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Original Article

# Assessment of zinc solubilization potential of zinc-resistant *Pseudomonas oleovorans* strain ZSB13 isolated from contaminated soil

## Avaliação do potencial de solubilização do zinco da cepa ZSB13 de *Pseudomonas oleovorans* resistente ao zinco isolada de solo contaminado

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**Abstract**

Zinc is an essential micronutrient that is required for optimum plant growth. It is present in soil in insoluble forms. Bacterial solubilization of soil unavailable form of Zn into available form, is an emerging approach to alleviate the Zn deficiency for plants and human beings. Zinc solubilizing bacteria (ZSB) could be a substitute for chemical Zn fertilizer. The present study aimed to isolate and characterize bacterial species from the contaminated soil and evaluate their Zn solubilizing potential. Zn resistant bacteria were isolated and evaluated for their MIC against Zn. Among the 13 isolated bacterial strains ZSB13 showed maximum MIC value upto 30mM/L. The bacterial strain with the highest resistance against Zn was selected for further analysis. Molecular characterization of ZSB13 was performed by 16S rRNA gene amplification which confirmed it as *Pseudomonas oleovorans.* Zn solubilization was determined through plate assay and broth medium. Four insoluble salts (zinc oxide (ZnO), zinc carbonate ($ZnCO_3$), zinc sulphite (ZnS) and zinc phosphate ($Zn_3(PO_4)_2$) were used for solubilization assay. Our results shows 11 mm clear halo zone on agar plates amended with ZnO. Likewise, ZSB13 showed significant release of Zn in broth amended with $ZnCO_3$ (17 and 16.8 ppm) and ZnO (18.2 ppm). Furthermore, Zn resistance genes czcD was also enriched in ZSB13. In our study, bacterial strain comprising Zn solubilization potential has been isolated that could be further used for the growth enhancement of crops.

**Keywords:** zinc solubilization, *Pseudomonas oleovorans*, zinc resistance bacteria, biofertilizers.

**Resumo**

O zinco é um micronutriente essencial necessário para o crescimento ideal das plantas. Ele está presente no solo em formas insolúveis. A solubilização bacteriana da forma indisponível de Zn no solo para a forma disponível é uma abordagem emergente para aliviar a deficiência de Zn em plantas e seres humanos. Bactérias solubilizadoras de zinco (ZSB) podem ser um substituto para fertilizantes químicos de Zn. O presente estudo teve como objetivo isolar e caracterizar espécies bacterianas de solo contaminado e avaliar seu potencial de solubilização de Zn. Bactérias resistentes ao Zn foram isoladas e avaliadas quanto ao seu MIC contra o Zn. Entre as 13 cepas bacterianas isoladas, ZSB13 apresentou valor máximo de MIC de até 30 mM/L. A cepa bacteriana com maior resistência ao Zn foi selecionada para análise posterior. A caracterização molecular de ZSB13 foi realizada por amplificação do gene 16S rRNA que o confirmou como Pseudomonas oleovorans. A solubilização do Zn foi determinada através de ensaio em placa e meio caldo. Quatro sais insolúveis (óxido de zinco (ZnO), carbonato de zinco (ZnCO3), sulfito de zinco (ZnS) e fosfato de zinco (Zn3 (PO4) 2) foram usados para o ensaio de solubilização. Nossos resultados mostram uma zona de halo clara de 11 mm em placas de ágar corrigidas com ZnO. Da mesma forma, ZSB13 mostrou liberação significativa de Zn em caldo alterado com ZnCO3 (17 e 16,8 ppm) e ZnO (18,2 ppm). Além disso, os genes de resistência ao Zn czcD também foram enriquecidos em ZSB13. Em nosso estudo, a cepa bacteriana compreendendo potencial de solubilização de Zn foi isolada e poderia ser usada posteriormente para o aumento do crescimento de safras.

**Palavras-chave:** solubilização de zinco, *Pseudomonas oleovorans*, bactéria resistente ao zinco, biofertilizantes.

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## 1. Introduction

Zinc is one of the key micronutrients that is required in small concentrations both for plants and animals for healthy growth (Saravanan et al., 2011). It is a necessary constituent involved in many physiological and metabolic actions in plants, humans and microorganisms (Greany, 2005). Although, it plays vital roles in membrane integrity, photosynthesis, pollen formation, immune system and protein synthesis (Hrynkiewicz and Baum, 2014; Turpeinen et al., 2002), but on the other side, increase of Zn in soil and environment to a certain limit is toxic. For human, it is toxic if Zn level is 100-500 mg per day (Manivasagaperumal et al., 2011).

In Pakistan, Zn concentration ranges from >0.1 to 1193mg/kg in soil except the polluted area where the maximum concentration of Zn in soil/dust is considered to be 29755mg/kg. (Radojevic and Bashkin, 2006; Muhammad et al., 2011). In general, Zn metal cannot be degraded to soluble form and persist in the environment. Its extensive use and low melting temperature caused contamination of environment, soil and freshwater due to divalent cation (Khalid et al., 2017). Notably, metal resistance has been reported in many microorganisms. Czc determinant encodes a multi-protein complex that is associated with resistance to cadmium, zinc and cobalt in bacteria. CzcA is one of the proteins characterized in several metal and Zn resistance bacteria such as *Cupriavidus metallidurans* CH34, *Pseudomonas putida* CD2 and *Gluconacetobacter diazotrophicus* PAl 5 (Intorne et al., 2012).

It is evident that excessive presence of Zn in soil have adverse effect but on the other way its deficiency also responsible for impaired plant and human growth (Abaid-Ullah et al., 2015). It has been reported that worldwide occurrence of Zn deficiency in crops is not due to scarcity of total Zn but it is due to low solubility of Zn in soils (Cakmak, 2008). Normally, zinc is found in soil in the forms such as smithsonite ($ZnCO_3$), sphalerite (ZnS), zincite (ZnO), franklinite ($ZnFe_2O_4$), wellemite ($Zn_2SiO_4$), and hopeite ($Zn_3(PO_4)_2{\cdot}4H_2O$) that are insoluble forms of Zn and could not be used by the plants. Low availability of Zn not only effect the crop growth but also lowers Zn level in seeds and grains, so affecting the nutritional quality that ultimately leads to Zn deficieny in human beings (Cakmak and Hoffland, 2012).

To overcome the Zn deficiency in soil for plant growth, normally chemical fertilizers are applied that causing environmental problems. Therefore, feasible alternative could be the use of soil microorganisms having Zn solubilization capacity. Certain bacteria including *Pseudomonas* sp, *Bacillus* sp., *Burkholderia cenocepacia* have been reported to transform insoluble forms of Zn to soluble form for enhanced availability and uptake by plants (Fasim et al., 2002; Abaid-Ullah et al., 2015; Pawar et al., 2015; Khande et al., 2017).

Zn solubilizing bacteria can solubilize Zn through various mechanisms like acidification, production of siderophores and oxidoreductive systems on cell membranes (Chang et al., 2005; Saravanan et al., 2011). These bacteria produce organic acids in soil which sequester the zinc cations and can also chelate zinc and enhance zinc solubility (Jones and Darrah, 1994). This study has been designed to identify and characterize Zn solubilizing bacteria and to investigate presence of Zn resistant genes.

## 2. Materials and Methods

### *2.1. Sample collection and metal analysis of samples*

Thirty five soil samples were collected from Paharang drain, Bawachak, Faisalabad (31°25'0"N/73°5'0"E). The major source of contamination is that industries discharge their waste in to Chenab river through Paharang drain originating from Chak Jhumra. This drain passes through Faisalabad with these industrial effluents. Soil samples were collected in sterile plastic containers at the depth of 10 cm at 600 meters distance intervals. Total 35 samples were collected in triplicate and transported to Laboratory for further use and stored at 4 °C. After collection, heavy metals were determined in the samples using the method previously described by Zeiner et al. (2007). Cadmium (Cd), nickel (Ni), zinc (Zn) and lead (Pb) were included for analysis. Samples were digested and metal analysis was performed using Atomic Absorption Spectrophotometer (AAS) (Zeeman Atomic Absorption Spectrophotometer, ZA3000 Series).

### *2.2. Isolation of zinc resistant bacteria*

Soil samples were taken in sterile water and to isolate bacteria from soil, samples were shaken at 150 rpm for 2 hr. After that, samples were settled for 5 min and one mL of the suspension was serially diluted upto $10^{-6}$. Each dilution was spread onto an LB plate supplemented with 1 mM Zn and incubated at 30 °C. The growing colonies were observed and again inoculated onto LB plates containing Zn to get pure culture.

### *2.3. Determination of Minimum Inhibitory Concentration (MIC)*

Minimum Inhibitory Concentration of Zn for selected isolated bacteria (based on their morphology) was determined by the plate dilution method (Ansari and Malik, 2007). The zinc salt $ZnSO_4{\cdot}7H_2O$ in varying concentrations ranging from 1 mM to 35 mM was used to determine MIC. Petri plate containing nutrient agar with varying concentrations of Zn was prepared and inoculated with 0.5 mL of overnight grown culture of bacterial isolate and incubated at 28-30 °C for 24h. The lowest concentration of the metal, where the growth of the microorganism was inhibited, considered as the MIC of the zinc against the bacterial isolates (Haroun et al., 2017).

### *2.4. Identification of bacterial isolate*

After selecting Zn resistant bacterial isolate, identification was done on the basis of cultural characteristics, Gram's staining and biochemical characteristics including oxidase reaction, catalase reaction, starch hydrolysis, fermentation tests and glucose utilization.

### *2.5. Bacterial genomic DNA Isolation and 16S ribosomal RNA (rRNA) sequencing*

After biochemical identification, molecular characterization of bacterial isolate on the basis of 16S rRNA gene sequencing was performed. Bacterial genomic DNA was isolated by ZR/Bacterial DNA Extraction Kit. From isolated DNA, amplification of 16S rRNA gene was performed by using Universal primer, 27 F: 5'-AGATTGATCTGGCTAGGGA-3' and 1492 R: 5'-TACGGTACCTTGTTACGCTT-3' (1500 bp product) (Vasas et al., 2013). The PCR was carried out with following conditions: initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C, 30 sec of annealing at 52 °C, and 40 sec of elongation at 72 °C. The last step was a final extension at 72 °C for 10 min. For ribotyping, commercial sequencing of PCR product was obtained from Macrogen (Korea). The sequences obtained were checked for base calling using FinchTV and contigs made using NCBI BLAST (two sequence alignments). Using NCBI blast analysis by the sequence of the 16S rRNA gene was submitted to the database of GenBank and compared with similar sequences. The phylogenetic tree of partial 16S rRNA was constructed using maximum likelihood method by the software MEGA version 6.0.

### *2.6. Zinc solubilization assay*

To determine the solubilization potential of selected bacterial isolate, four insoluble zinc salts like zinc carbonate ($ZnCO_3 4H_2O$), zinc sulphide (ZnS), zinc oxide (ZnO) and zinc phosphate ($Zn(PO_4)_2 H_2O$) were used.

For qualitative solubilization, Mineral Salt medium (MSM) was prepared. The media was prepared by adding NaCl 1 g, $CaCl_2$ 0.1 g, $MgSO_4$ 0.5 g, $KH_2PO_4$ 1 g, $K_2HPO_4$ 1 g, yeast extract 4 g and agar 16-18 g in one litre $dH_2O$ and pH was maintained at 7.2. MS media was prepared containing 0.1% conc. of each insoluble zinc salt. Petri plates containing MS media were inoculated by pour plate method with bacterial strain and incubated at 30 °C for 48 hrs (Binder, Germany). After that, clear zone around the colonies was observed (Khanghahi et al., 2018).

For quantitative solubilization of zinc, MS broth was prepared with 0.1% zinc concentration for each zinc salts at pH 7. 1 mL of bacterial culture was inoculated into each flask containing MS Broth. The flasks were incubated at 30 °C for 72 hrs on shaking at 150 rpm (Thermo Scientific™, UK). After 72 hrs incubation, culture broth was centrifuged at 10,000 rpm for 10min. Concentration of Zn in supernatant was measured with atomic absorption spectrophotometer (Shimatzu AAS-7000). After 72 hrs incubation, pH of the medium was also measured.

### *2.7. Detection of resistant genes in strain ZSB13*

Analysis of Zn resistance encoding genes were performed with primers encoding for Zn (*CzcA*, *CzcB* and *CzcD*). Primers used for *czcA* F 5-GTTTGAACGTATCATTAGTTTC-3, R 5-GTAGCCATCCGAAATATTCG-3 with 1885 bp. *czcD* F 5-CAGGTCACTGACACGACCAT-3, R 5-CATGCTGATGAGATTGATGATC-3 with 1000 bp. *czcB* F 5-CTATTTCGAACAAACAAAAGG-3, R 5-CTTCAGAACAAAACTGTTGG-3 with 1520 bp. The PCR conditions used were initial denaturation at 95 °C for 5 min followed by 35 cycles at denaturation: 95 °C for 30 sec, annealing: 55 °C for 30 sec, amplification: 72 °C for 2 min and final extension at 72 °C for 7 min (Babalola and Ayangbenro, 2019). Electrophoresis was done with amplified PCR products on 1.5% gel.

## 3. Results

### *3.1. Metal analysis in samples and isolation of zinc resistant bacteria*

Heavy metal analysis of soil samples through atomic absorption spectrophotometer revealed the presence of different heavy metals in the samples. Concentrations of different heavy metals are: Zn 129.7 ± 0.30, Pb 54. ± 0.07, Cd 11.0 ± 0.03 and Ni 36.5 ± 0.09. Out of 35 samples, only 13 samples exhibited positive growth on medium containing Zn. 13 isolated bacteria using spread plate procedure labeled as ZSB1 to ZSB13 exhibited resistance to Zn.

### *3.2. Determination of Minimum Inhibitory Concentration (MIC)*

13 bacterial isolates were used to determine the MIC. Isolates were inoculated on the nutrient agar plates having zinc 0.5, 1, 2, 5, 6, 8, 10, 12, 15, 20, 25, 30, 35mM. With increasing concentration of zinc, the growth of bacterial isolates decreased. The maximum zinc level where bacteria showed growth was 30mM. Isolate ZSB13 showing MIC upto 30 mM.

### *3.3. Identification of bacterial isolate*

Based on the morphological and biochemical characteristics ZSB13 was found to be gram negative, rod shaped, non-spore forming, non-capsulated and non-motile (Table 1).

### *3.4. Molecular identification of bacterial isolate based on 16S ribosomal RNA (rRNA) sequencing*

The 16S rRNA sequence of the strain ZSB13 was amplified by PCR. Ribotyping confirmed bacterial strain ZSB13 as *Pseudomonas oleovorans* and was submitted to the database of GenBank with the accession number (GenBank accession no.: MN396696.1).

The strain displayed highest level of similarity 99.30% with *Pseudomonas mendocina* strain Y12 (Accession number KP324955.1). In order to determine the relationship between strain ZSB13 and the other *Pseudomonas* species, the phylogenetic tree based on 16S rRNA sequence was constructed. So, based on the results of morphological, biochemical characteristics and 16S rRNA sequence analysis, strain ZSB13 was identified as *Pseudomonas oleovorans* ZSB13 shown in (Figure 1).

### *3.5. Zinc solubilization assay*

Zn solubilization potential of bacterial strain ZSB13 was evaluated by clear zone against four insoluble zinc salts ($ZnCO_3 4H_2O$, ZnS, ZnO and $(Zn(PO_4)_2 . H_2O)$. Maximum Zn solubilization zone observed on ZnO medium was 11 mm followed by 10 mm for $ZnCO_3$ as shown in Figure 2.

**Table 1.** Morphological and Biochemical characteristics of strain ZSB13.

| Characteristics | Strain ZSB13 |
|---|---|
| **Morphological observations** | |
| Colony color | off white (on nutrient agar) |
| Gram nature | Gram-negative |
| Cell shape | Coccus |
| Colony shape | Round |
| Motility | Positive |
| Spore formation | Negative |
| **Biochemical tests** | |
| Catalase test | Positive |
| Urease test | Negative |
| Gelatin hydrolysis test | Positive |
| Carbohydrate test | Negative |
| MRVP test | Negative |
| Citrate test | Positive |
| Blood agar test | Positive |
| Chocolate agar test | Positive |

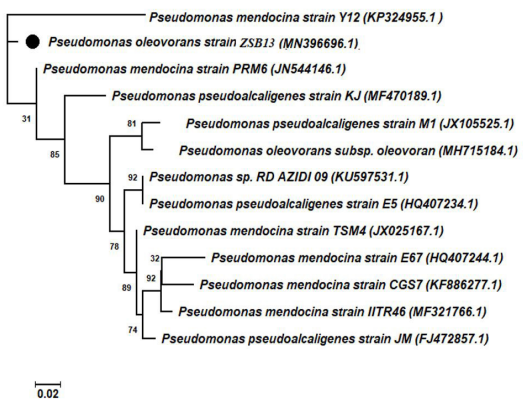


**Figure 1.** Neighbour-Joining phylogenetic tree of 16S rRNA gene sequences of *Pseudomonas oleovorans* strain ZSB13 isolated from contaminated soil.

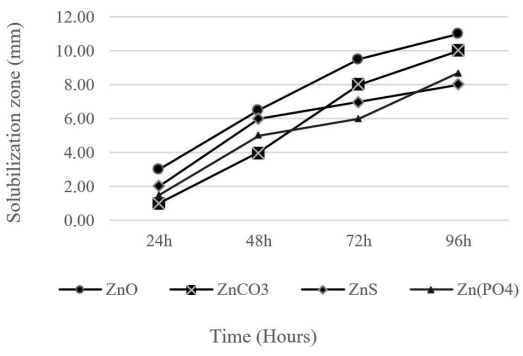


**Figure 2.** Zn solubilization potential of bacterial strain ZSB13.

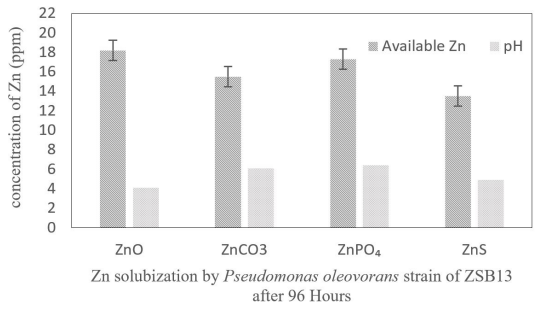


**Figure 3.** Available zinc (ppm) released by ZB13 in broth medium containing different zinc salts after 96 hrs.

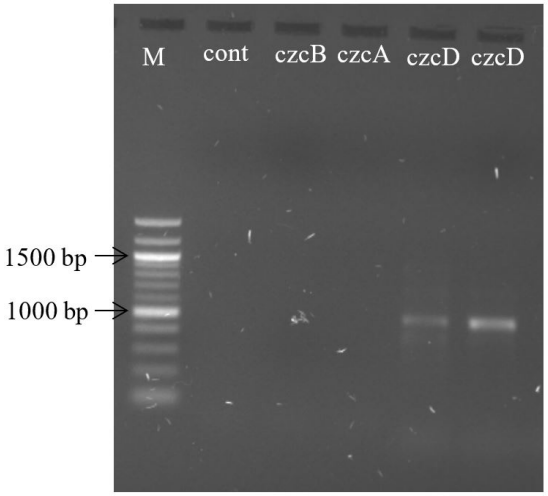


**Figure 4.** PCR products of czcA, czcB and czcD genes: M, gene ruler, cont: negative control (czcD 1000bp).

Quantitative assay for zinc solubilization revealed that strain ZSB was able to dissolve in 18.2, 15.5, 17.3 and 13.8 ppm from ZnO, $ZnCO_3$, $Zn(PO_4)_2$ and ZnS respectively in liquid medium as shown in Figure 3. However, maximum solubilization was observed in ZnO in liquid assay.

### *3.6. Determination of resistant genes in strain ZSB13*

Zn resistant genes were detected in ZSB13 and our results showed the presence of only czcD while others Zn resistance genes i.e., czcA and czcB were not observed in *Pseudomonas oleovorans* ZSB13 (Figure 4).

## 4. Discussion

In the present study efforts were made to isolate Zn resistant bacterial strains. In the present investigation, bacterial strains isolated from Paharang drain, Bawachak, Faisalabad showed a promising solubilizing efficiency for ZnO. After initial screening, 13 bacterial isolates were recovered on nutrient agar supplemented with 1 mM concentration of zinc from 35 soil samples. Morphological characterization of isolates showed as Gram-negative species. Gram negative bacteria have two layer of cell

membrane enabling them to resist and grow at higher metal concentration than gram positive bacteria (Khanghahi et al., 2018). MIC of selected bacterial strains were evaluated and one strain ZSB13 showed maximum growth on nutrient agar incorporated with 30 mM of Zn. Lee et al. (2009) evaluated the MIC value upto 11.5 mmol/L of zinc for *Pseudomonas. putida* strain 06909. Muzammil et al. (2021) also isolated cadmium resistant bacteria (*Bacillus cereus* GCFSD01) from Paharang drain.

Morphological and biochemical characteristics showed the ZSB13 was gram negative, rod shaped and non-spore forming bacteria. Molecular characterization confirmed the strain as *Pseudomonas oleovorans* ZSB13. It has been reported in literature that most of the Zinc solubilizing bacteria belong to different genera like *Pseudomonas, Bacillus, Enterobacter, Xanthomonas, Stenotrophomonas,* and *Acinetobacter* etc (Hussain et al., 2015; Gandhi and Muralidharan, 2016; Sunithakumari et al., 2016). Saravanan et al., (2003) studied *Bacillus* sp. and *Pseudomonas* sp. for their Zn solubilizing potential in broth assays and found that solubilization potential vary in different bacterial isolates.

In our study, the selected strain was identified as *Pseudomonas oleovorans* that can transform unavailable forms of Zn salts into available forms through solubilization as PGPB strains (Scagliola et al., 2016). In literature, many species of Pseudomonas like *P. putida, P. fluorescens, P. aeruginosa* have been studied for their Zn solubilizing potential (Goteti et al., 2013) but according to our knowledge, this is the first report on zinc solubilization potential of *Pseudomonas oleovorans.* The selected zinc solubilizing bacterial strain was screened for solubilization of insoluble zinc compounds and in our study, we used four different insoluble sources of Zinc like ZnO, $ZnCO_3$, ZnS and $Zn_3(PO_4)_2$.

In our study, it has been observed that *Pseudomonas oleovorans.* ZSB13 showed maximum solubilization potential for ZnO. These finding are in accordance with previous studies in which ZSB strains showed the highest solubilizing efficiency for ZnO compared to $ZnCO_3$ and $Zn_3(PO_4)_2$ (Gandhi and Muralidharan, 2016; Khanghahi et al., 2018). The zone of solubilization is ranged from 16 mm to 6 mm for insoluble compounds at 96 hrs. The halo zone showing the solubilization could be due to decrease of medium pH. In contrast to our results Sharma et al. (2014) found greater solubilization zone for $Zn_3(PO_4)_2$) and Vidyashree (2016) found it for $ZnCO_3$. However, size of the solubilization zone could vary with the carbon source provided during the assay (Saravanan et al., 2008) and it could be altered with strains used.

The selected *Pseudomonas* strain ZSB13 was also evaluated for qualitative liquid assay containing insoluble Zn compounds. The broth assay also confirmed the ability of *Pseudomonas* ZSB13 to solubilize the Zn compound and here again we observed maximum solubization for ZnO. Different mechanisms could be involved in solubilisation of zinc that include excretion of organic acids, proton extrusion and production of inorganic acids such as sulphuric acid, gluconic acid and nitric acid (Desai et al., 2012). Di Simine investigated the Zn phosphate solubilization by *Pseudomonas fluorescens* and identified gluconic acids producedion in medium. The solubilization potential may be vary for different Zn salts as already reported in literature (Abaid-Ullah et al., 2015). In the present study, we found decrease in pH of the medium in all cases of the insoluble salt that gave the idea that solubilization could be due to production of organic acids. Although significant decrease in pH has been observed for ZnS but highest solubilization was for ZnO. No significant relation between pH and solubilization has been established. Although in our study, production of acids like gluconic acid has not been evaluated but its relation with the decrease in pH is evident (Desai et al., 2012). *Pseudomonas oleovorans* ZSB13 capable of solubilizing zinc. are also needed to evaluate for further use of these bacteria for bioremediation. It is therefore likely to express genes that confer metal resistance. In the present study, czc determinants (czcA, czcB and czcD) were used for analysis of metal-resistant determinant of ZSB13. It is also evident from literature that some metal resistance bacteria could harbor also antibiotics resistance genes with metal resistance genes (Rave et al., 2019). Therefore, with the evaluation of metal resistance genes, some antiobiotics resistance genes. The resistance ability of *Pseudomonas oleovorans* ZSB13 for zinc was first evaluated in an MIC assay. The results revealed that ZSB13 was tolerant to Zn.

## 5. Conclusions

On the basis of this study, it was concluded that native bacterial strain ZSB13 *Pseudomonas oleovorans* isolated from soil possessed Zn solubilization potential. However, further investigation need to use of this strain for plant growth promotion and to detoxify contaminated soil, but it may be a potential candidate as bioremediation agent development and as a substitute to synthetic fertilizers.

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