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Isolation and characterization of Zinc solubilizing bacteria from Bhitarkanika mangrove forest of Odisha, India

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ABSTRACT

Zinc is a crucial element for optimal plant growth. Some bacteria that solubilize zinc are potential substitutes for zinc supplements because they transform applied inorganic zinc into usable forms. As zinc cannot be produced by the plants by themselves, bacteria play important role in growth increasing of plants by providing zinc. This investigation was done to examine the potential of zinc-solubilizing bacteria isolated from mangrove forests of Odisha. In this study, seven zinc solubilizing bacteria (ZSB) were isolated, of which ZSB2 was found to have maximum zinc solubilizing efficiency of 111.51% on solid medium and solubilizes 6.55 mg/L of Zn in liquid medium. The bacterium was identified as *Enterobacter kobei* by 16S r-RNA sequencing. Along with zinc solubilization, some ZSB isolates showed positive response towards siderophore production. The study bears significance for application in plant growth promotion.

1. Introduction

Zinc is an essential micronutrient required in small but critical amount for optimum plant growth. It acts as a cofactor for many enzymes, thus have a key role for plant growth, development and defense mechanism. Plants can absorb zinc as a divalent cation, but the amount of soluble zinc in soil solution is relatively less (Kabata and Pendias, 2001). Zinc is mainly present as ore, minerals and insoluble compounds in the soil (Alloway, 2008). Due to unavailability of zinc in soil, many plants show zinc deficiency, one of the most common micronutrient deficiencies. There are many techniques that have been used for a long time to treat zinc deficiency. Zinc fertilizers have been used in the form of zinc sulphate (White and Broadly, 2005) or Zn-EDTA (Karak *et al.*, 2005), but their use affect the economy and environment, and these fertilizers change into insoluble complex forms within 7 days of application (Rattan and Shukla, 1991).

Zinc-solubilizing bacteria can serve as an alternative to zinc supplements by converting applied inorganic zinc into useable forms. They solubilize zinc by various mechanisms such as acidification and chelation. The microbes secrete organic acids to the soil which sequester the zinc cations by lowering the pH of the soil (Alexander, 1997). Further the anions can also chelate zinc to enhance its solubility via zinc chloride, zinc phosphate, zinc carbonate etc. (Jones and Darrah, 1994). Other mechanisms include oxido-reduction reactions on cell membrane with chelated ligands (Chang *et al.*, 2005), production of protons or siderophores (Saravanan *et al.*, 2011). *Azotobacter, Azospirillum, Bacillus, Serratia, Rhizobium, Gluconacetobacter, Pseudomonas* and facultative thermophilic iron oxidizers are a few Zn-solubilizing bacterium genera that have been identified so far (Deepak *et al.*, 2013; Saravanan *et al.*, 2007). Many PGPR have been reported to be efficient zinc solubilizers. By populating the rhizosphere and converting complicated zinc compounds into simpler

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ones, these bacteria aid in the improvement of plant growth and development. Moreover, these zinc solubilizing bacteria with PGPR activity were successfully utilized for enhanced nutrition and zinc biofortification in maize, soyabean, wheat (Khande *et al.*, 2017; Kamran *et al.*, 2017) and rice (Vaid *et al.*, 2014).

Mangroves are typical ecological niches found in the inter-tidal zones of river deltas with waterlogged saline conditions which facilitates the growth of diverse bacterial. Further, the delta being the slowest stretch of river flow accumulates the major portion of effluents and nutrient carried over by the river system and thus impacts the soil physico-chemical parameters and microbial diversity. The microbial diversity is the backbone of nutrient recycling and have significant role in the sustainability of such ecosystem. Most of the Indian soil are deficient of micronutrients which results in to low crop productivity and reduced nutritional quality (Sukla *et al.*, 2021). Therefore, the present study focused on the isolation and characterization of zinc solubilizing bacteria from the diverse habitat of mangrove forest of Odisha with an aim for future utilization of bioresource for agricultural yield.

2. Materials and methodology

The soil samples were collected from Bhitarkanika mangrove forests of Kendrapara district, Odisha. Various physicochemical parameters were measured to determine the soil properties following standard methods (Banerjee, 2018).

2.1. Isolation of Zn solubilizing bacteria (ZSB) from soil samples

The spread plate method was used to count and isolate Zn-solubilizing bacteria from soil samples on Bunt and Rovira medium (Bunt and Rovira, 1955) containing 0.1% insoluble zinc oxide (ZnO). The zinc solubilizing bacteria displayed a clear hollow zone on the plate, and the bacteria were isolated as zinc solubilizers and purified further to make pure cultures. The bacteria showing distinct solubilization zone were named as ZSB1 to 7 respectively and selected for further study.

2.2. Growth and morphological characteristics of the ZSB isolates

The population of the ZSB isolates were evaluated by counting their colony abundance from the master plate and was expressed in colony forming units (CFU) as follows. All the selected isolates were examined morphologically through their colony morphology, cell shape and gram staining reaction (Barthalomew and Mittewer, 1950).

Colony forming unit/ml =

$$\frac{No\ of\ colonies\ x\ dilution\ factor}{Volume\ of\ inoculum}$$

2.3. Determination of zinc solubilizing efficiency and biochemical characterization of ZSB isolates

All the 7 isolates were tested for solubilization efficiency by plate assay using modified Bunt and Rovira agar medium containing 0.1% of ZnO as insoluble source. After inoculation, the plates were incubated for 48hrs at 37^0C. The hollow zone on the plates indicated solubilizing efficiency of bacteria. The diameter of the colony growth and clear zone created by it was measured and then the solubilization efficiency was calculated as per Khanghahi *et al.* (2018). Indole test and Siderophore production tests were examined for the bacteria by using Kovac's reagent and $FeCl_3$ test (Dave *et al*., 2006).

Solubilizing efficiency (%) =

$$\frac{Diameter\ of\ holozone\ -\ diameter\ of\ colony\ growth}{Diameter\ of\ colony\ growth}\ x\ 100$$

Zinc solubilization capacity was also tested under liquid culture conditions using Bunt and Rovira medium containing 0.1% ZnO as the sole source of Zn. To determine the effect of ZSB on pH of growth media, samples were withdrawn at regular intervals and pH of the broth were measured. An uninoculated media sample was also kept as control. The bacterial culture and control were centrifuged and filtered. To measure the titrable acidity of the broth, 4ml of supernatant was titrated against 0.01% NaOH using phenopthalene as indicator (Nenwani *et al.*, 2010). The measurement of soluble Zinc ion in the culture was done through AAS. The sample was prepared by digestion with triacid mixture (prepared by nitric acid, sulphuric acid and perchloric acid in the ratio of 4:2:1) in a glass bottle. Five ml of triacid mixture was added to 2ml of filtered broth and heated for digestion until the solution became transparent. Then the volume was made up to 20ml (White *et al.*, 1997) and Zn concentration was measured by using an AAS (ISE 3000AA, Thermo Fisher).

2.4. Molecular characterization of ZSB isolate

The bacteria showing maximum solubilization efficiency was further identified by 16S rRNA sequencing. Bacterial DNA was extracted from a loop full of overnight grown cells on nutrient agar by using a QIAmp DNA mini kit (Qiagen, Duesseldorf, Germany) as per manufacturers' protocol. The purity and concentration of DNA were examined by electrophoresis on agarose gel (1%) and quantified with a

Nanodrop 2000c spectrophotometer (Thermo Fischer Scientific Inc, Walthum, MA, USA). Two universal primers 27f and 907r were used for PCR amplification using a thermocycler (Bio-rad T100, USA) with initial 5 min denaturation at 95°C, followed by 35 cycles of 1 min denaturation, 1 min annealing at 55°C, 2 min extension at 72°C, and a final extension of 10 min at 72°C. The PCR product was resolved in 1.2% agarose gel along with ethidium bromide staining followed by visualized using a gel documentation system (Bio-rad Gel Doc XR+, USA).

The PCR product was purified using a DNA purification kit (Illustra GFX PCR DNA and Gel Band purification kit, GE Healthcare, UK) as described in the manufacturer's protocol. Purified amplicon then sequenced by outsourcing. The DNA sequences thus obtained were compared with known sequences through BLAST (http://www.ncbi.nlm.nih.gov/blast). Phylogenetic analysis was conducted based on neighbor joining method by using MEGA-X.

3. Results

In this study attempts were made to isolate zinc solubilizing bacteria from soil samples of Bhitarkanika mangrove soil. After examining the physicochemical analysis of collected soil samples the pH was found in the range of 7.2 to 7.8. The soil samples were clay type and found to have high water holding capacity as well as moisture content. The water holding capacity was observed between 38 % to 49 % and the moisture content were recorded between 33.86 to 34.2%.

Seven zinc solubilizing bacteria were isolated from the mangrove soils showing distinct solubilization zone and having morphologically different colonies. The selected isolates were characterized based on morphological and biochemical properties as presented in Table 1. The population density of the ZSB isolates in the mangrove soil ranged from $16x10^5$ to $28x10^6$ CFU/g soil. Three ZSBs showed siderophore production ability, whereas all bacteria showed negative response to indole test (Table 1). The ZSB isolates were tested for their efficiency to solubilize the insoluble zinc on solid medium and ZSB2 showed the highest zinc solubilization efficiency i.e 111.51% whereas, ZSB7 showed the least solubilization efficiency of 22.21% (Fig. 1).

A gradual decrease in the pH of the culture broth was observed under liquid culture conditions up to 6 days of incubation, after which it remained constant (Fig. 2). Maximum decrease of pH was observed for ZSB2 on 5^{th} days of incubation, whereas ZSB7 showed least change in culture pH. With decreasing pH, the titrable acidity of the culture medium was found to be increased gradually for each ZSB isolates. The maximum titrable acidity of 3.34 ml was recorded by ZSB2 on 6^{th} day of incubation followed by ZSB4 and minimum acidity was observed by ZSB7 (Fig. 3). ZSB2 showing maximum solubilization efficiency and titrable acidity was tested for zinc solubilization in liquid medium containing zinc oxide as a sole source. Fig. 4 showed a significant ($P<0.05$) increase in zinc ion concentration in each day (except 6^{th} day) in the culture medium with a maximum value of 6.55 mg/L on 5^{th} day of incubation. The value showed 2.5 times higher available zinc ion concentration in the ZSB2 inoculated culture on 5^{th} day of incubation than the control (uninoculated) medium. Further the ZSB2 bacterial isolate was identified as *Enterobacter kobei* by 16SrRNA sequencing showing 95% similarities with the data available in the NCBI gene bank (Fig 5).

Table-1

Morphological characterization of ZSB isolates

Sl. No	Isolates	Gram staining Type	Population density (CFU/g soil)	Nature	Colony Forms	Elevation	Margins	Optical Density	Colony colour	Sidero-phore
1	ZSB1	-ve	$17x10^6$	Dull	Irregular	Flat	Undulated	Opaque	Yellow	+
2	ZSB2	+ve	$19x10^6$	Sticky	Circular	Raised	Round	Opaque	White	+
3	ZSB3	+ve	$22x10^6$	Sticky	Irregular	Flat	Round	Opaque	White	-
4	ZSB4	+ve	$16x10^6$	Dull	Circular	Raised	Undulated	Opaque	Off white	++
5	ZSB5	-ve	$28x10^6$	Dull	Circular	Raised	Undulated	Opaque	Pale Yellow	-
6	ZSB6	+ve	$24x10^6$	Sticky	Circular	Flat	Round	Opaque	Pale Yellow	-
7	ZSB7	-ve	$22x10^6$	Dull	Irregular	Raised	Undulated	Transparent	White	-

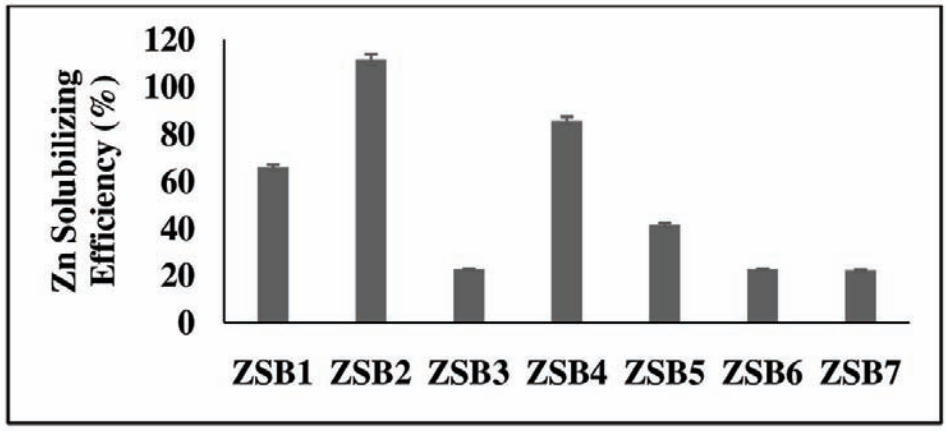

Fig 1. Solubilizing efficiency of ZSB isolates on solid media.

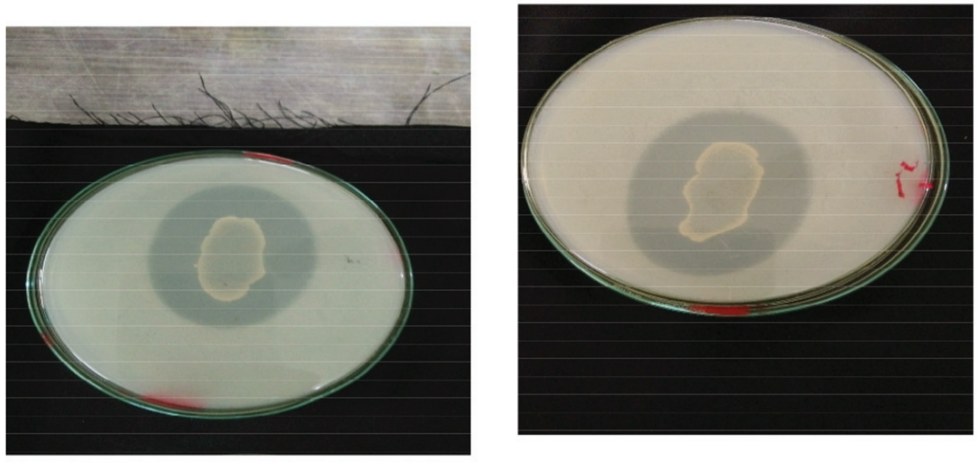

Plate 1: Solubilization zone produced by ZSB isolates (A) ZSB2 (B) ZSB4 on Bunt and Rovira solid medium.

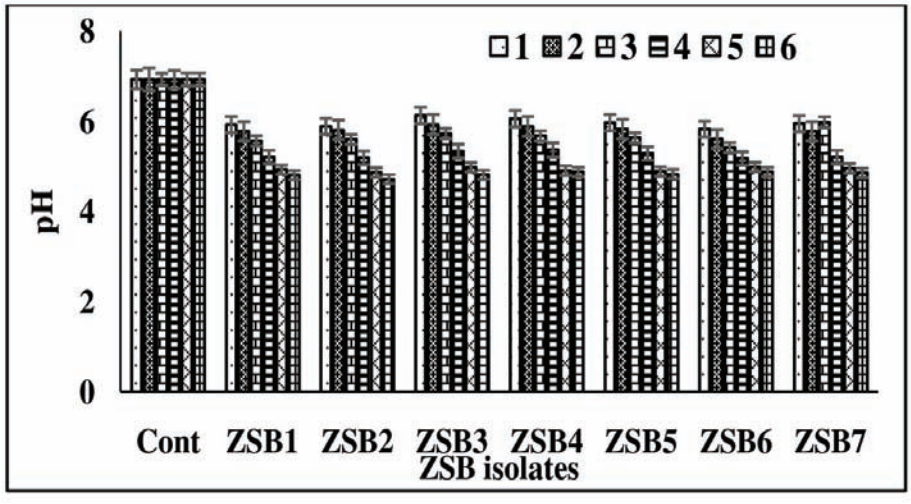

Fig 2. Change in pH of culture broth by ZSB isolates.

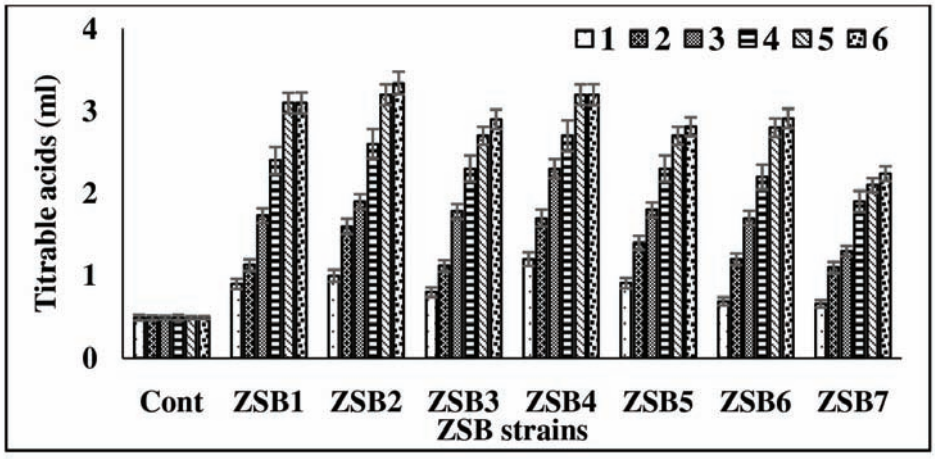

Fig 3: Amount of titrable acids produced by ZSB isolates on different days of incubation.

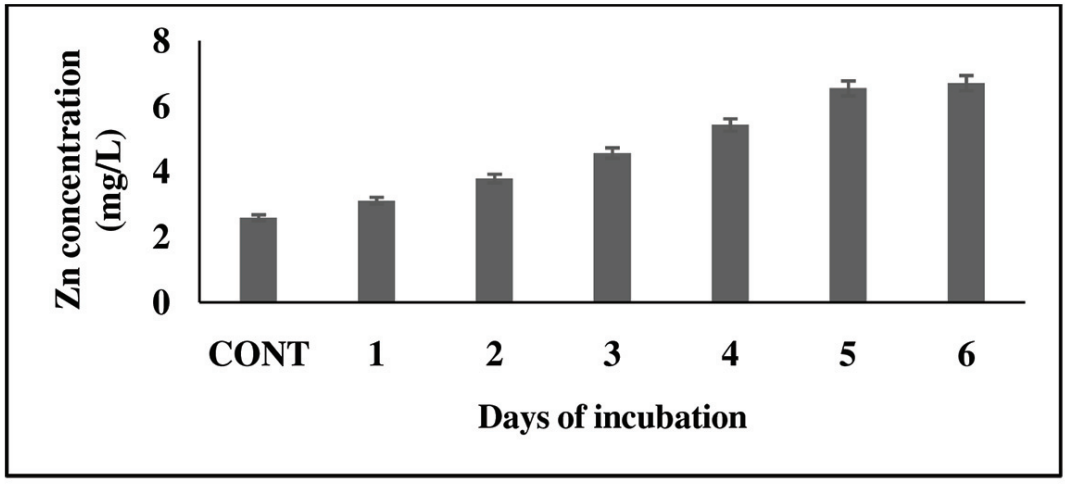

Fig 4: Concentration of soluble Zn ion in the culture broth of ZSB2.

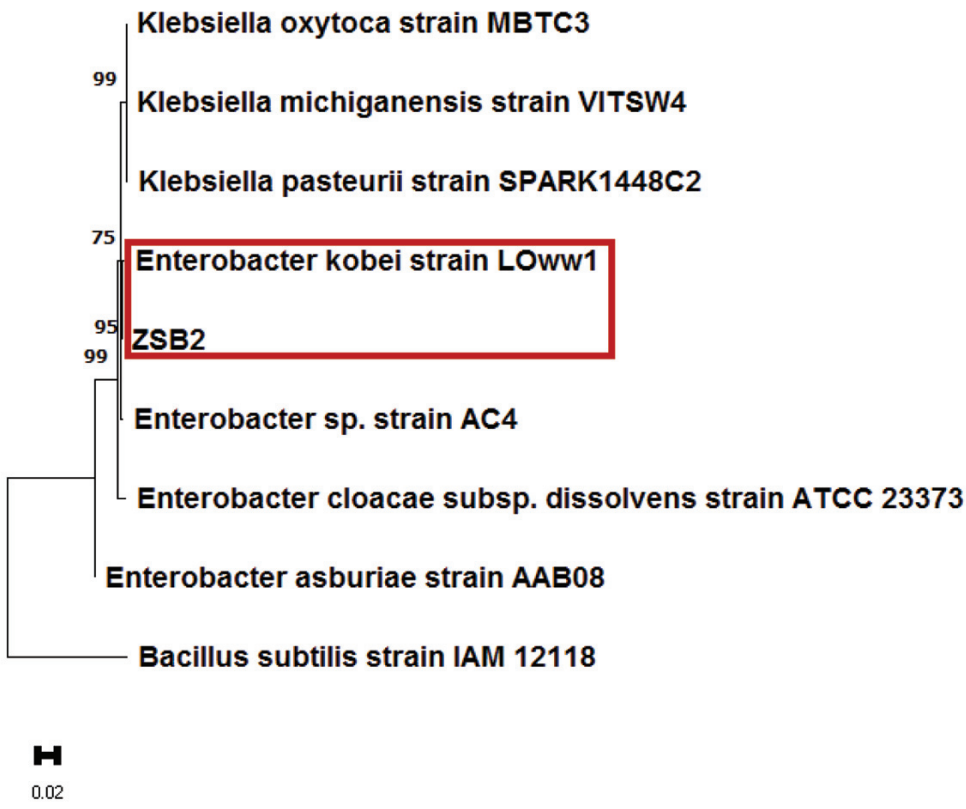

Fig 5: 16S rRNA sequencing of ZSB2 identified as *Enterobacter kobei.* Phylogenetic tree showing the relationships among the isolated bacteria and between representatives of other related taxa. The tree was constructed by using the software package MEGAX.0 and the distance matrix inferred by the neighbour-joining method using Jukes–Cantor model. The number at the branching points indicate the levels of bootstrap support based on data for 1000 replicates; values greater than 50% are only presented. The scale bar indicates 0.02 substitutions per nucleotide position.

Discussion

Bhitarkanika mangrove is located in the east coast is a second largest tropical mangrove ecosystem in India. A diverse range of microorganisms are present in mangrove areas due to its estuary environment to maintain the nutrient cycling and ecological balances. On the basis of physicochemical properties of soil, the microbial diversity varies from place to place. In this study, the soil samples were clay type and the pH ranges from 7.2 to 7.8. Our results corroborate with the reports of Mishra *et al.* (2012) where a pH variation of 6-8 was mentioned due to seasonal fluctuation. Banerjee *et al.* (2018) reported that silt and clay soil have a positive correlation whereas, sandy soil has negative correlation with the organic carbon content of the soil. High organic carbon content leads to high microbial diversity and population than that of sandy soil as evident from our result (Table 1). This variation in population size might be due to other soil factors such as soil nutrients, pH, moisture and salinity (Vikram *et al.*, 2007).

There is several literature suggesting a variety of soil microbes which can solubilize zinc from insoluble mineral sources to available form, thereby increasing availability to crop and produce plant growth promoting substances like auxin which is known to stimulate the growth of crop plants (Sedhegi *et al.*, 2012). In our study out of several colonies grew on the Bunt and Rovira medium, 7 best isolates were selected on the basis of their zinc solubilizing efficiency and colony morphology on solid medium. Among the 7 isolates, ZSB2 showed maximum solubilization efficiency (Fig 1, Plate 1). In our study with increase in incubation period, a decrease in pH and increase in titrable acid of the culture medium by all the seven ZSB isolates was observed (Fig. 2 and 3). It might be due to production of organic acid. A drop in pH of the broth with insoluble zinc compounds has been argued by many authors. Fasim *et al.* (2002) reported a decrease in the pH due to bacterial growth was associated with the secretion of gluconic acid, which was attributed to release Zn ions from Zn metal. Production of H^+ and organic acids (gluconic acid and 2-keto gluconic acid) was found to be

the most important mechanism for heterotrophic metal (Zn) solubilization (Bennett *et al.,* 1978) however, few bacteria solubilize Zn by secreting siderophores (Saravanan *et al.*, 2011). In this study, ZSB1, ZSB2 and ZSB4 showed better solubilization efficiency than other strains, this could be due to the dual mechanism of organic acid production and siderophore production by the isolates as evident from Table 1 and Figure 2,3. Among them ZSB2 showed maximum efficiency both in solid as well as liquid medium (Fig. 1, 5) and identified as *Enterobacter kobei* by 16SrRNA sequencing with 95% similarities with *Enterobacter kobei* strain LOww1 by neighbour-hood joining method (Fig 5).

4. Conclusion

From this study it was concluded that the mangrove soil has a rich diversity of zinc solubilizing bacteria. Among the isolates, highest Zn solubilization efficiency was observed by ZSB2 which was further identified as *Enterobacter kobei* by 16 S rRNA sequencing. A 2.5-fold rise in available Zn concentration was detected from the culture broth of *E. kobei* as compared to the uninoculated culture. Along with Zinc solubilization the isolate also showed siderophore production, thus may be considered as a suitable candidate for plant growth promotion after evaluating its actual potential.

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References

Alexander, M. (1997). Introduction to Soil Microbiology, John Wiley and Sons, New York.

Alloway, B. J. (2004). Zinc in soils and crop nutrition. IZA Publishers, International Zinc Association, Brussels, 1-116.

Banerjee, K., Bal, G. and Mitra, A. (2018). How soil texture affects the organic carbon load in the mangrove ecosystem? A case study from Bhitarkanika, Odisha. *In*: Singh VP, Yadav, S. & Yadava, R, N. (eds.), Environmental Pollution, Springer, Singapore, pp. 329–341.

Barthalomew, J. W. and Mittewer, J. (1950). A simplified bacterial strain. Stain Tech. 25:153.

Bennett, J. C. and Tributsch, H. (1978). Bacterial leaching on pyrite crystal surfaces. J. Bacteriol. 134: 310–317.

Broadley, M. R., White, P.J., Hammond, J. P., Zelco, I. and Lux, A. (2007). Zinc in plants. New Phytologist 173: 677-702.

Bunt, J. S. and Rovira, A. D. (1955). Microbiological studies of some subantarctic soils. Journal of Soil Science. 6(1): 119-128.

Chang, H. B., Lin, C. W. and Huang, H. J. (2005). Zinc induced cell death in rice (*Oryza sativa* L.) roots. Plant Growth Regul. 46: 261–266.

Dave, B. P., Anshuman, K. and Hajela, P. (2006). Siderophores of halophilic archaea and their chemical characterization. Ind. J. Exp. Biol. 44: 340–344.

Deepak, J., Geeta, N., Sachin, V. and Anita, S. (2013). Enhancement of wheat growth and Zn content in grains by zinc solubilizing bacteria. Int. J. Agr. Environ. Biotechnol. 6: 363–370.

Fasim, F., Ahmed N., Parsons R. and Gadd, G. M. (2002). Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. FEMS Microbiol. Lett. 213: 1–6.

Jones, D. L. and Darrah, P. R. (1994). Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. Plant & Soil 166: 247–257.

Kabata, A. and Pendias, H. (2001). Trace elements in soils and plants, CRC Press, London.

Kamran, S., Izzah, S., Deeba, N. B., Muhammad, R., Kauser, A. M., and Samina, M. (2017). Contribution of Zinc solubilizing bacteria in growth promotion and Zinc content of wheat. Front Microbiol. 8: 2593.

Karak, T., Singh U. K., Das S., Das D. K. and Kuzyakov Y. (2005). Comparative efficacy of $ZnSO_4$ and Zn-EDTA application for fertilization of rice (*Oryza sativa* L.). Arch. Agron. Soil Sci. 51, 253–264.

Khande, R., Sushil K. S., Ramesh, A. and Mahaveer, P. S. (2017). Zinc solubilizing *Bacillus* strains that modulate growth, yield and zinc biofortification of soybean and wheat. Rhizosphere 4: 126–138.

Khanghahi, M.Y., Ricciuti, P., Allegretta, I., Terzano, R. and Crecchio, C. (2018). Solubilization of insoluble zinc compounds by zinc solubilizing bacteria (ZSB) and optimization of their growth conditions. Environ. Sci. Pollut. Res. 25:25862-25868.

Mishra, R. R., Swain, M. R., Dangar, T. K. and Thatoi, H. (2012) Diversity and seasonal fluctuation of predominant microbial communities in Bhitarkanika, a tropical mangrove ecosystem in India. Int. J. Trop. Biol 60: 909–924.

Nenwani, V., Doshi, P., Saha, T. and Rajkumar, S. **(**2010). Isolation and characterization of a fungal isolate for phosphate solubilization and plant growth promoting activity. J. Yeast Fungal Res. 1:9-14.

Sadeghi, A., Karimi, E., Dahaji, P. A., Javid, M. G., Dalvand, Y. and Askari, H. (2012). Plant growth promoting activity of an auxin and siderophore producing isolate of Streptomyces under saline soil conditions. World J. Microbiol. Biotechnol. 28:1503–1509.

Saravanan, V. S., Kumar, M. R. and Sa, T. M. (2011). Microbial zinc solubilization and their role on plants, in bacteria in agrobiology: Plant nutrient management, (Maheshwari, D. K., Ed.), Springer, Berlin, pp. 47–63.

Saravanan, V. S., Madhaiyan, M. and Thangaraju, M. (2007). Solubilization of zinc compounds by the diazotrophic, plant growth promoting bacterium *Gluconacetobacter diazotrophicus*. Chemosphere 66: 1794–1798.

Shukla, A. K., Behera, S. K. and Prakash, C. (2021). Deficiency of phyto-available sulphur, zinc, boron, iron, copper and manganese in soils of India. Sci. Rep. 11: 19760.

Vaid, S. K., Kumar, B., Sharma, A., Shukla, A. K. and Srivastava, P. C. (2014). Effect of zinc solubilizing bacteria on growth promotion and zinc nutrition of rice. J. Soil Sci. Plant Nut. 14: 889–910.

Vikram, A. and Hamzehzarghani, H. (2008). Effect of phosphate solubilizing bacteria on nodulation and growth parameters of greengram (*Vigna radiata* (L.) Wilczec.). Res. J. Microbiol. 3: 62–72.