



RESEARCH ARTICLE

Isolation, characterisation and evaluation of native zinc-solubilising bacteria from the sorghum rhizosphere

Aglin Sherin S¹, Anbukkarasi Kaliyaperumal^{2*}, T S Raja Rajeshwaran¹ & R Jeyajothi³

¹Department of Soil Science, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Baburayanpettai, Chengalpattu 603 201, Tamil Nadu, India

²Department of Natural Resource Management, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Baburayanpettai, Chengalpattu 603 201, Tamil Nadu, India

³Department of Agronomy, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Baburayanpettai, Chengalpattu 603 201, Tamil Nadu, India

*Correspondence email - anbukkak@srmist.edu.in

Received: 07 June 2026; Accepted: 13 July 2026; Available online: Version 1.0: 01 September 2026

Cite this article: Sherin SA, Anbukkarasi K, Rajeshwaran TSR, Jeyajothi R . Isolation, characterisation and evaluation of native zinc-solubilising bacteria from the sorghum rhizosphere. Plant Science Today. 2026;13(sp5): 01–10. <https://doi.org/10.14719/pst.15912>

Abstract

Zinc is an important micronutrient required for plant growth and development. However, a large proportion of soil zinc occurs in insoluble forms that are poorly available to plants, leading to zinc deficiency. To alleviate the zinc deficiency, zinc-solubilising bacteria (ZSB) are employed. This study aimed to isolate, characterise and assess the zinc solubilisation potential of different genera from the rhizosphere soil of sorghum. A total of 10 ZSB isolates were obtained using Bunt and Rovira medium amended with 0.1 % zinc oxide (ZnO). The isolates were screened for their ability to solubilise insoluble 0.1 % ZnO using a plate assay. Among the 10 isolates, ZNS2 exhibited the highest zinc solubilisation efficiency (224.99 %), followed by ZNS7 (212.49 %) in zinc oxide. In the quantitative assay, ZNS2 released the highest available zinc (299.79 mg L⁻¹) in ZnO-amended medium. Zinc solubilisation was associated with a significant reduction in pH of the medium, suggesting organic acid-mediated solubilisation. The isolates ZNS1, ZNS2, ZNS7, ZNS8 and ZNS9 were non-haemolytic (γ-haemolysis) on blood agar, indicating non-pathogenicity. Based on the 16S rRNA sequencing, isolates ZNS1, ZNS2, ZNS7, ZNS8 and ZNS9 were identified as *Niallia nealsonii*, *Pseudomonas fluorescens*, *Micrococcus luteus*, *Klebsiella pneumoniae* and *Paenibacillus lautus*, respectively. To the best of our knowledge, *N. nealsonii*, *M. luteus* and *P. lautus* are reported here for the first time as zinc solubilising bacteria. These isolates demonstrated considerable potent for improving zinc availability and may contribute to reducing dependence on chemical zinc fertilisers. These findings suggest that native rhizobacterial isolates could serve as sustainable, low-cost bioinoculants for improving zinc bioavailability and addressing zinc-related malnutrition. Further studies are required to evaluate their zinc-solubilising efficiency under soil conditions and their effects on crop growth and zinc uptake.

Keywords: biofertiliser; rhizobacteria; sorghum; zinc biofortification; zinc solubilisation; zinc solubilising bacteria

Introduction

Zinc is an indispensable micronutrient and plays a critical role in numerous metabolic and physiological processes in the plant system (1–3). It is essential for the synthesis of tryptophan, which is a precursor for indole acetic acid production (4) and helps in the synthesis of secondary metabolites that alleviate abiotic stress (5). Although it is required in small amounts, its deficiency in crops leads to physiological effects such as interveinal chlorosis, reduced IAA production in shoot apical meristems and young leaves, decreased leaf size and stunted growth. It also manifests visually as spotting on older leaves along with an increased susceptibility to bacterial and fungal infections (6). Deficiency of zinc in soil also results in reduced crop yields and decreased nutritional quality (7), which ultimately causes nutritional defects in humans, especially children (8–10). It is estimated that approximately 50 % of Indian soil is deficient in zinc (11), mainly due to various environmental and soil conditions such as salinity, high pH, high temperature, high amounts of organic matter, phosphorus and iron availability

(12). Currently, zinc deficiency is listed as a major risk factor for human health. As plants serve as a main source of zinc in the human diet, zinc deficiency affects women and children (13, 14) and causes over 400000 deaths each year (15).

Plants only absorb zinc in its divalent (Zn²⁺) and chelated form from the soil solution. Most of the zinc exists in soil as minerals such as smithsonite (ZnCO₃), zincite (ZnO), hopeite (Zn₃(PO₄)₂·4H₂O) and sphalerite (ZnS), which are sparingly soluble (16,17). Inorganic fertilisers such as zinc sulphate (ZnSO₄) have been widely used to manage zinc deficiency due to their low cost and high solubility (18). However, zinc fertilisers are often ineffective, as 96–99 % of the applied ZnSO₄ rapidly converts into insoluble forms within 7 days of application under alkaline, calcareous soil conditions characterised by high pH (> 7.5), high CaCO₃ content and high available phosphorus levels. Furthermore, the long-term application of zinc-based chemical fertilisers leads to disturbance of soil ecology, changes in soil microbial activity and deterioration of soil fertility (19).

Hence, the use of zinc-solubilising microorganisms, especially bacteria and fungi, is an effective alternative strategy to enhance zinc bioavailability in soil and improve plant growth (19). Zinc-solubilising microorganisms reside in the rhizosphere and transform complex, inaccessible forms into easily accessible forms by the synthesis of organic acids, enabling plants to absorb zinc and contributing to better growth and development (20). A previous study reported that the generation of protons and organic acids might contribute to the solubilisation of insoluble zinc compounds by *Gluconacetobacter diazotrophicus* (21). Similarly, *Bacillus glycinifermentans* CRS-9 and *Bacillus tequilensis* CRS-38 produce gluconic acid and 2-ketogluconic acid to transform the inaccessible form of zinc compounds to an accessible form (22). Additionally, other mechanisms such as siderophore production, proton extrusion and oxidoreductive processes on the cell membrane (23) and the production of chelated ligands, phytohormones and vitamins (24) encourage zinc solubilisation.

Recently, many researchers have reported that *Pseudomonas aeruginosa*, *G. diazotrophicus*, *Bacillus aryabhattai*, *Serratia liquefaciens*, *S. marcescens* and *B. thuringiensis*, *Bacillus spp.*, *Pseudomonas striata*, *Pseudomonas fluorescence*, *Burkholderia cenocepacia*, *Pseudomonas protegens* RY2 and *Bacillus altitudinis* have been able to solubilise zinc under different soil conditions, thus helping to alleviate Zn deficiency in crops (11, 20, 21, 25–29).

In addition to zinc solubilisation, zinc-solubilising bacteria (ZSB) also helps enhance plant growth and yield through biological nitrogen fixation (BNF), synthesis of phytohormones, aid in production of the siderophores and antibiotics, ACC deaminase activity, exopolysaccharide production and antifungal compounds. Several research studies have also observed the effect of ZSB on soil zinc availability and zinc uptake in soybean, rice, wheat, green gram, etc. (11, 18, 30, 31). The availability of zinc in soil and its concentrations in plant tissues is affected by the composition and abundance of microbial communities in the rhizosphere (32). Therefore, the inoculation of efficient bacterial strains might be helpful to enhance both soil micronutrient zinc availability and plant uptake. The use of native microorganisms as a bioinoculant may be appropriate because they are competent and adapted to their specific local climate and soil conditions. In another study, the native ZSB *B. paramycooides* strain-1 improved zinc uptake by 17 % in rice and enhanced the water-soluble and exchangeable Zn fractions in deficient soils by 22–24 % (33, 34).

Sorghum (*Sorghum bicolor* (L.) Moench) is commonly cultivated in arid and semi-arid climatic conditions as it can tolerate drought conditions. With respect to micronutrients, sorghum is highly sensitive to zinc deficiency, which can cause interveinal chlorosis, stunted growth, delayed maturity and reduced grain size. Improving zinc nutrition in sorghum is therefore critical for sustaining productivity under stress conditions. Zinc seed priming in sorghum mitigates the toxicity of salinity by increasing photosynthetic pigments, better leaf water status, osmolyte accumulation and higher potassium accumulation (5). The inoculation of selected ZSB, namely *Bacillus sp.* (ZM20), *B. aryabhattai* (ZM31 and S10) and *Bacillus subtilis* (ZM63), improved root length, shoot length, plant fresh and dry biomass and could be potential bio-inoculants for biofortification of maize to overcome the problems of malnutrition (7).

Therefore, the application of ZSB strains in crops could be an alternative to chemical zinc fertiliser and address the malnutrition problem through increased nutrient concentration in edible parts of

plants (35). The study hypothesised that bacteria isolated from the sorghum rhizosphere can solubilise various forms of inorganic zinc compounds in vitro and that characterising their solubilisation potential and would identify candidates for future soil and plant bioassays. With this in view, the present study was designed to isolate native zinc-solubilising rhizobacteria from the sorghum rhizosphere and evaluate their zinc solubilisation potential through qualitatively, using plate assays to determine the solubilisation index and efficiency based on halo zone formation and quantitatively, using atomic absorption spectrophotometry to measure soluble zinc released in broth culture. Finally, the promising isolates were identified based on morphological, biochemical and molecular approaches.

Materials and Methods

Collection of samples

Rhizosphere soil samples were collected from sorghum (*S. bicolor*) cultivated at the College Farm, SRM College of Agricultural Sciences, Baburayanpettai, Chengalpattu, Tamil Nadu, India (12.378887° N, 79.731607° E) during May–June 2025. Ten healthy sorghum plants were randomly selected and carefully uprooted to retain the soil adhering to the roots. The prevailing meteorological conditions during the sampling period represented the typical tropical climate of the region, with a maximum air temperature of 40 °C, a minimum air temperature of 28 °C, mean relative humidity of 73 % at 07:22 hr and 36 % at 14:22 hr, soil temperatures of 33 °C, 34 °C, 35 °C and 44 °C at depths of 5, 10, 15 and 20 cm, respectively, an average daily sunshine duration of 9.15 hr and a mean daily evaporation rate of 7.2 mm, while no rainfall was recorded during the sampling period. Approximately 50 g of rhizosphere soil was aseptically collected from each plant into sterile polyethylene bags, transported to the laboratory and stored at 4–7 °C until the isolation of ZSB was carried out (36, 37). The physicochemical characteristics of the rhizosphere soil included a pH of 7.8, electrical conductivity of 0.20 dS m⁻¹ (1 : 2.5 soil-water suspension), organic carbon content of 0.80 % (chromic acid wet digestion method), HCl-extractable zinc of 2.7 mg kg⁻¹ and DTPA-extractable available zinc of 1.2 mg kg⁻¹. The prevailing meteorological conditions during the sampling period represented the typical tropical climate of the region, with a maximum air temperature of 40 °C, a minimum air temperature of 28 °C, mean relative humidity of 73 % at 07:22 hr and 36 % at 14:22 hr, soil temperatures of 33 °C, 34 °C, 35 °C and 44 °C at depths of 5, 10, 15 and 20 cm, respectively, an average daily sunshine duration of 9.15 hr and a mean daily evaporation rate of 7.2 mm, while no rainfall was recorded during the sampling period.

Isolation of zinc-solubilising rhizobacteria

Serial dilution and plating techniques were employed using Bunt and Rovira medium (glucose 20.0 g, peptone 1.0 g, yeast extract 1.0 g, (NH₄)₂SO₄ 0.50 g, K₂HPO₄ 0.40 g, MgCl₂ 0.10 g, FeCl₃ 0.01 g, distilled water 1000 mL, pH 6.7) supplemented with 0.1 % ZnO for the isolation of ZSB. In this method, 10 g of rhizosphere soil were added into 90 mL of sterilised distilled water, giving a 10⁻¹ dilution. The 10⁻¹ dilution was serially diluted to 10⁻¹. One mL of the aliquot from each 10⁻⁵ and 10⁻⁷ dilution was spread individually on Bunt and Rovira medium augmented with 0.1 % ZnO and plates were incubated at 28 ± 2 °C for 8 days (38, 39). After incubation, bacterial colonies that exhibited a clear zone with different morphologies were selected and transferred into nutrient broth. Isolates showing distinct colony

characters were preserved in a 15 % glycerol stock at the temperature of -20 °C for further analysis.

Screening for zinc solubilisation potential of rhizobacteria

The isolates exhibited a clear zone in Bunt and Rovira medium containing 0.1 % ZnO was further screened to assess zinc solubilisation potential via qualitative and quantitative assays.

Qualitative assessment of zinc solubilisation using solid medium

Zinc solubilisation potential was assessed qualitatively by plate assay. Active cultures were spot inoculated on the Bunt and Rovira media augmented with 0.1 % ZnO (16, 38) and were incubated for the 8 days at 28 ± 2 °C. Following incubation, isolates that showed the clear zone around the colonies were designated as positive for zinc solubilisation. Each treatment was kept in triplicate. The diameter of colony and halo zones were measured at 2nd, 4th, 6th and 8th days of incubation for the investigation of zinc solubilisation index (ZSI) and zinc solubilisation Efficiency (ZSE) using the following formulae (27)

Zinc solubilisation index (ZSI) =

$$\frac{\text{Colony diameter} + \text{Halo zone diameter}}{\text{Colony diameter}} \quad (\text{Eq. 1})$$

Zinc solubilisation efficiency % (ZSE) =

$$\frac{\text{Halo zone diameter}}{\text{Colony diameter}} \times 100 \quad (\text{Eq. 2})$$

Quantitative assessment of zinc solubilisation in liquid medium

The bacterial isolates exhibiting the highest ZSI and efficiency in the plate assay were further evaluated quantitatively in liquid medium following the method of previous researchers (25). An aliquot of 0.1 mL (approximately 10^8 CFU mL⁻¹) of overnight cultures was inoculated into 100 mL of the Bunt and Rovira broth amended with 0.1 % ZnO and incubated in the rotary shaker at the 120 rpm for 8 days. The experiment was conducted in triplicate. Uninoculated broth with 0.1 % ZnO served as the control. After incubation, 10 mL of broth was centrifuged at 10000 rpm for 10 min and the supernatant solution was filtered through the Whatman No. 1 filter paper. Then, 2 mL of clear filtrate was diluted to 100 mL with sterile distilled water and the concentration of soluble zinc was determined through atomic absorption spectrophotometry at a wavelength of 213.9 nm (AAS, AA-6300, Shimadzu).

Determination of pH

To study the effect of bacterial isolates on pH, the pH variation in inoculated and uninoculated media were monitored at 2nd, 4th, 6th and 8th days of incubation using a pH meter (36). A 100 µL of overnight cultures (approximately 10^8 CFU mL⁻¹) were inoculated into the 100 mL of Bunt and Rovira broth amended with 0.1 % ZnO and incubated in the rotary shaker at 120 rpm for up to 8 days. Uninoculated broth with 0.1 % ZnO served as the control. After incubation, the pH of the broth was recorded after the 2nd, 4th, 6th and 8th days of incubation. Experiments were performed in triplicate.

Pathogenicity test

The pathogenicity test was performed for all ZSB isolates to distinguish non-pathogenic isolates from potential pathogens. All ZSB were spot inoculated onto blood agar base (Himedia) media containing 5 % v/v with the sterile sheep blood and incubated for 48 hr. After incubation, pathogenicity was observed as beta-haemolysis (clear zones), alpha-

haemolysis (green zones) and gamma-haemolysis (no clear zones) (40).

Morphological and biochemical characterisation

The morphological characterisation of the five promising isolates was carried out by observing colony morphology (colour, shape, size, margin, elevation and surface texture) on nutrient agar media after 48 hr at 28 ± 2 °C and cell morphology (shape, Gram reaction and motility) by light microscopy. Gram staining was performed by a standard process of four-step method using crystal violet, Gram's iodine, acetone-alcohol decolouriser and safranin counterstain. Biochemical characterisation was performed using standard protocols: catalase activity (3 % H₂O₂); indole production in tryptone broth with Kovacs' reagent after 48 hr at 37 °C; methyl red and Voges-Proskauer tests in MR-VP broth after 5 days at 37 °C; gelatin liquefaction in nutrient gelatin after 7 days at 22 °C; starch hydrolysis on starch agar with iodine flooding after 72 hr; casein hydrolysis on skim milk agar after 72 hr and lipolysis on tributyrin agar after 72 hr. All tests were performed in triplicate as per the methods as described previously (41).

Molecular identification of bacterial isolates

The five promising bacterial isolates were further identified through the partial sequencing of the 16S rRNA gene. The bacterial isolates were grown in the nutrient broth at temperature of 25 °C for 24 hr. The pellets were collected by the centrifugation at the 10000 rpm at temperature 4 °C. The extraction of genomic DNA was done as per the protocol stated by previous researchers (42). Pure DNA was dissolved in 20 µL 1× TAE buffer solution. The quality of the genomic DNA was assessed at A260/A280 nm using Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -20 °C for further processing. The bacterial 16S rRNA genes were amplified by the polymerase chain reaction (PCR) using two universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTGTTCAGACTT-3'). Reactions were performed in the Mastercycler gradient (Eppendorf, India) in the following conditions: 94 °C for 4 min for the early denaturation stage, followed by 35 cycles of the 94 °C for 45 sec, 61 °C for 45 sec, 72 °C for the 2 min and the final extension at 72 °C for 7 min. The partial sequencing was performed by the Barcode Biosciences India Pvt. Ltd., Bengaluru, Karnataka, India and to be compared with the sequences in the NCBI database. The phylogenetic tree was constructed by using MEGA11 software. The nucleotide sequences were deposited in GenBank.

Statistical analysis

All experiments were conducted using a completely randomised design (CRD) with 3 replications (n = 3) for each treatment/isolate. Statistical analyses were performed using RStudio (Version 2024.12.1+563, Posit Software, Boston, MA, USA) running R version 4.4.2 (R Foundation for Statistical Computing, Vienna, Austria). One-way analysis of variance (ANOVA) was performed and treatment means were compared using Duncan's multiple range test (DMRT). Differences among treatment means were considered statistically significant at $p \leq 0.05$.

Results

Isolation and screening of bacterial isolates for zinc solubilisation

A total of 10 ZSB were isolated from the rhizosphere soil of the sorghum crop. These strains were purified and named ZNS1 to ZNS10 and stored in 15 % of glycerol stock at a temperature of

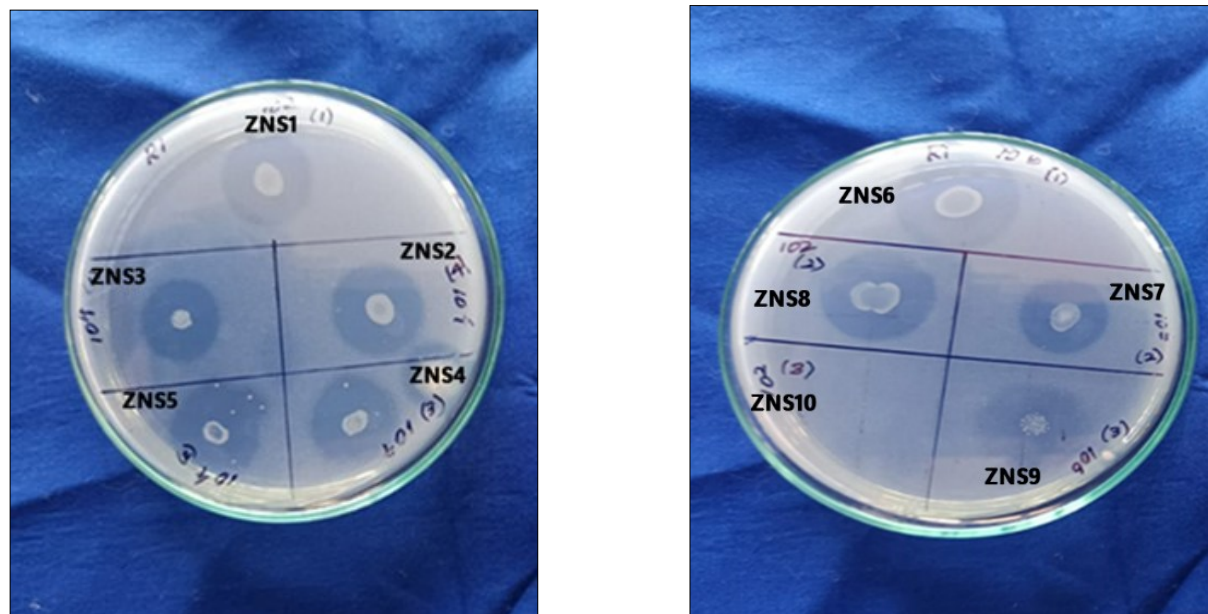


Fig. 1. Formation of halo zone by zinc-solubilising bacterial isolates in 0.1 % zinc oxide.

-20 °C for further process and experimentation. Zinc solubilisation ability was qualitatively estimated by the plate assay. The formation of halo zone indicates the solubilisation ability of the isolates. Halo zone formation begins at 24 hr after inoculation and continued to enlarge for up to 8 days, after which it stabilised (Fig. 1). The solubilisation index was analysed on the 2nd, 4th, 6th and 8th days.

The results showed the ZSI of the 10 bacterial isolates ranged between 2.37 and 3.28 after 8 days of incubation. Isolates ZNS2 and ZNS7 exhibited maximum SI of 3.28 and 3.12, respectively (Table 1). Bacterial isolate ZNS2 demonstrated the highest solubilisation efficiency (224.99 %), followed by ZNS7 (212.49 %) and ZNS8 (188.87 %) in ZnO-amended medium. The lowest zinc oxide solubilisation

efficiency was recorded in isolate ZNS3 (137.48 %) (Table 2).

Quantitative estimation of zinc solubilisation

The bacterial isolates were further assessed for the zinc solubilisation potential quantitatively in a broth medium supplemented with 0.1 % ZnO after 8 days of incubation by atomic absorption spectrophotometry. All isolates were positive for solubilising ZnO in liquid medium, except isolates ZNS5 and ZNS10. The maximum concentration of zinc released was observed by isolate ZNS2 (299.79 mg L⁻¹), followed by ZNS7 (222.34 mg L⁻¹) on the 8th day of incubation (Fig. 2).

Effect of zinc sources on pH of broth medium

Inoculation of the bacterial isolates in broth amended with 0.1 %

Table 1. Evaluation of the solubilisation index of zinc-solubilising bacterial isolates using solid medium containing ZnO at different time intervals

Isolates	2 d	4 d	6 d	8 d
ZNS1	1.67 ± 0.03d	1.99 ± 0.03c	2.20 ± 0.01c	2.50 ± 0.01e
ZNS2	1.74 ± 0.02c	2.20 ± 0.04ab	1.99 ± 0.01d	3.28 ± 0.03a
ZNS3	1.85 ± 0.02b	1.75 ± 0.01e	2.00 ± 0.01d	2.37 ± 0.01g
ZNS4	1.72 ± 0.02cd	1.88 ± 0.03d	1.78 ± 0.02e	2.87 ± 0.01c
ZNS5	–	–	–	–
ZNS6	1.50 ± 0.01e	2.12 ± 0.03b	2.37 ± 0.02b	2.56 ± 0.01d
ZNS7	1.55 ± 0.01e	2.00 ± 0.03c	2.44 ± 0.02b	3.12 ± 0.01b
ZNS8	2.00 ± 0.01a	2.29 ± 0.03a	2.63 ± 0.05a	2.89 ± 0.01c
ZNS9	1.85 ± 0.02b	2.12 ± 0.02b	2.63 ± 0.07a	2.44 ± 0.01f
ZNS10	–	–	–	–

Data represented as means ± standard error, n = 3. Means with similar letter(s) in each column did not differ significantly ($p \leq 0.05$).

Table 2. Evaluation of the zinc solubilisation efficiency (%) of zinc-solubilising bacterial isolates using solid medium containing ZnO at different time intervals

Isolates	2 d	4 d	6 d	8 d
ZNS1	66.75 ± 2.85d	99.37 ± 2.87c	120.00 ± 1.15c	149.97 ± 1.44e
ZNS2	74.45 ± 1.61c	120.09 ± 4.24ab	98.92 ± 1.44d	224.99 ± 0.36a
ZNS3	85.32 ± 2.29b	75.00 ± 0.72e	100.42 ± 7.10d	137.48 ± 1.17g
ZNS4	71.60 ± 1.89cd	87.98 ± 2.81d	77.86 ± 8.77e	187.49 ± 0.81c
ZNS5	–	–	–	–
ZNS6	50.00 ± 0.63e	111.67 ± 3.03b	137.49 ± 1.03b	155.54 ± 0.57d
ZNS7	54.99 ± 1.19e	100.47 ± 2.92c	144.44 ± 1.31b	212.49 ± 0.63b
ZNS8	100.02 ± 0.83a	128.57 ± 2.97a	162.99 ± 1.24a	188.87 ± 0.78c
ZNS9	84.89 ± 2.30b	112.05 ± 1.72b	163.25 ± 1.22a	144.43 ± 1.00f
ZNS10	–	–	–	–

Data represented as means ± standard error, n = 3. Means with similar letter(s) in each column did not differ significantly ($p \leq 0.05$).

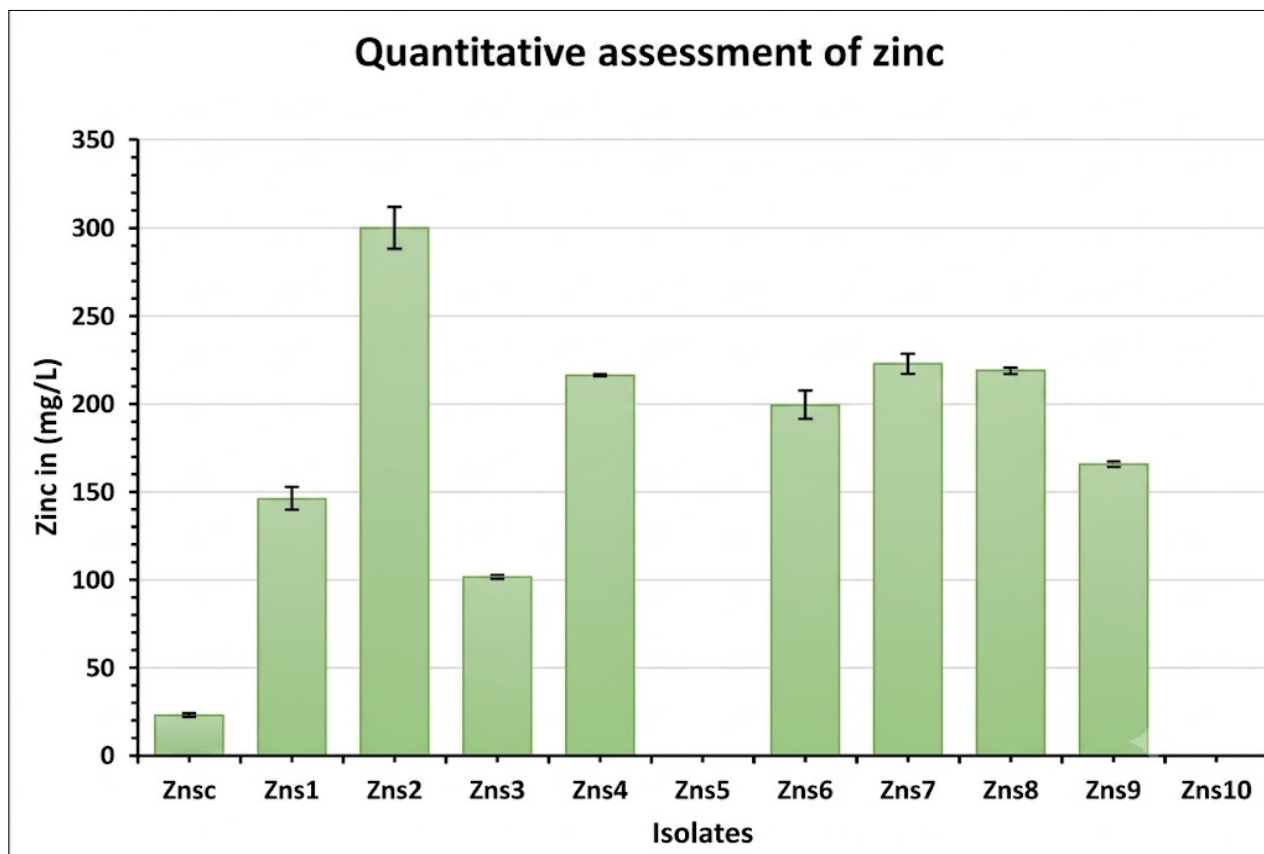


Fig. 2. Quantitative assessment of zinc-solubilising potential by potential bacteria using atomic absorption spectrophotometry.

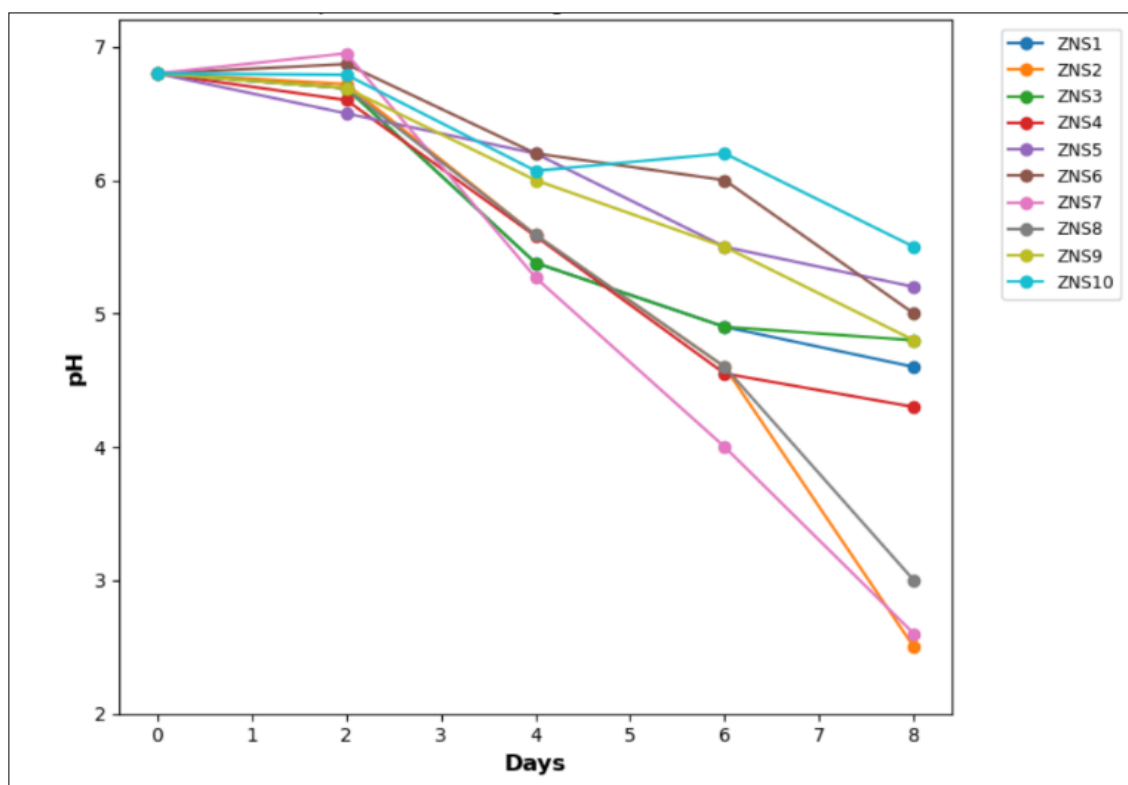


Fig. 3. Assessment of pH decrease by zinc-solubilising isolates in 0.1 % zinc oxide liquid medium at different days of incubation.

ZnO resulted in significant reduction in pH of the culture medium compared to uninoculated control, indicating the production of organic acid and active zinc solubilisation by the isolates (Fig. 3). In ZnO-amended broth, all isolates caused a progressive and pronounced decline in pH; the greatest acidification was observed with ZNS2 (final pH 2.50) and ZNS7 (final pH 2.60) on day 8, followed by ZNS8, confirming their ability to solubilise ZnO through

acidification mechanisms.

Pathogenicity of isolates

Five potential isolates (ZNS1, ZNS2, ZNS7, ZNS8 and ZNS9) were found to be non-pathogenic as they did not exhibit haemolytic activity on blood agar and therefore, they may be considered safer candidates for agricultural applications (Fig. 4). Therefore, it can be

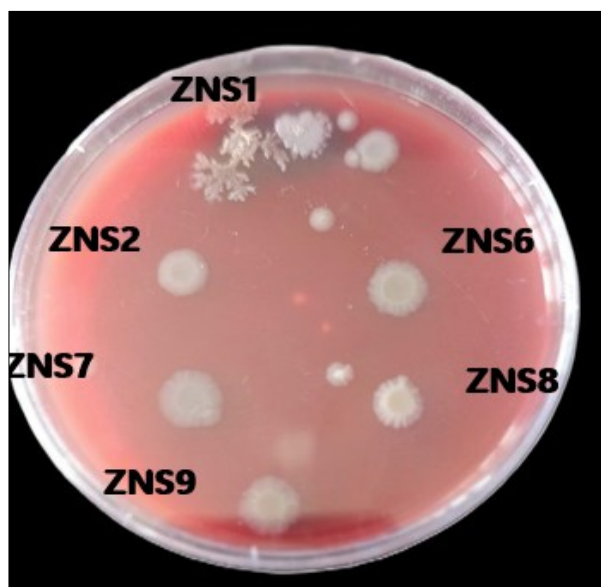


Fig. 4. Plates showing non-haemolytic nature of the promising zinc-solubilising bacterial isolates.

concluded that five promising isolates which can be used as PGPR for the plant growth without causing any human pathogenicity.

Morphological and biochemical characterisation of potential zinc solubilising bacteria isolates

Based on their consistently high performance across qualitative (solubilisation index and efficiency) and quantitative (soluble zinc released) zinc solubilisation assays, together with pronounced acidification of the medium and confirmed non-haemolytic (γ -haemolysis) behaviour on blood agar, five potent isolates, ZNS1, ZNS2, ZNS7, ZNS8 and ZNS9, were selected as the most promising zinc solubilising and subjected to detailed morphological and biochemical characterisation, summarised in Table 3 and 4. All isolates produced small- to medium-sized colonies with predominantly creamy to white colouration. Among the five isolates, one isolate (ZNS2) was Gram-negative, motile and rod-shaped. The ZNS1 and ZNS9 were Gram-positive, rod-shaped bacteria. Isolates ZNS7 and ZNS8 showed positive and variable Gram reactions, respectively. All selected isolates were positive for catalase, gelatin liquefaction, casein hydrolysis, citrate utilisation and H_2S production.

Table 3. Morphological characterisation of potential zinc-solubilising bacterial isolates

Morphological properties	ZNS1	ZNS2	ZNS7	ZNS8	ZNS9
Colony size	Small	Medium	Medium	Small	Medium
Colony colour	Creamy	Creamy	White	Cream yellow	White
Colony margin	Entire	Entire	Entire	Entire	Entire
Colony shape	Circular	Irregular	Circular	Circular	Circular
Surface	Dry, Translucent	Dry, Opaque	Moist, Opaque	Dry, Translucent	Moist, Opaque
Motility	Motile	Motile	Non-motile	Motile	Motile
Gram reaction	Positive	Negative	Positive	Gram variable	Positive
Cell shape	Rod	Rod	Cocci	Rod	Rod
Elevation	Flat	Flat	Raised	Flat	Raised

Table 4. Biochemical characterization of potential zinc-solubilising bacterial isolates

Biochemical Characteristics	ZNS1	ZNS2	ZNS7	ZNS8	ZNS9
Catalase	+	+	+	+	+
Indole test	+	-	-	+	-
Methyl red	+	+	-	-	-
Voges-Proskauer	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+
Starch hydrolysis	-	-	+	+	-
Casein hydrolysis	+	+	+	+	+
Lipolysis	+	-	-	+	-
Citrate utilisation	+	+	+	+	+
H_2S production	+	+	+	+	+
Haemolysis activity	-	-	-	-	-

Two isolates (ZNS1 and ZNS2) showed a positive methyl red test, while the remaining isolates were negative. The ZNS7 and ZNS8 showed positive amylase activity.

Taxonomic identification of promising isolates using 16S rRNA sequences

The partial 16S rRNA sequences for five potential isolates ZNS1, ZNS2, ZNS7, ZNS8 and ZNS9 were sequenced and analysed using the BLAST tool. The identified sequences were deposited in the GenBank. The accession numbers, strains with maximum of homology and percentage homology were summarised in the Table 5. Isolate ZNS1 was identified as *Niallia nealsonii*; ZNS2 as *Pseudomonas fluorescens*; ZNS7 as *Micrococcus luteus*; ZNS8 as *Klebsiella pneumoniae*; and ZNS9 as *Paenibacillus lautus*. The phylogenetic tree for the five isolates was constructed using the MEGA 11 and is depicted in Fig. 5.

Discussion

Zinc plays an important role in facilitating the physiological and metabolic activities in plants. Although required in the trace amounts, zinc deficiency is widespread and adversely affects both crop productivity and human nutrition. Many strategies, including genetic biofortification, agronomic biofortification and microbial biofortification, are employed to overcome zinc deficiency. Among these strategies, biofortification of food through the application of zinc-solubilising microorganisms can be a sustainable and environmentally sound approach. Zinc-solubilising microorganisms help to convert the inaccessible form of zinc into an easily available form for plant uptake. Keeping in view the importance of zinc-solubilising bacterial isolates on the growth and production of sorghum, this study aimed to isolate, evaluate and characterise potential ZSB from the sorghum rhizosphere. A total of 10 zinc solubilising rhizobacterial isolates were obtained from the sorghum rhizosphere, among which five bacterial isolates showed maximum solubilisation efficiency in 0.1 % ZnO. These strains were identified as *N. nealsonii* (ZNS1), *P. fluorescens* (ZNS2), *M. luteus* (ZNS7), *K. pneumoniae* (ZNS8) and *P. lautus* (ZNS9), which can effectively solubilise insoluble zinc oxide. These five promising strains were selected for further studies because they exhibited the

Table 5. Identification of zinc-solubilising bacterial strains based on 16S rRNA sequencing

Isolate code	NCBI strain	Similarity (%)	E value	GenBank Accession No.	Identified as
ZNS1	<i>Niallia nealsonii</i> strain HNXS-W9	99.54	0.0	PZ273399.1	<i>Niallia nealsonii</i>
ZNS2	<i>Pseudomonas fluorescens</i> strain PPA42	99.41	0.0	PZ244064.1	<i>Pseudomonas fluorescens</i>
ZNS7	<i>Micrococcus luteus</i> strain ANRFB29	99.69	0.0	PZ244065.1	<i>Micrococcus luteus</i>
ZNS8	<i>Klebsiella pneumoniae</i> strain 216-A	99.5	0.0	PZ273398.1	<i>Klebsiella pneumoniae</i>
ZNS9	<i>Paenibacillus lautus</i> strain B682/17	99.7	0.0	PZ273400.1	<i>Paenibacillus lautus</i>

NCBI: National Centre for Biotechnology Information; E value: expectation value obtained from BLAST sequence alignment.

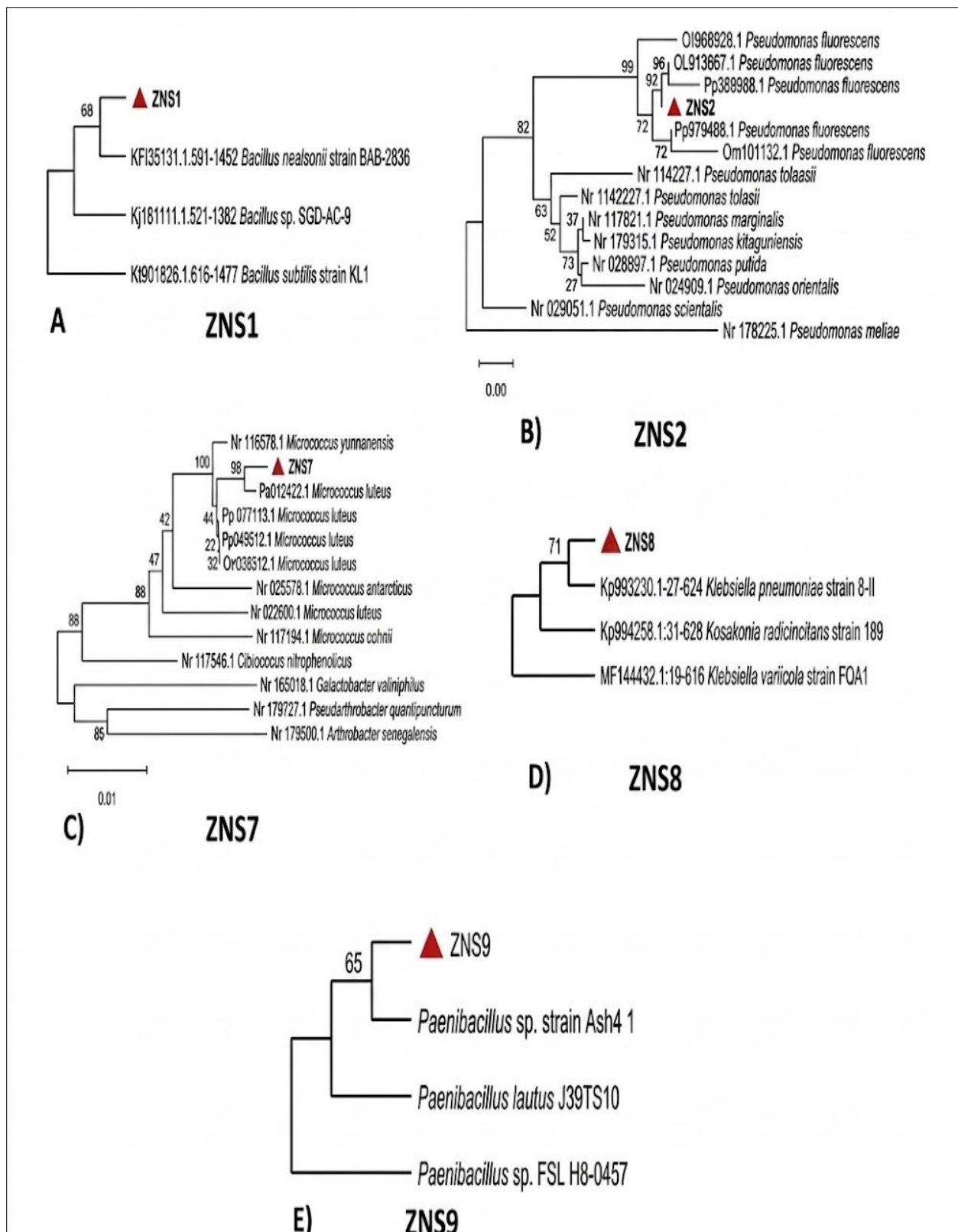


Fig. 5. Phylogenetic analysis. A: *Niallia nealsonii* strain HNXS-W9 (ZNS1); B: *Pseudomonas fluorescens* strain PPA42 (ZNS2); C: *Micrococcus luteus* strain ANRFB29 (ZNS7); D: *Klebsiella pneumoniae* strain 216-A (ZNS8); E: *Paenibacillus lautus* strain B682/17 (ZNS9) generated from 16S rRNA sequences using MEGA 11.

highest zinc solubilisation index, solubilisation efficiency, high soluble zinc released and high soluble zinc content. In addition, these isolates did not display haemolytic activities on blood agar.

Numerous species of *Pseudomonas* species, including *P. fluorescens*, *P. protegens*, *Pseudomonas japonica*, *P. aeruginosa*, *P. fragi*, *Klebsiella pneumoniae*, are reported for their role in zinc solubilisation by many researchers (43–47). However, in our study, *N. nealsonii*, *M. luteus* and *P. lautus* were reported for the first time to possess zinc solubilisation potential.

All 10 bacterial isolates were screened for their ability of zinc-solubilising in plate assay. Isolate ZNS2 exhibited in the highest zinc solubilisation efficiency (224.99 %), followed by ZNS7 (212.49 %) and ZNS8 (188.87 %) in medium augmented with 0.1 % ZnO insoluble compounds. Similar results were observed by previous researchers that the strain of *P. fluorescens*, able to solubilise all insoluble zinc compounds effectively (43). Similarly, *P. aeruginosa* FA-9 showed a clear zone diameter of 63 mm on agar plate amended with different zinc ores (48). *Pseudomonas putida* showed the highest zinc solubilisation index of 3.31 in medium containing ZnO (4). Another study reported that ZnJ11 *K. pneumoniae* showed maximum zinc solubilisation efficiency (525 ± 2.9) followed by ZnJ10 (383 ± 3.1) in three zinc sources by plate assay (47).

Since the plate assay does not represent a true indicator of zinc solubilisation ability (39), all 10 isolates were further assayed in liquid medium amended with 0.1 % ZnO to assess soluble Zn release after 8 days of incubation. Among ten isolates, *P. fluorescens* (ZNS2) released 299.79 mg L⁻¹, followed by *M. luteus* (ZNS7) 222.34 mg L⁻¹. Similarly, (48, 49) examined that 8 out of 40 strains of *P. fluorescens* released soluble Zn from ZnO in the range of 28–625 mg L⁻¹. (27) Also reported that *P. protegens* released a maximum available zinc of 142.35 mg L⁻¹ after 15 days of incubation.

Isolate ZNS10 did not produced significant results in both plate and broth assay. However, isolate ZNS 5 showed measurable halo zones on solid Bunt and Rovira medium (Fig. 1) but did not yield detectable soluble zinc in the corresponding broth assay. The halo formation by the isolate ZNS5 may be due to rapid diffusion of organic acid through the agar matrix, resulting in localised drop in pH which produce visible halo on the plate. However, in the broth assay, strains with large halos on plates might simply produce weaker, volatile, or transient organic acids that are released below the detection limit of atomic absorption spectrophotometry. This is consistent with previous reports noting that the plate assay is not always a reliable quantitative predictor of solubilisation performance in liquid culture which is why broth assays were used in this study.

The secretion of the organic acid is one of the most common mechanisms is used by ZNB to produce available zinc from insoluble Zinn sources. Gluconic acid is the most prevalent acid for zinc solubilisation among various organic acids produced by bacterial strains. (21, 16). In this current study, it showed a significant decrease in the pH, indicating that the all-bacterial strains have the capacity to secrete organic acids, leading to the reduction of pH from the 6.8 to less than of 2.5 in media with zinc oxide after 8 days. The acidic environment showed the solubilisation of insoluble zinc compounds in the soil. Isolate ZNS2 *P. fluorescens* showed the decrease of pH scale of (2.5) in ZnO-amended media of the 8th day. Similar findings were documented by previous researchers wherein *P. fluorescens* (Ur21) exhibited the highest reduction of pH (4.2) compared to the control at pH 6.8 (4).

The identification of efficient zinc-solubilising strains highlights their potential as microbial inoculants for improving zinc availability in zinc-deficient soils. *Pseudomonas fluorescens* and *K. pneumoniae* are reported for zinc solubilisation in plate and broth media. However, to our knowledge, zinc solubilisation potential of *N. nealsonii*, *M. luteus* and *P. lautus* has not been previously reported. Furthermore, all five potential strains showed γ -haemolysis in the haemolysis test, indicating that these five most prominent isolates could be potential candidates to overcome zinc deficiency and reduces the uses of the conventional fertilisers in agricultural field areas. It is also important to note that *K. pneumoniae* (ZNS8) is recognised as an opportunistic human pathogen, most associated with nosocomial infections in immunocompromised individuals and its identification among the zinc-solubilising isolates in this study warrants careful consideration before any practical application. In the present work, to ensure the safety of the *K. pneumoniae* ZNS8, it was tested for haemolytic activity on blood agar and was found to be non-haemolytic (γ -haemolysis), which is a preliminary indicator of non-pathogenicity. Moreover, several environmental and rhizosphere strains of *K. pneumoniae* distinct from clinical lineages have been reported to promote plant growth without association with human disease (46). Nonetheless, in line with biosafety best practice such as capsular serotyping, biofilm formation, virulence genes screening or antibiotic susceptibility profiling may be included in future work before recommending ZNS8 as a biofertiliser. The present study was conducted under laboratory conditions using ZnO as the sole zinc source. Validation under greenhouse and field conditions is necessary to determine the effectiveness of these isolates in natural soil environments.

Conclusion

Ten bacterial strains were isolated from the sorghum rhizosphere. Five isolates exhibited superior zinc-solubilisation ability in ZnO-amended medium and were selected for further characterisation. Strain ZNS2 demonstrated the greatest zinc solubilising ability, releasing the highest amount of soluble zinc in ZnO-amended medium. Based on zinc solubilisation potential and haemolytic activity, five promising bacterial strains were identified as *Niallia nealsonii*, *Pseudomonas fluorescens*, *Micrococcus luteus*, *Klebsiella pneumoniae* and *Paenibacillus lautus*. The detection of zinc solubilisation activity in *N. nealsonii*, *M. luteus* and *P. lautus* expands the diversity of bacterial taxa associated with zinc mobilisation. These isolates represent promising candidates for development as microbial inoculants aimed at improving zinc nutrition and reduce dependence on chemical zinc fertilisers. Furthermore, the specific biosafety risks such as virulence genes, antibiotic resistance gene may be included as a future line of work. Future studies may also focus on evaluating zinc release under soil conditions and assessing the effects of these isolates on plant growth, zinc uptake and crop productivity under greenhouse and field conditions with particular emphasis on zinc bioavailability, plant growth promotion and crop biofortification.

Acknowledgements

The authors would like to extend their heartfelt gratitude to the Department of Natural Resource Management (Microbiology) and the Department of Soil Science, SRM College of Agricultural Sciences,

Chengalpattu, Tamil Nadu, for providing the facilities and support required to carry out this research.

Authors' contributions

ASS carried out the isolation, screening and characterisation of zinc-solubilising bacteria, performed the formal analysis, curated the data and prepared the original draft of the manuscript. KA conceived and designed the study, developed the methodology, validated the results, supervised the research, provided resources, administered the project and reviewed and edited the manuscript. TSRR contributed to the visualisation of the results, supervised the research and provided resources. RJ contributed to the visualisation of the results, supervised the research, provided resources and participated in project administration. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) to assist with language editing, grammar improvement and refinement of the manuscript text. The authors carefully reviewed and edited all AI-assisted content and take full responsibility for the accuracy, integrity and originality of the final manuscript.

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