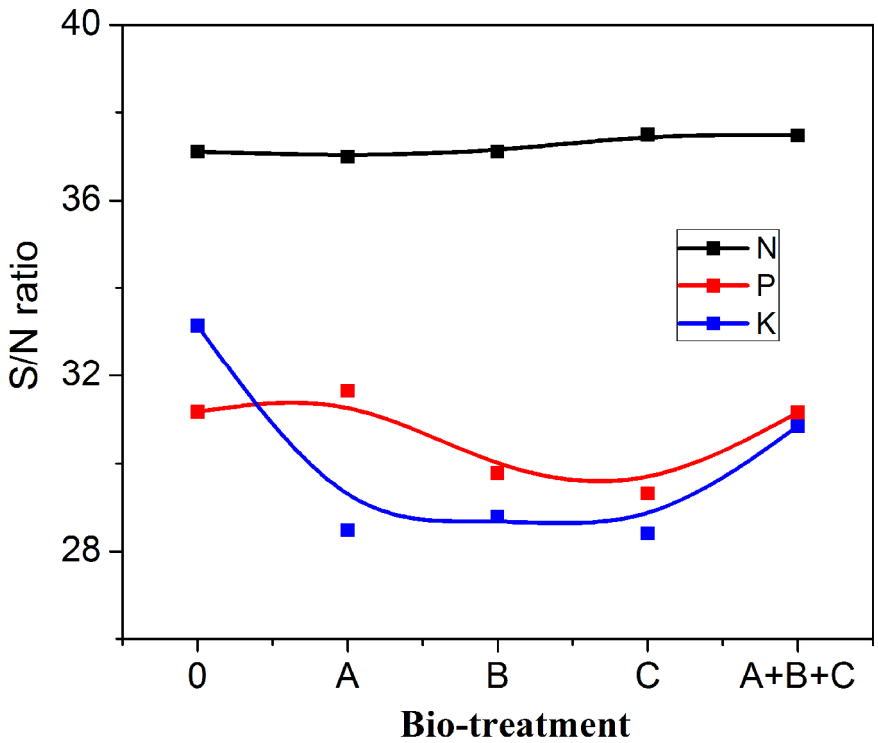

Fig. 9. Effect of biosurfactant producing bacteria on the NPK uptake by watercress (S/N is signal to noise ratio)

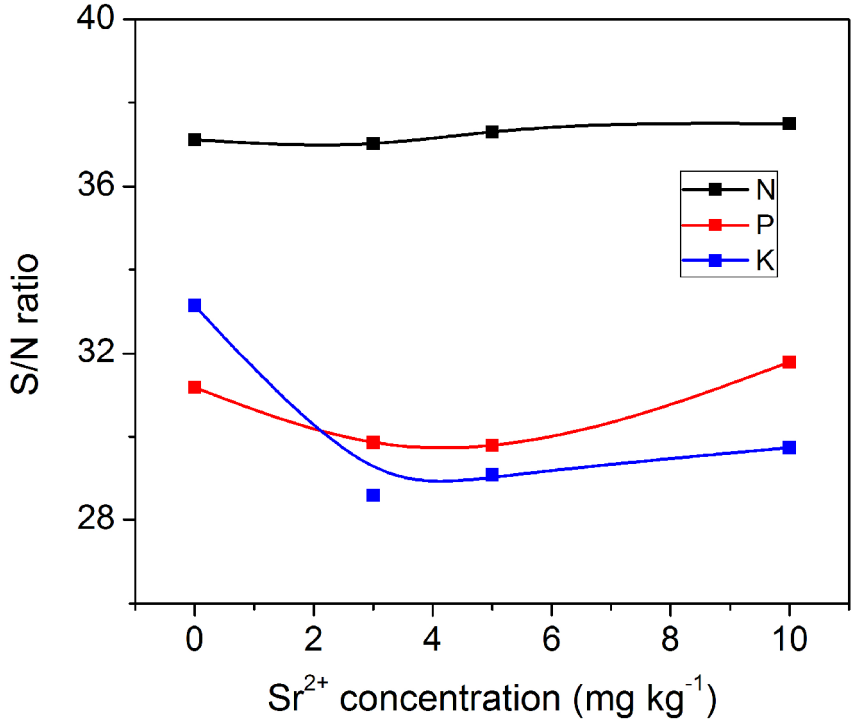

Fig. 10. Effect of Sr^{2+} doses on NPK uptake by watercress (S/N is signal to noise ratio)

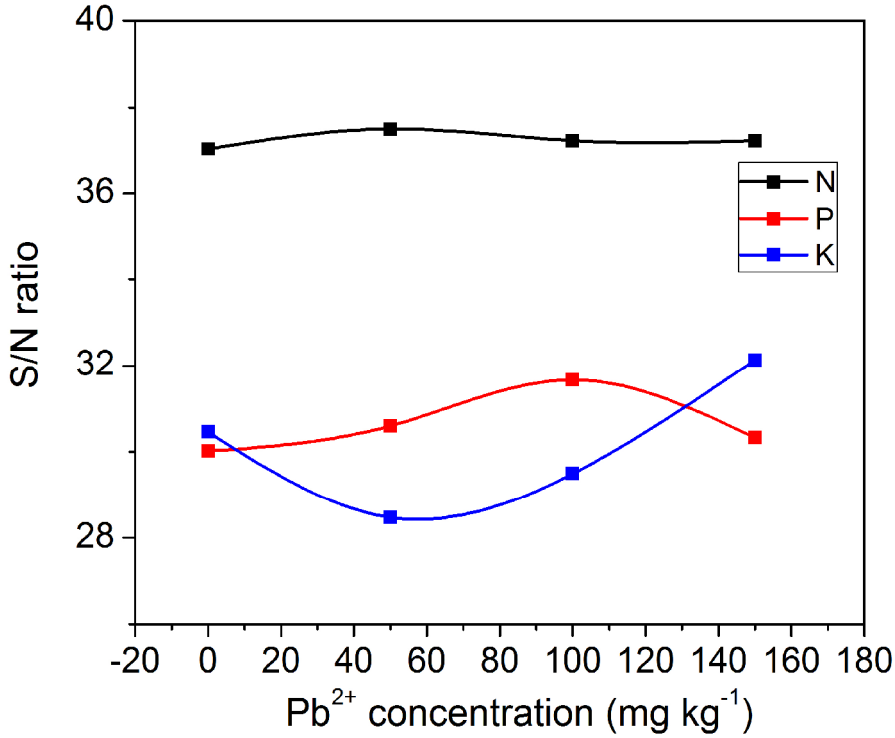

Fig. 11. Effect of Pb^{2+} doses on NPK uptake by watercress (S/N is signal to noise ratio)

At high concentrations of Pb^{2+} doses (150 mg kg^{-1}) the phosphorous uptake by watercress was reduced to be close with what was observed at Pb^{2+} doses of 50 mg kg^{-1}. The potassium uptake by watercress was deceased when Pb^{2+} doses increased from 50 mg kg^{-1} to 100 mg kg^{-1}. Noticeably, potassium uptake by watercress was

quit high at 150 mg kg^{-1} of Pb^{2+} doses. Fig. (12) shows the contribution percent of biosurfactant producing bacteria, Sr^{2+} concentrations, and Pb^{2+} concentrations towards NPK uptake by watercress determined by Taguchi approach. The most influential factors affected NPK uptake by watercress were found to take the following sequence biosurfactant producing bacteria > Sr^{2+} doses > Pb^{2+} doses. Tables (14-16) show the analysis of variance (ANOVA) for the effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses. The exchangeable form of Pb^{2+} were not significant towards nitrogen and phosphorous uptake by watercress. The effects of mixture addition (A+B+C) and Sr^{2+} doses on potassium uptake by watercress were significant at α (risk) 0.01.

It's clear seen from Figs. (8 and 12) that the contribution percent of biosurfactant producing bacteria was the highest during Pb^{2+} uptake by watercress and (N, P, and K) uptake by plant compared with Sr^{2+} and Pb^{2+} doses. Based on literature review, the effects of biosurfactant on plant morphology and physiology aspects are absent. Ongoing researches are intended to investigate the impact of surfactant substances produced from either naturally or artificially sources and the results will be published soon. Regarding the effect of naturally produced surfactant on Sr^{2+} availability, we believe that the high mobility of Sr^{2+} can be exploited as an assistant tool during soil electrokinetic remediation using perforated cathode pipe SEKR (PCPSS). The outlet discharge will be treated simultaneously using electrolysis, electrodialysis, electrodeionization, and adsorption as it previously discussed in our previous work and the obtained date will be published soon (Bi *et al*., 2011, Almeria *et al*., 2012a; Almeria *et al*., 2012b; Abou-Shady *et al*., 2012a; Abou-Shady *et al*., 2012b; Peng *et al*., 2013; Abou-Shady *et al*. 2018).

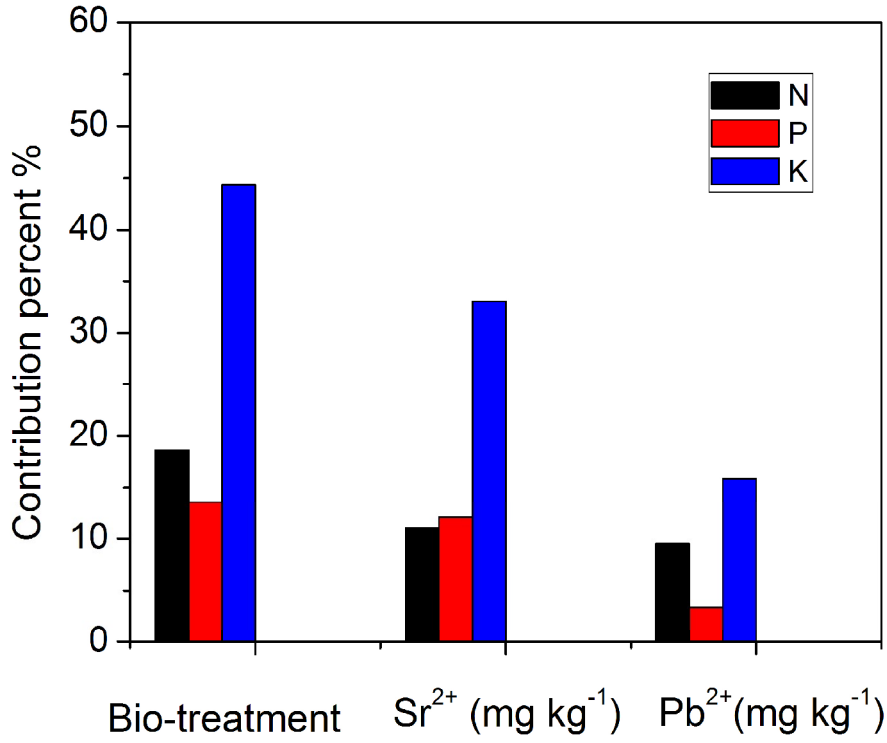

Fig. 12. The contribution percent of biosurfactant producing bacteria, Sr^{2+} concentrations, and Pb^{2+} concentrations towards NPK uptake by watercress

Table 14. ANOVA analysis shows the significant effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses on nitrogen uptake by watercress

Factors	DOF	SS	V	F	P
Bio-treatment	4	60.76389	15.19097	0.382383	18.60852
Sr^{2+}	3	36.01074	12.00358	0.302151	11.02804
Pb^{2+}	3	31.12793	10.37598	0.261181	9.53271
Error	5	198.6355	39.72711		
Total	15	326.5381			

Table 15. ANOVA analysis shows the significant effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses on phosphorous uptake by watercress

Factors	DOF	SS	V	F	P
Bio-treatment	4	335.451	83.86274	0.239354	13.5848
Sr^{2+}	3	298.4892	99.49641	0.283974	12.08796
Pb^{2+}	3	83.51467	27.83822	0.079454	3.382104
Error	5	1751.856	350.3712		
Total	15	2469.311			

Table 16. ANOVA analysis shows the significant effects of bacteria species, Sr^{2+} doses, and Pb^{2+} doses on potassium uptake by watercress

Factors	DOF	SS	V	F	P
Bio-treatment	4	1191.675	297.9187	8.250617	44.37122
Sr^{2+}	3	886.8599	295.62	8.186954	33.02163
Pb^{2+}	3	426.6154	142.2051	3.938255	15.88474
Error	5	180.5433	36.10866		
Total	15	2685.694			

CONCLUSIONS

The effects of biosurfactant producing microorganisms on heavy metal containing polluted soils were studied in terms of leaching columns and agriculture experiments designed using Taguchi approach (L_{16}OA). The results showed that, the biosurfactant producing bacteria controlled the mobility of Pb^{2+} and Sr^{2+}in the treated soils. Pb^{2+} was adsorbed/ precipitated in the treated soils and in contrast Sr^{2+}. Results obtained from Taguchi approach indicated that, the mechanisms of biosurfactant producing bacteria on Pb^{2+} was very complicated and interfusion with each other. Generally, the most factors affected the available form of Pb^{2+} and Pb^{2+} existed in organic form was Pb^{2+} concentrations, however for exchangeable form, carbonate form, sulfate form, and plant content the most influenced factors was biosurfactant producing bacteria. The most influential factors affected NPK uptake by watercress were found to take the following sequence biosurfactant producing bacteria > Sr^{2+} doses > Pb^{2+} doses.

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الملخص العربي

اضافة الكائنات الدقيقة المنتجة للمركبات الحيوية النشطة سطحيا لمعالجة التلوث بالعناصر الثقيلة بمنطقة الجبل الاصفر

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تزايدت فى الاونة الاخيرة تركيزات الملوثات وخصوصا العناصر الثقيلة فى الاراضى الموجودة فى المناطق الجافة ونصف الجافة المجاورة للمناطق الصناعية، حيث ان ظروف الجفاف السائدة اجبرت المزارعين على اعادة استخدام مياة الصرف كبديل متاح لمياة الرى فى الزراعة. تمثل العناصر الثقيلة خطرا لكل من الانسان والحيوان اذا تم امتصاصها بواسطة النباتات المروية اذا انتقلت من الجذور الى الاوراق و باقى النبات. فى هذا البحث تم دراسة تاثير البكتريا المنتجة للمركبات النشطة سطحيا كمصدر رخيص لتقليل ذائبية الرصاص للنباتات المنزرعة فى منطقة الجبل الاصفر – مصر. ذلك لان منطقة الجبل الاصفر تعتبر من المناطق التى تعتمد على مياة الصرف الغير معالجة كمصدر للرى. تم عزل البكتريا المنتجة للمركبات النشطة سطحيا من عدة مزارع بمنطقة الجبل الاصفر، هذة المناطق مزروعة لفترات تتراوح مابين ٢٠ و ٤٠ و ٦٠ سنة وتم تعريفها وهذة البكتريا كالاتى *Pseudochrobactrum lubricantisa* (A), *Lysobacter novalis* (B), and *Aspergillus niger* (C). واوضحت النتائج المتحصل عليها ان اضافة البكتريا المنتجة للمركبات الحيوية النشطة سطحيا قللت من تيسر الرصاص اثناء اجراء تجربة الاعمدة، وعلى العكس فان هذة الاضافات البكترية زادت من ذائبية الاسترانشيم فى هذة الاراضى. اكثر العوامل تاثير على الصورة الميسرة والمرتبطة بالمادة العضوية من الرصاص كانت تركيز الرصاص المضاف. بالنسبة للرصاص المتواجد فى الصور المتبادلة و المرتبط بالكربونات و والمرتبط بالكبريتات و الموجود داخل النبات فان اضافة البكتريا المنتجة للمركبات النشطة سطحيا كانت اكثر العوامل تاثيرا على تزايد تركيزاتها. كما اوضحت النتائج ان اكثر العوامل تاثير على امتصاص النتروجين والفوسفور والبوتاسيوم كانت البكتريا المنتجة للمواد النشطة سطحيا > اضافات الاسترانشيوم > اضافات الرصاص