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## Isolation of polysaccharide producer and heavy metal tolerant local rhizobial isolates

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*Rhizobium*; Isolation;  
Exopolysaccharide  
production; Heavy metal  
tolerance

### Abstract

**Background:** Rhizobial bacteria is an important species among the soil bacteria that inter a relationship with leguminous plant and fix nitrogen symbiotically. Importance of this relation not only for soil as soil fertilizer but also to keep our environment without pollution.

**Methods:** Survey was conducted to collect different strains of rhizobial from different area in Nineveh Governate in Iraq. Isolation and biochemical tests were done under laboratory conditions. Determination of exopolysaccharide and tolerance of heavy metal was conducted also. Data obtained was recorded after cultivation and incubation of rhizobial strains.

**Results:** The rhizobial bacteria were isolated from the following leguminous plants: *Vigna unguiculata* L., *Trifolium alexandrinum*, *Trigonella foenum-graecum* L., *Leucaena leucocephala* L., *Medicago sativa* L., *Phaseolus vulgaris* L., *Tribulus terrestris* L. and *Vicia faba* L. Maximum exopolysaccharide production were reached to 3.70 gm/L by the isolate *R. leguminosarum* bv. *Viciae* RM25, after two days of incubation. The maximum cell dry weight was 2.90 gm/L. by the isolate *E. meliloti* RM14, after two days of incubation. Maximum reduction in pH were 4.30 by strain *E. meliloti* RM5, after two days incubation. All the local isolated rhizobia were tolerated to nickel chloride for the studied concentrations: 100, 500, 1000 and 5000 µg/ml. Also were tolerance to 100 and 500 µg/ml of zinc sulfate and copper sulfate. 1000 µg/ml concentration of zinc sulfate were also tolerated by all rhizobial isolates.

**Conclusion:** Rhizobium bacteria possess several mechanisms that allow them to tolerate heavy metal exposure. These mechanisms include the expression of efflux pumps, the presence of metal-resistance plasmids, the production of EPS, and the ability to adapt to environmental factors. Further research is needed to fully understand the mechanisms behind the heavy metal tolerance in *Rhizobium* and to explore the potential applications of these bacteria in bioremediation of heavy metal polluted soils.



## Introduction

*Rhizobium* is a soil bacterium that forms a symbiotic relationship with leguminous plants. This relationship allows the bacteria to fix atmospheric nitrogen and convert it into a form that can be used by the plant [1]. Heavy metal pollution is becoming increasingly common and poses a threat to the survival of many plant species. However, some strains of rhizobia have been reported to possess the ability to withstand heavy metal exposure [2, 3].

Rhizobial bacteria have been found to accumulate heavy metals such as lead and cadmium in their cells. This suggests that the bacteria are able to tolerate heavy metal exposure by either sequestering it in cellular compartments or by actively pumping it out of the cells. The latter mechanism involves the expression of efflux pumps that are capable of expelling heavy metals from the cell. In *Rhizobium leguminosarum* bv. *Viciae*, a metal resistance determinant has been identified that is responsible for the efflux of heavy metals [4, 5].

Several studies have reported that heavy metal tolerance in *Rhizobium* is associated with the presence of plasmids that carry metal-resistance genes. These genes encode proteins that are involved in metal uptake, sequestration, and detoxification. For example, a study by Lakzain *et al.*, [6] found that a plasmid transfer among isolates of *R. leguminosarum* bv. *Viciae* in heavy metal contaminated soils. Thakur and Singh [7] revealed that the disappearance of symbiotic plasmids is a direct indicator of metal toxicity and absence of nitrogen fixing efficacy of the bacterial strain.

The ability of rhizobia to adapt to heavy metal exposure is also influenced by the growth stage of the bacteria. Sazykin *et al.*, [8] revealed that heavy metals influence the bacterial community of soils. The heavy metal tolerance in *Rhizobium* is also influenced by environmental factors such as pH, temperature, and oxygen availability. In study found that the pH of the growth medium had a significant impact on the metal tolerance of *Rhizobium leguminosarum* bv. *Viciae*. Also, the study found that the bacteria were more tolerant to cadmium and lead at pH 6.5 compared to pH 7.5 and 8.5. This suggests that the pH of the soil can play a role in selecting for metal-tolerant *Rhizobium* strains [9].

Rhizobial bacteria are known to produce exopolysaccharides (EPS) that can play a major role in metal tolerance. EPS are long chains of sugars that can bind to heavy metals and prevent them from entering the cell [10]. In addition, EPS can also act as a barrier that prevents the heavy metals from binding to the cell wall and causing damage [11]. The purpose of this study is to explore the ability to isolate local rhizobial

strains that have the ability to tolerate heavy metal as well as produce exopolysaccharides.

## Methods

### Leguminous plants collection:

Different leguminous plants of as follows: Cowpea, Clover, Fenugreek, Leucaena, Alfalfa, Green bean, Puncturevine and Broad were collected from different farms and area in Nineveh Governorate/Iraq. After collecting leguminous plant species were immediately transported to the laboratory of Microbial Molecular Biology for analysis for isolation and characterization of the strains. Procedure of Vincent [12] were followed to isolate different bacterial isolates from root nodules of collected plants.

### Nodules sampling and rhizobia isolation:

Nodules of leguminous plants were carefully detached from the root system plants and washed gently several times under tap water to remove sterile distilled water to remove soil particles. The nodule surface was sterilized by immersion in 3% NaOCl solution for a period of 2-min, followed by 70% ethanol for 5-min, and six consecutive washing in sterilized distilled water. Each sterilized surface nodule from different leguminous plants was aseptically crushed with a sterile glass rod in a test tube that contained 1 ml sterile normal saline (0.85% NaCl). One loopful of the nodule suspension was streaked on Petri dishes that contained yeast extract mannitol (YEM) agar medium with the following components (gm/mL): mannitol, 10; K<sub>2</sub>HPO<sub>4</sub>, 0.5; NaCl, 0.1, MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2, and agar, 16. Incubation was done for 3-4 days at 30°C. A single colony from each culture was selected and recultured on fresh YEMA plates [13]. The purified cultures were maintained on YEM agar slants, stored at 4°C in refrigerator for further experiments.

### Cultural conditions for exopolysaccharide production:

#### *Rhizobial inoculum preparation:*

The inoculum of rhizobial strains were prepared with transfer a loopful of rhizobial bacteria growth from trypton yeast solid plates to 100 ml conical flask containing 20 ml of YEM liquid medium with 6.8 pH. Incubation was done in shaker incubator with irrigation 150 rpm with temperature 28±2 °C for 24 hrs.

#### *Preparation for exopolysaccharide production:*

Preparation of inoculates of rhizobial isolates were done by transfer of loopful rhizobial culture growing on trypton yeast medium to conical flask size 100 ml with 10 ml of yeast extract mannitol broth medium. Incubation was done at 150 rpm/minute and 30°C for two days. Transfer 2% of inoculum to 20 mL yeast extract mannitol broth medium were done. Conical

flasks containing the inoculated medium were transferred to shaker incubator with temperature 30°C and 150 rpm/minute for 2 days [14].

**Determination of final pH, biomass and exopolysaccharide, biomass:**

Final pH valuation of fermented broth culture using pH meter model PW 9421 Philips. Biomass determinations were done according to Duta *et al.* [15] i.e., by centrifugation of fermented rhizobial cultures by centrifuge with 6000 rpm/minute for 20 minutes. The pellet was collected and dried in oven on 80°C for one day. Whereas exopolysaccharide determination, addition of 10 mL of cold ethanol to 5 mL of supernatant were done. Mixed properly and the mixture were put in centrifuge tubes for centrifugation at 6000 rpm/minute for 30 minutes. Supernatant were canceled and pellet (exopolysaccharide) were collected. Wet exopolysaccharides were dried by transfer to oven at 80°C for 1 day. Triplicates were done for each rhizobial isolate. Biomass determinations were done by [15].

**Tolerance of heavy metal:**

Experiment of tolerance of heavy metal by isolated rhizobial strains were conducted according to Teresa *et al.* [16], in this procedure wells were done on tryptone yeast extract (TY) solid medium containing the following components (g/L): tryptone, 5; yeast extract, 3; CaCl<sub>2</sub>, 0.87 and agar, 15. Petri dishes were incubated at 28±2 °C for 24 -48 hours. The tested heavy metals were as follows: Pb(CH<sub>3</sub>COO)<sub>2</sub>, ZnSO<sub>4</sub>, CuSO<sub>4</sub> and NiCl<sub>2</sub> with concentrations: 100, 500, 1000 and 5000 µ/mL. The diameter of inhibition zone (clear zone) was measured in millimeter (mm) after 2 days of incubation. Isolated stains were considered as resistant when no inhibition zones were observed around wells.

**Screening of rhizobial isolates for exopolysaccharide production:**

According to Sridevi and Mallaiah [17] the rhizobial extreme producer isolates were selected preliminarily by naked eye according to cultural appearance, i.e., mucoid culture with sticky texture.

**Statistical analysis:**

The values obtained in this research work were statistically analyzed for standard deviation (S. D.) using computer program [18].

**Results**

**Isolation of local rhizobial strains:**

Extreme exopolysaccharide producer rhizobial isolates with tolerance to heavy metal salts was the goal of this study. Results revealed a success of isolation of twenty-five local rhizobial isolates from different leguminous

plants. Among these isolates eight were selected as high exopolysaccharide producer according to cultural appearance on yeast extract mannitol agar medium. The local isolates named as follows: *Ensifer fredii* bv. *fredii* RM1, *Rhizobium leguminosarum* bv. *trifolii* RM3, *Ensifer meliloti* RM5, *Rhizobium grahamii* RM12, *Ensifer meliloti* RM14, *Rhizobium leguminosarum* bv. *phaseoli* RM16, *Rhizobium* sp. RM23 and *Rhizobium Vicia* RM25. These rhizobial isolates were isolated from the following leguminous plants: *Vigna unguiculata* L., *Trifolium alexandrinum*, *Trigonella foenum-graecum* L., *Leucaena leucocephala* L., *Medicago sativa* L., *Phaseolus vulgaris* L., *Tribulus terrestris* L. and *Vicia faba* L. The leguminous plants were collected from different farms and areas in Nineveh Governorate/Iraq. Purified cultures of eight rhizobial isolates , i. e., appearance of colonies of bacteria were regular, circular and transparent.

**Production of biomass and exopolysaccharide by local isolated isolates:**

Table 1 shows biomass production as well as exopolysaccharide production and final pH. The maximum exopolysaccharide production was 3.76 gm/L by the isolate *R. leguminosarum* bv. *Viciae* RM25 and the minimum exopolysaccharide production was 0.48 gm/L by the isolate by the isolate *R. leguminosarum* bv. *trifolii* RM3. Maximum dry cell weight was 3.50 gm/L by *Ensifer meliloti* RM5 and minimum dry cell weight was 0.21 gm/L by isolate *R. grahamii* RM12. Initial pH was 6.8 and after two days of incubation was decreased, the maximum decrease was 4.30 by the isolate *Ensifer meliloti* RM5. The minimum decrease in pH was by the isolate *Ensifer fredii* bv. *fredii* which reached 5.70. [19] found that the maximum exopolysaccharide production was 6.31 gm/ L by the isolate *R. leguminosarum* bv. *Viciae* WS18 after two days of incubation, when mannitol used as sole carbon source.

Isolate No.	C.D.W. gm/L	E.P.S. gm/L	Final pH
RM1	0.25 ±(0.16)	0.84 ± (0.90)	5.70 ± (0.94)
RM3	0.23 ±(0.24)	0.48 ± (0.77)	5.00 ± (0.52)
RM5	3.50 ±(0.33)	3.66 ± (0.63)	4.30 ± (1.16)
RM12	0.21 ±(0.09)	0.64 ± (0.22)	5.40 ± (0.89)
RM14	2.90 ±(1.01)	3.56 ± (0.85)	4.80 ± (0.22)
RM16	2.10 ±(0.07)	2.23 ± (0.53)	4.50 ± (0.63)
RM23	0.95 ±(0.29)	1.16 ± (0.98)	4.90 ± (0.55)
RM25	2.60 ±(1.10)	3.76 ± (0.12)	5.20 ± (0.71)

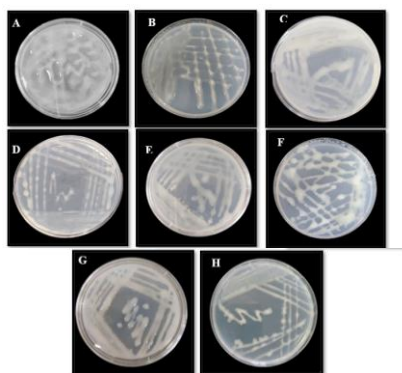
**Table 1:** Cell dry weight, exopolysaccharide production and final pH after two days incubation of local rhizobial isolates.

Other studies also revealed that the mannitol supported a maximum exopolysaccharide production [20, 21]. Ten strains of *Rhizobium* were isolated from *Vigna mungo* L. According to the research, production of exopolysaccharides is highly dependent on microorganisms, under specific conditions, media composition, as well as the genus and species of bacteria [10]. Howieson *et al.*, [22] revealed that the

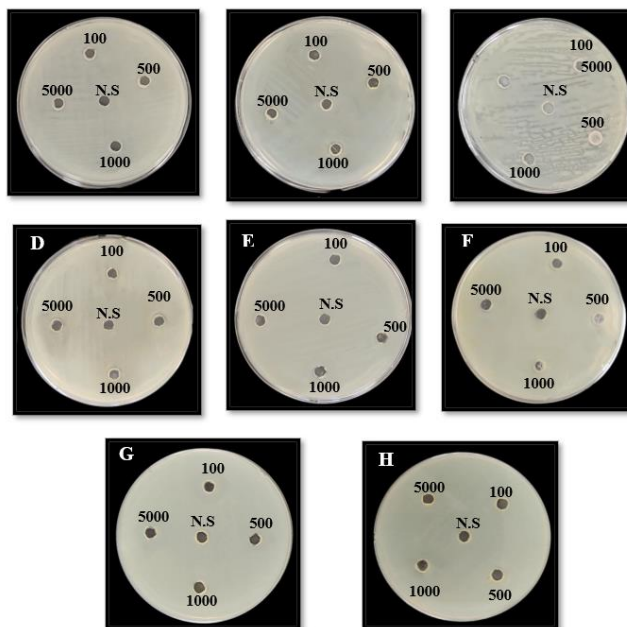
decrease in fermented cultures due to the production of acidic intermediates through incomplete oxidation of sugars in nutrient media.

**Tolerance of rhizobial isolates to heavy metal:**

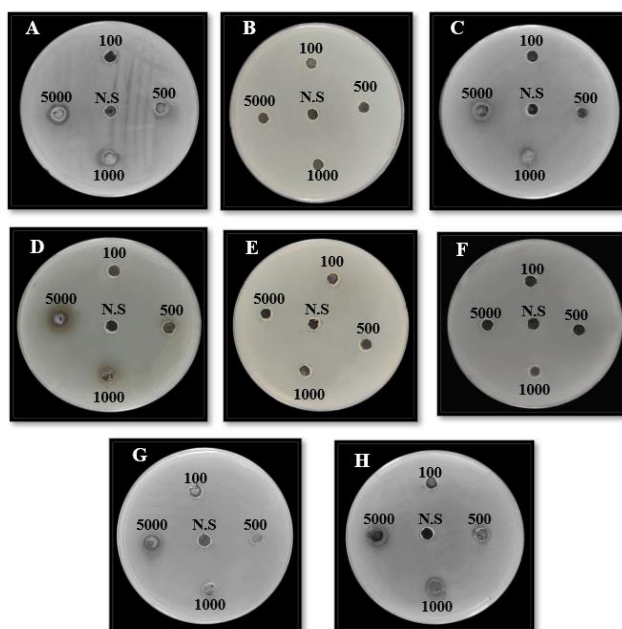
Results of this study Table 2 and Fig 2-5 showed tolerance of most local isolated rhizobia to studied heavy metals. Results revealed that all the eight studied rhizobial bacteria were tolerated nickel chloride with studied concentrations 100, 500, 1000 and 5000 µg/ml. Also, all the isolates were tolerated 100 and 500 µ/ml of zinc sulfate and copper sulfate, respectively. Lead acetate with 100 µg/ml were tolerated by all isolated rhizobial strains. Tolerance of all rhizobial isolates to 1000 µg/ml of zinc sulfate were noticed. For other values were varied from minimum inhibition zone (4 mm) against 5000 µg/ml copper sulfate by the isolate *R. leguminosarum* bv. *phaseoli* RM16 and against 500 µg/ml lead acetate by the isolate *R. leguminosarum* bv. *Viciae* RM25 to maximum inhibition zone (24mm) against 5000 µg/ml copper sulfate by the isolate *R. leguminosarum* bv. *trifolii* RM3. Shen *et al.*, [23] isolated twenty rhizobial strains showed different levels of tolerance to copper, nickel and zinc. Liu *et al.* [24] results indicated novel evidence for further exploring more functional microbes from the nickel-mine soil. Rahal and Chekireb [25] analyzed the diversity of 50 strains isolated from *Trifolium* sp. Nodules growing on Pb- Zn mine soil. Mechanisms mediating resistance to Cobalt and Nickel have been identified in many metals' resistant *rhizobium* through the identification of orthologues of metal resistance genes characterized in *C. metallidurans* CH34 [26]. Microorganisms tolerant of metal contaminants are known to have two mechanisms by which they protect themselves from the toxic effects of heavy metals. Exclusion, as mediated by the exopolysaccharide capsule around the cell, has been shown to prevent cells uptake of metals [27].



A. *Ensifer fredii* bv. *fredii* RM1, B. *R. leguminosarum* bv. *trifolii* RM3, C. *Ensifer meliloti* RM5, D. *R. grahamii* RM12, E. *Ensifer meliloti* RM14, F. *R. leguminosarum* bv. *phaseoli* RM16, G. *Rhizobium* sp. RM23, H. *R. leguminosarum* *Vicia* RM25  
**Figure 1:** Culture of local rhizobial isolates on YEM.



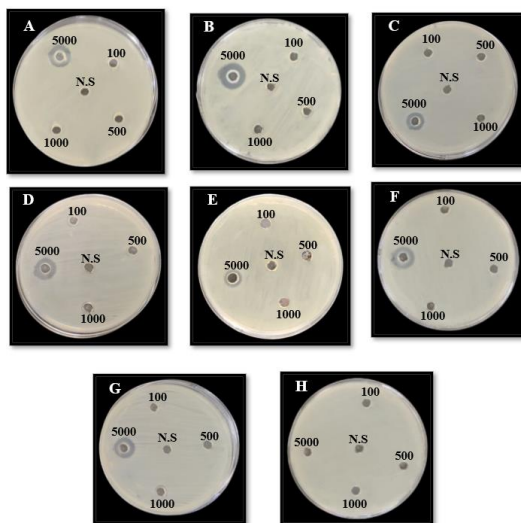
A. *Ensifer fredii* bv. *fredii* RM1, B. *R. leguminosarum* bv. *trifolii* RM3, C. *Ensifer meliloti* RM5, D. *R. grahamii* RM12, E. *Ensifer meliloti* RM14, F. *R. leguminosarum* bv. *phaseoli* RM16, G. *Rhizobium* sp. RM23, H. *R. leguminosarum* *Vicia* RM25  
**Figure 2:** Heavy metal tolerance of local rhizobial isolates NiCl<sub>2</sub> on Mueller-Hinton medium with concentration 100, 500, 1000 and 5000 µg\ ml. N.S. : Normal saline.



A. *Ensifer fredii* bv. *fredii* RM1, B. *R. leguminosarum* bv. *trifolii* RM3, C. *Ensifer meliloti* RM5, D. *R. grahamii* RM12, E. *Ensifer meliloti* RM14, F. *R. leguminosarum* bv. *phaseoli* RM16, G. *Rhizobium* sp. RM2, H. *R. leguminosarum* *Vicia* RM25.  
**Figure 3:** Heavy metal tolerance of local rhizobial isolates Pb (CH<sub>3</sub>COO)<sub>2</sub> on Mueller-Hinton medium with concentration 100, 500, 1000 and 5000 µg\ ml. N.S. : Normal saline.

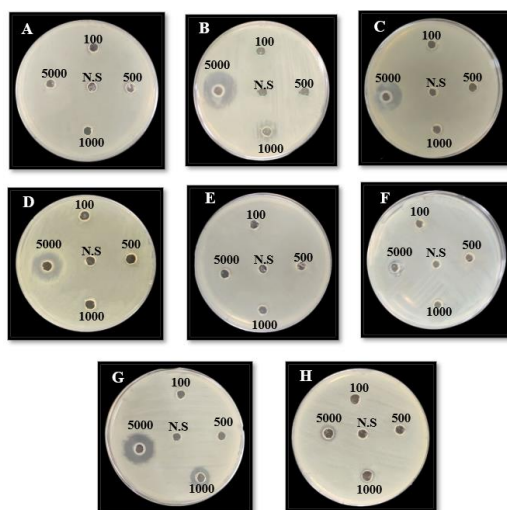
Isolate No.	NiCl <sub>2</sub> (µg/ml)				Pb (CH <sub>3</sub> COO) <sub>2</sub> (µg/ml)				ZnSO <sub>4</sub> (µg/ml)				CuSO <sub>4</sub> (µg/ml)			
	100	500	1000	5000	100	500	1000	5000	100	500	1000	5000	100	500	1000	5000
RM1	00	00	00	00	00	7	9	11	00	00	00	13	00	00	00	00
RM3	00	00	00	00	00	00	00	00	00	00	00	14	00	00	14	24
RM5	00	00	00	00	00	00	00	8	00	00	00	10	00	00	00	15
RM12	00	00	00	00	00	00	7	12	00	00	00	11	00	00	00	17
RM14	00	00	00	00	00	00	00	00	00	00	00	8	00	00	00	00
RM16	00	00	00	00	00	00	00	00	00	00	00	12	00	00	00	4
RM23	00	00	00	00	00	00	8	14	00	00	00	14	00	00	10	20
RM25	00	00	00	00	00	4	6	13	00	00	00	00	00	00	8	10

Table 2: Tolerance of local rhizobial isolates to heavy metals.



A. *Ensifer fredii* bv. *fredii* RM1, B. *R. leguminosarum* bv. *trifolii* RM3, C. *Ensifer meliloti* RM5, D. *R. grahamii* RM12, E. *Ensifer meliloti* RM14, F. *R. leguminosarum* bv. *phaseoli* RM16, G. *Rhizobium* sp. RM23, H. *R. leguminosarum* *Vicia* RM25.

Figure 4: Heavy metal tolerance of local rhizobial isolates ZnSO<sub>4</sub> on Mueller-Hinton medium with concentration 100, 500, 1000 and 5000 µg/ml. N.S. : Normal saline.



A. *Ensifer fredii* bv. *fredii* RM1, B. *R. leguminosarum* bv. *trifolii* RM3, C. *Ensifer meliloti* RM5, E. *Ensifer meliloti* RM14, D. *R. grahamii* RM12, F. *R. leguminosarum* bv. *phaseoli* RM16, G. *Rhizobium* sp. RM23, H. *R. leguminosarum* *Vicia* RM25

Figure 5: Heavy metal tolerance of local rhizobial isolates CuSO<sub>4</sub> on Mueller-Hinton medium with concentration 100, 500, 1000 and 5000 µg/ml. N.S. : Normal saline.

## Discussion

Ali and Orf [10] from Egypt isolated rhizobial isolates produced exopolysaccharide [17] results revealed that the ability of isolation of 16 stains of *Rhizobium* from root nodules of *Sesbania sesban* grown in deferent area in Indian farms and they found that the strain SS5 was the best among the isolates in exopolysaccharide production. [19] found that the maximum exopolysaccharide production was 6.31 gm/ L by the isolate *R. leguminosarum* bv. *Viciae* WS18 after two days of incubation, when mannitol used as sole carbon source. Other studies also revealed that the mannitol supported a maximum exopolysaccharide production [20, 21]. Ten strains of *Rhizobium* were isolated from *Vigna mungo* L. According to the research, production of exopolysaccharides is highly dependent on microorganisms, under specific conditions, media composition, as well as the genus and species of bacteria [10, 22] revealed that the decrease in fermented cultures due to the production of acidic intermediates through incomplete oxidation of sugars in nutrient media. Shen T et al., [23] isolated twenty rhizobial strains showed different levels of tolerance to copper, nickel and zinc [24] results indicated novel evidence for further exploring more functional microbes from the nickel mine soil. [25] analyzed the diversity of 50 strains isolated from *Trifolium* sp. Nodules growing on Pb-Zn mine soil. Mechanisms mediating resistance to Cobalt and Nickel have been identified in many metal resistant *rhizobium* through the identification of orthologues of metal resistance genes characterized in *C. metallidurans* CH34 [26]. Microorganisms tolerant of metal contaminants are known to have two mechanisms by which they protect themselves from the toxic effects of heavy metals. Exclusion, as mediated by the exopolysaccharide capsule around the cell, has been shown to prevent cells uptake of metals [27].

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## Author Contributions

Mohammed Jasim is a Researcher was achieved this research work under guidance of Dr. Raad Hassani Sultan.

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