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# Characterization of Zinc-Solubilizing *Bacillus* Isolates and their Potential to Influence Zinc Assimilation in Soybean Seeds

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**One hundred thirty-four putative *Bacillus* isolates were recovered from soybean rhizosphere soils of Nimar region to select effective zinc solubilizers for increased assimilation of zinc (Zn) in soybean seeds. These isolates were screened *in vitro* for zinc-solubilization ability on Tris-minimal agar medium supplemented separately with 0.1% zinc in the form of zinc oxide, zinc phosphate, and zinc carbonate. Of all, 9 isolates and a reference *Bacillus cereus* ATCC 13061 were characterized and identified as *Bacillus* species based on Gram-positive reaction, endospore-forming cells, and the presence of iso-$C_{15:0}$ and anteiso-$C_{15:0}$ as predominant fatty acids. On plate assay, two isolates KHBD-6 and KHBAR-1 showed a greater diameter of solubilization halo and colony diameter on all the three zinc compounds. The isolates KHBD-6, KHBAR-1, BDSD-2-2C, and KHTH-4-1 and the reference strain ATCC 13061 had higher soluble zinc concentration in liquid medium supplemented with zinc phosphate and zinc carbonate compounds as compared with the other isolates and uninoculated control. Evaluation under microcosm conditions showed that inoculation of isolates KHBD-6 (57.34 µg/g), KHBAR-1 (55.67 µg/g), and strain ATCC 13061 (53.10 µg/g) significantly increased the Zn concentration in soybean seeds as compared with the other isolates and uninoculated control (47.14 µg/g). This study suggests the occurrence of zinc-solubilizing *Bacillus* in soils of Nimar region and isolates KHBD-6 and KHBAR-1 were found to be promising zinc solubilizers for increased assimilation of Zn in soybean seeds.**

**Keywords:** *Bacillus*, FAMEs, rhizosphere, soybean, zinc solubilization

Soybean has established itself as one of the major oilseed crops in India. The majority of the soybean area is in Madhya Pradesh of central India (59% of total area) and is largely grown on Vertisols and associated soils under rainfed situations [3]. Nimar region, located in central India, is characterized by low rainfall, high temperature, and nutrient-deficient shallow soils, resulting in suboptimal crop yields. There is a considerable potential to abridge the yield gap between the actual and potential yields through adoption of appropriate improved resource nutrient management strategies [1]. Zinc is an essential micronutrient that plays a vital role in various metabolic processes in plants, and its deficiency adversely affects the growth and development of crop plants [2]. The crop and soil management practices mine large amounts of zinc from the native pool of the soil. For example, a harvest of 6.5 ton grain/ ha/yr removed 416 g Zn/ha/yr in soybean-wheat cropping systems [16, 34]. More than 50% of Indian soils are deficient in zinc [32] and warrants remedial measures to increase the zinc availability in these soils and sustain the growth in agricultural production. The available zinc content in Indian soils is low; however, the total zinc content is substantially high and exists in fixed forms such as smithsonite ($ZnCO_3$), sphalerite (ZnS), zincite (ZnO), franklinite ($ZnFe_2O_4$), wellemite ($Zn_2SiO_4$), and hopeite ($Zn_3(PO_4)_2{\cdot}4H_2O$) [13], which are sparingly soluble. Consequently, large inputs of zinc fertilizers are required to be added to the soil to meet the zinc needs of crops. The strategy of ameliorating the low zinc fertility constraints and improve availability, especially in Nimar region, requires ecologically sound, efficient, and cost-effective alternatives. It is plausible that exploitation of zinc mineralizing and solubilizing bacteria may aid in overcoming zinc deficiency.

Zinc-solubilizing microorganisms can solubilize zinc from inorganic and organic pools of total soil zinc and can be utilized to increase zinc availability to plants. Fungi have been extensively studied for solubilization of insoluble zinc compounds both *in vitro* and *in vivo* [7, 38]. However, only some bacterial species of the genera *Acinetobacter*, *Bacillus*, *Gluconacetobacter*, and *Pseudomonas* have been reported [4, 5, 20, 22, 23]. Occurrence of zinc-solubilizing *Pseudomonas* in Vertisols of Malwa region of central India

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has been reported in previous studies [30]; however, the Nimar region is unexplored for rhizobacteria having Zn-solubilizing ability and assimilation of zinc by soybean. Therefore, the present study was carried out to isolate and characterize native zinc-solubilizing bacilli from soybean rhizosphere of Nimar region, and evaluate their zinc-solubilizing potential and zinc acquisition by soybean from the native pool of soil. Bacilli were isolated assuming that these endospore-forming bacteria will survive even under the extreme climatic situation prevailing in Nimar region.

## Materials and Methods

### Collection of Soil Samples and Isolation of *Bacillus*

Soil samples from soybean rhizosphere were collected from 16 locations encompassing the districts of Burhanpur, Badwani, Khandwa, and Khargone located in Nimar region of Madhya Pradesh, during *kharif* (rainy season June to September, 2007). Nimar region falls between the latitude 21°00' to 22°38' N and longitude in between 74°21' to 77°28' E under the central Plateau and Hill Region belonging to agro-ecological zone 5 of India. The soils in these districts are classified as isohyperthermic, montmorillonitic, and Typic Haplusterts. The details regarding the crop and soil management practices along with soil pH and organic carbon of samples are presented in Table 1. The pH (soil:water ratio, 1:2.5) and organic carbon content in these soils were analyzed using standard methods [10]. The soil samples were stored at 4°C in sterile containers until used for isolation. To isolate *Bacillus*, 1.0 g of rhizosphere soil sample was suspended in 9 ml of sterile distilled water in test tubes. The tubes were placed in a hot-water bath at 60°C for 60 min to kill all vegetative cells of microbes, leaving only spore-forming bacilli in suspension. Immediately after cooling the tubes, the suspensions of different soil samples were diluted to $10^{-3}$-folds dilution, and thereafter a 100 µl aliquot was spread on nutrient agar medium and the plates were incubated at 30°C for 24–48 h. Putative *Bacillus* isolates were selected based on colony morphology, namely, colony form, elevation, margin, texture, and pigmentation. Isolates were re-streaked on nutrient agar medium for assessing their purity. All the isolates were stored as glycerol stock at –20°C.

### Selection of Effective Zinc-Solubilizing *Bacillus* Isolates

To select effective zinc-solubilizing bacilli, *Bacillus* isolates were evaluated on Tris-minimal agar medium supplemented with D-glucose and different insoluble zinc compounds [5]. The Tris-minimal medium was separately amended with zinc oxide (1.244 g/l = 15.23 mM), zinc phosphate (1.9882 g/l = 5.0 mM), and zinc carbonate (1.728 g/l = 5.2 mM) at a concentration of 0.1% Zn. After sterilization and plating, freshly grown bacterial cultures were spot inoculated in triplicates on the media using sterile toothpicks. The spotted plates were incubated at 28°C for 7 days in the dark to observe clear halo zone formation around colonies. The diameter of the halo zone around the colony and the colony diameter were measured after 7 days and subsequently the plates were flooded with methyl red solution to observe acid production by bacteria. The change of clear zone to red is an indication of acid production.

For liquid broth assay, 50 ml of liquid Tris-minimal medium supplemented with 0.1% zinc in the form of zinc phosphate and zinc carbonate separately was used. One ml aliquots of each culture with a cell load of $10^8$ CFU/ml were inoculated in each flask [5]. Tris-medium supplemented with the zinc compounds but without bacterial inoculation served as uninoculated control. Three replications of each treatment were maintained. All the flasks were incubated at 28°C in an orbital shaker at 120 rpm for 10 days and the pH of the samples was recorded. Subsequently, aliquots of the medium were

**Table 1.** pH and organic carbon content of rhizosphere soils of soybean cultivated under different agricultural management practices in Nimar region.

| Districts | Locations | Cropping system | Nutrient management | pH of soil | Organic carbon (%) |
|---|---|---|---|---|---|
| Burhanpur (BR) | 1. Borgaon (BR) | Soybean-wheat-cotton | FYM and urea | 7.74 | 0.42 |
| | 2. Dhupghata (DH) | Soybean-wheat-cotton | DAP and urea | 7.91 | 0.82 |
| Badwani (BD) | 3. Sendhwa(SD) | Soybean-wheat-cotton | FYM and urea | 7.37 | 0.52 |
| | 4. Pansemal (P) | Maize-soybean-wheat | FYM and urea | 6.54 | 0.60 |
| | 5. Badwani | Soybean-wheat-cotton | FYM and DAP | 8.71 | 0.57 |
| | 6. Khetia (KH) | Soybean-wheat-cotton | FYM and urea | 7.29 | 1.39 |
| | 7. Niwali (N) | Soybean/cotton-wheat | FYM, urea, and DAP | 7.30 | 0.68 |
| Khandwa (KD) | 8. Morghadi (MR) | Soybean-wheat-cotton | FYM, urea, and DAP | 8.04 | 0.49 |
| | 9. Kumathi (KUM) | Soybean-wheat-cotton | FYM, urea, DAP, and potash | 8.16 | 0.69 |
| Khargone (KH) | 10. Choli (CH) | Soybean-wheat-cotton | Urea | 7.80 | 0.55 |
| | 11. Ojhara (OJ) | Soybean-wheat | FYM and IFFCO | 8.53 | 0.78 |
| | 12. Baduad (BD) | Soybean-wheat-cotton | FYM and IFFCO | 8.25 | 0.75 |
| | 13. Manihar (M) | Soybean-wheat-cotton | Nil | 7.95 | 0.69 |
| | 14. Kasarwad (KS) | Soybean-wheat-cotton | IFFCO | 8.34 | 0.84 |
| | 15. Thangaon (TH) | Soybean-wheat-cotton | Urea | 8.43 | 0.53 |
| | 16. Barley (BAR) | Soybean-wheat- cotton | Urea | 8.33 | 0.57 |

Farm yard manure (FYM) was applied to soybean phase and fertilizers in wheat and cotton phase; Nil: No nutrient applied; IFFCO is a complex fertilizer containing N:$P_2O_5$:$K_2O$ at 12:32:16; soils belong to Vertisols and associated soils (vertic Inceptisol)

centrifuged at 8,000 rpm for 8–10 min to remove cells debris and the clear supernatant was collected and fed directly into an Atomic Absorption Spectrophotometer (Perkin Elmer Model AS 100) for determination of total soluble zinc in the clear broth.

**Phenotypic Characterization and FAMEs Analysis of *Bacillus* Isolates**

Nine putative *Bacillus* isolates were characterized morphologically and biochemically based on methodologies described in *Bergey's Manual of Determinative Bacteriology* and identified putatively as *Bacillus* [17]. The bacterial isolates were subjected to whole-cell fatty acids methyl esters (FAMEs) profiling for further confirmation of bacterial identity. The whole-cell cellular fatty acids of the bacterial isolates were derivatized to methyl esters and analyzed by gas chromatography (GC) using the Sherlock Microbial Identification System (MIDI, Inc., Newark, DE, USA). The procedures and protocols used for growing the cultures and instrument specifications are described by Sasser [26]. The fatty acids from the *Bacillus* culture grown on tryptic soya agar for 24 h at 28°C were extracted by saponification in dilute sodium hydroxide [15% (w/v)] and followed by derivatization with dilute hydrochloric acid and methanol solution [HCl:MeOH, 1:0.85 (v/v)] to give methyl esters, and extracted with an organic solvent consisting of hexane and methyl tert-butyl ether (MTBE; 1:1). Finally, the organic phase was base washed with mild sodium hydroxide (0.5 M). The resulting organic extract was analyzed by GC (Agilent Technologies, Model 7890A) equipped with a 7683 series autosampler and 7683 B series injector using FID, HP-Ultra 2 fused silica capillary column of 25 m × 0.2 mm × 0.33 µm with hydrogen as the carrier gas (fuel gas), nitrogen as the "make up" gas, and air to support the flame. The GC oven temperature was programmed from 170 to 270°C at 5°C increase per minute with a 2 min hold at 300°C. The fatty acids were identified and quantified by comparison with the retention times and peak areas obtained for the authentic standards mixture. Fatty acid profiles were compared with the profiles in the Sherlock bacterial fatty acid reference library RTSBA6 6.10 of MIDI [25, 27].

**Evaluation of Zinc-Solubilizing *Bacillus* for Zinc Assimilation in Soybean Seeds**

A microcosm experiment was conducted during *Kharif* 2008 in soils belonging to Sarol series (Isohyperthermic, montmorillonitic, Typic Haplusterts) collected from Research Farm, Directorate of Soybean Research, Indore, Madhya Pradesh, India. The experimental soil had the following characteristics: pH 8.2, OC 4.6 g/kg, clay content 56.2%, DTPA-Zn 1.24 µg/g, and available P 5.82 µg/g. The soil was air dried and passed through a 2 mm sieve and filled in polythene bags (black color, 10 kg capacity) and moisture content was brought to field capacity. No fertilizer was applied to the experimental soil. Eleven treatments, namely, isolates KHBD-2-2A, BDKH-3, BD-3-1B, BDSD-2-2C, BDN-5, KHTH-4-1, KHBAR-1, KHBD-6, KDMR-1-1, ATCC 13061, and an uninoculated control, were evaluated in a completely randomized design with six replications. Cultures were grown for 24 h and cells were harvested by centrifugation at 6,000 rpm for 8 min. The pellets were suspended in 0.85% sterile saline solution and the cell concentration of each bacterial isolate was adjusted to $10^8$ CFU/ml. The soybean seeds (JS 335) were surface sterilized with a three-step sterilization procedure: a 30 s wash in 90% ethanol, followed by a 3 min wash in 3.0% NaOCl, and 2 times rinse in sterile distilled water. Four sterilized seeds per bag were sown by placement on the soil surface in a 0.5 cm deep hole and subsequently 1.0 ml aliquots of bacterial suspension were placed over each seed. Uninoculated control was maintained in a similar manner but seeds were applied only with 0.85% saline solution without bacteria. After germination, two plants were maintained in each polythene bag. Plants were irrigated as and when required. Plants were harvested at maturity (90 days after germination). The Zn content in seeds was determined through an Atomic Absorption Spectrophotometer after acid digestion of the seeds [10].

**Statistical Analysis**

Analysis of variance was carried out to test the significance of treatment (inoculants) difference. The least significant differences (LSD) were used to separate the treatment means using the Duncan Multiple Range Test (DMRT) (COSTAT statistical software; Cohart, Berkeley, CA, USA).

## RESULTS AND DISCUSSION

One hundred and thirty-four putative *Bacillus* isolates were recovered from 16 rhizosphere soil samples. These isolates were subjected to screening for zinc solubilization on three insoluble zinc compounds. In general, 31%, 27%, and 19% isolates were found positive towards zinc solubilization on the media containing zinc oxide, zinc phosphate, and zinc carbonate, respectively. Different colony characteristics of bacilli were observed on Tris-minimal medium supplemented with different zinc compounds. This result is in agreement with Saravanan *et al*. [24], wherein they reported induction in cell pleomorphism in *G. diazotrophicus* under zinc metal-incorporated medium. Based on the initial solubilization assay, 9 potential isolates were selected for further study. All these isolates were able to form a clear halo zone with varying size on all the three zinc compounds included in this study. So far, only bacterial species belonging to genera *Acinetobacter*, *Bacillus*, *Gluconacetobacter*, and *Pseudomonas* were reported to be zinc solubilizer as they form a clear halo zone [4, 20–22].

**Solubilization of Zinc Compounds on Agar Medium**

Based on preliminary screening, all 9 potential isolates and *B. cereus* (ATCC 13061) as a standard strain were assessed for measuring the magnitude of zinc solubilization on Tris-minimal media supplemented with three zinc compounds at 0.1% zinc concentration. These isolates were from locations having comparatively higher organic carbon content in soils (0.49–1.39%). The diameter of solubilization halo and colony diameter produced by these isolates at 0.1% insoluble zinc are presented in Table 2. It was observed that all the isolates except BDKH-3 and KHBAR-1 showed solubilization halos after 24 h (1 day) incubation on medium supplemented with zinc oxide (data not shown). Isolate KHBD-6 produced a clear halo zone on zinc oxide with a solubilization diameter of 28 mm and was found to be significantly greater as

**Table 2.** Diameter of colony and solubilization zone formed by *Bacillus* isolates and *B. cereus* on Tris-minimal medium supplemented with insoluble zinc compounds.

| Isolates/Standards | Diameter (mm) of colony and solubilization zone after 7 days on | | | | | |
|---|---|---|---|---|---|---|
| | Zinc oxide | | Zinc phosphate | | Zinc carbonate | |
| | Col. diam. | Halo zone | Col. diam. | Halo zone | Col. diam. | Halo zone |
| KHBD-2-2A | $8^{de}$ | $14^{bcd}$ | $13^{cd}$ | $15^{d}$ | $8^{bc}$ | $10^{cd}$ |
| BDKH-3 | $10^{cd}$ | $13^{cd}$ | $11^{d}$ | $14^{de}$ | $7^{c}$ | $8^{d}$ |
| BD-3-1B | $6^{e}$ | $14^{bcd}$ | $7^{e}$ | $12^{ef}$ | $6^{c}$ | $9^{cd}$ |
| BDSD-2-2C | $11^{bc}$ | $15^{bc}$ | $17^{ab}$ | $18^{c}$ | $10^{b}$ | $14^{b}$ |
| BDN-5 | $6^{e}$ | $9^{e}$ | $7^{e}$ | $11^{f}$ | $6^{c}$ | $8^{d}$ |
| KHTH-4-1 | $8^{de}$ | $13^{cd}$ | $15^{bc}$ | $19^{c}$ | $6^{c}$ | $8^{d}$ |
| KHBAR-1 | $13^{b}$ | $16^{b}$ | $18^{a}$ | $22^{b}$ | $8^{bc}$ | $10^{cd}$ |
| KHBD-6 | $16^{a}$ | $28^{a}$ | $15^{bc}$ | $30^{a}$ | $17^{a}$ | $27^{a}$ |
| KDMR-1-1 | $8^{de}$ | $15^{bc}$ | $12^{d}$ | $15^{d}$ | $8^{bc}$ | $11^{c}$ |
| *B. cereus* | $8^{de}$ | $12^{d}$ | $18^{a}$ | $22^{b}$ | $7^{c}$ | $10^{cd}$ |
| LSD (P = 0.01) | 2.2 | 2.1 | 2.4 | 2.6 | 2.0 | 2.4 |

Means are value of three replications; Means followed by the same letter in a column are not significantly different but by different letters are significantly different (P= 0.01) using the Duncan multiple range test.

compared with the other isolates evaluated (Table 2). With the exception of BDN-5, all the isolates showed a solubilization diameter greater than 10 mm with zinc oxide. The results revealed that the efficiency to solubilize zinc oxide varied between strains and are in consonance with the findings of other researchers [5, 23]. In general, the colony diameter and solubilization halo zone of all bacterial isolates had achieved maximum growth on solid medium containing zinc phosphate, indicating utilization of both zinc and phosphate by the bacteria. It is known that all organisms require both Zn and P for optimum growth and energy. On the medium containing zinc phosphate, a halo zone was observed only after 72 h (3 days) of incubation (data not shown). Isolate KHBD-6 had shown a double halo zone on zinc phosphate medium (hazy outer side and clear inner side around colony), which is a unique feature and was not observed with the other isolates evaluated in this study. The halo zone produced by KHBD-6 was significantly greater (30 mm) as compared with the solubilization halo produced by the other isolates (Table 2). It was observed that isolates KHBD-6, KHBAR-1, and ATCC 13061 produced greater solubilization halos (> 20 mm) as compared with the other isolates. Moreover, these isolates also produced a greater colony diameter in comparison with rest of the isolates. The sizes of halo zones produced by bacilli on zinc phosphate were greater than that of zinc oxide and this is in contrast to the findings of Fasim *et al*. [5], wherein they observed higher solubilization zones on zinc oxide as compared with zinc phosphate.

The solubilization potential on zinc carbonate revealed that only a few isolates had the capacity to solubilize zinc carbonate (Table 2), and a clear halo zone was observed on the 4$^{th}$ day after inoculation, mainly due to slow growth of bacteria (data not shown). The isolate KHBD-6 was found to be an effective solubilizer of zinc from zinc carbonate with a solubilization halo of 27 mm, followed by BDSD-2-2C, KDMR-1-1, KHBAR-1, and KHBD-2-2A. The higher zinc-solubilizing potential of these isolates against a recalcitrant source with high stability like zinc carbonate assumes significance in terms of zinc nutrition to crop plants, as Vertisols and associated soils are abundant in carbonates and hydroxy carbonates of zinc. Isolates KHBD-6 and KHBAR-1 showed greater diameters of solubilization halo and colony diameter on all the three zinc compounds. In the present study, the maximum clear solubilization halo was observed after the 7$^{th}$ day of inoculation in all the three zinc compounds, and thereafter there was no appreciable increase in the size of solubilization halo (data not shown).

In the current study, the isolates were found to be comparatively more efficient in solubilizing zinc from zinc phosphate as compared with zinc oxide and zinc carbonate. These results are in contrast to earlier reports [5, 37]. The above results show a wide variation in zinc solubilization by *Bacillus* isolates recovered from rhizosphere soil samples collected from different agricultural and soil management practices on nutrient agar medium. This can be attributed to location specificity in terms of varying organic carbon content, and species and strains variability. Previous studies conducted in plate assay with *G. diazotrophicus* strains evaluated for zinc solubilization revealed strain-level variability [15, 23]. Therefore, the present study indicated that *Bacillus* species have greater potential to solubilize zinc phosphate as compared with zinc oxide or zinc carbonate.

It was observed that *Bacillus* isolates that produced clear halo zones on zinc phosphate-supplemented medium also

turned red with methyl red indicator, indicating acidification of the medium and solubilization of the compound through acid production, whereas zones produced on zinc oxide and zinc carbonate also turned to mild red color. The mechanisms of acquisition of the zinc by *Bacillus* isolates from insoluble zinc compounds might be a consequence of production of organic acids of microbial origin leading to solubilization of zinc and thereby influencing the bioavailability of zinc. Such solubilization of zinc compound mediated through production of organic acids by *Bacillus* and subsequent release of zinc in the external environment and bioaccumulation of zinc inside cells of *Bacillus* species has been reported by earlier workers [22, 23].

**Solubilization of Zinc Compounds in Liquid Broth Medium**

All the 9 isolates were grown in liquid broth supplemented with two different compounds (zinc phosphate and zinc carbonate) added separately. These isolates showed a shift in pH of the broth after 10 days of incubation. In general, there was a decline in the pH with inoculation ranging from 7.10 to 4.21 and 7.20 to 6.50 with zinc phosphate and zinc carbonate, respectively (data not shown). All the isolates showed a reduction in pH as compared with uninoculated control. Our result corroborates with the study of Di Simine *et al*. [4], wherein they reported that *P. fluorescens* 3a reduced the pH in zinc phosphate-supplemented medium as a consequence of gluconic acid production. The change in pH of broth supplemented with zinc carbonate by inoculation of *Bacillus* was not appreciable and can be attributed to the intrinsic buffering potential of the zinc carbonate, and is in agreement with the study of Franz *et al*. [6], who reported such buffering in medium containing zinc carbonate inoculated with *Penicillium simplicissimum.* Previous studies also reported acidification as a main process of solubilization of zinc phosphate by bacteria and fungi [5, 28]. However, reports also suggest that phosphate solubilization by bacteria and actinomycetes is governed by siderophore production, organic acid production, and proton extrusion, and these are dependent on metabolic activities of the microorganisms [9, 29].

The present findings revealed that inoculation of *Bacillus* isolates increased soluble zinc content in liquid broth as compared with uninoculated control. In case of zinc phosphate, the soluble zinc concentration increased with inoculation of *Bacillus* isolates (3.45–4.65 μg/ml broth) as compared with 2.23 μg/ml in uninoculated control (Table 3). Similarly, the soluble zinc concentration also increased in broth supplemented with zinc carbonate (2.86–3.65 μg/ml broth) as compared with 1.98 μg/ml in uninoculated control. Isolates KHBD-6, KHBAR-1, and BDSD-2-2C significantly increased the soluble zinc concentration in liquid media supplemented with zinc phosphate and zinc carbonate as compared with the other isolates and uninoculated control

**Table 3.** Soluble zinc content in Tris-minimal liquid broth supplemented with zinc phosphate and zinc carbonate, and zinc assimilation by seeds of soybean in response to inoculation of zinc-solubilizing *Bacillus* isolates.

| Isolates/Standards | Soluble zinc content (μg/ml broth) | | Zinc content (μg/g seed) |
|---|---|---|---|
| | Zinc phosphate | Zinc carbonate | |
| KHBD-2-2A | 4.09$^{c}$ | 3.21$^{b}$ | 50.20$^{e}$ |
| BDKH-3 | 4.08$^{c}$ | 2.86$^{d}$ | 51.69$^{cd}$ |
| BD-3-1B | 3.45$^{d}$ | 2.95$^{cd}$ | 48.10$^{f}$ |
| BDSD-2-2C | 4.22$^{c}$ | 3.26$^{b}$ | 52.56$^{c}$ |
| BDN-5 | 3.58$^{d}$ | 3.10$^{bc}$ | 53.13$^{c}$ |
| KHTH-4-1 | 4.17$^{c}$ | 3.05$^{bcd}$ | 51.02$^{de}$ |
| KHBAR-1 | 4.65$^{ab}$ | 3.13$^{bc}$ | 55.67$^{b}$ |
| KHBD-6 | 4.87$^{a}$ | 3.65$^{a}$ | 57.34$^{a}$ |
| KDMR-1-1 | 3.98$^{c}$ | 3.11$^{bc}$ | 52.01$^{cd}$ |
| *B. cereus* | 4.53$^{b}$ | 3.11$^{bc}$ | 53.10$^{c}$ |
| Uninoculated control | 2.23$^{e}$ | 1.98$^{e}$ | 47.12$^{f}$ |
| LSD | 0.24* | 0.22* | 1.39** |

*Means are value of three replications; Means followed by the same letter in a column are not significantly different, but by different letters are significantly different (P = 0.01) using the Duncan multiple range test.
**Means are value of six replications; Means followed by the same letter in a column are not significantly different, but by different letters are significantly different (P = 0.05) using the Duncan multiple range test.

(Table 3). The increase in soluble zinc content with inoculation of *Bacillus* isolates suggested involvement of *Bacillus* isolates in zinc solubilization. The data also revealed that inoculation of *Bacillus* isolates increased soluble zinc in broth supplemented with zinc phosphate to a greater extent as compared with zinc carbonate. This result is in consonance with the report of Saravanan *et al*. [23], wherein they also observed more soluble zinc content in zinc phosphate-supplemented LGI medium with the inoculation of *G. diazotrophicus* strain.

**Identification of *Bacillus* Isolates**

The morphology of colonies observed was white, orange, and yellow in color and the texture was opaque, shiny, smooth or wrinkled, and mucoid or dry. Comprehensive study of the 9 isolates showed that most of the isolates were Gram positive, endospore-forming, and positive for catalase, oxidase, and nitrate reduction, with the exception of isolates BD-3-1B and BDN-5, which were found to be Gram positive, endospore-forming but positive only for catalase (Table 4). On the basis of morphological and biochemical characteristics, it was assumed that all the isolates belong to the genus *Bacillus* [11, 33]. The whole-cell fatty acid profile of the 9 isolates indicated to contain prominently iso-$C_{15:0}$, anteiso-$C_{15:0}$, and anteiso-$C_{17:0}$, whereas $C_{14:0}$, iso-$C_{14:0}$, iso-$C_{16:0}$ $C_{16:0}$ anteiso-$C_{16:0}$, and iso-$C_{17:0}$ were present only in minor quantities and identified at species

**Table 4.** Morphological and biochemical characteristics of zinc-solubilizing *Bacillus* isolates.

| Characteristics | Strains/Standards | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | KHBD-2-1A | BDKH-3 | BD-3-1B | BDSD-2-2C | BDN-5 | KHTH-4-1 | KHBAR-1 | KHBD-6 | KDMR-1-1 | *B. cereus* |
| Appearance | D | S | D | S | S | S | S | Sm | S | Sm |
| Form | C | C | C | C | C | C | C | Ir | C | C |
| Margin | Sr | Fil | L | Un | E | Un | Ir | Ir | Fil | L |
| Elevation | F | F | F | F | R | Um | F | F | F | F |
| Pigmentation | W | W | Y | W | O | W | W | Y | W | W |
| Gram reaction | + | + | + | + | + | + | + | + | + | + |
| Spore staining | + | + | + | + | + | + | + | + | + | + |
| Catalase | + | + | + | + | + | + | + | + | + | + |
| Oxidase | + | + | - | + | - | + | + | + | + | + |
| Nitrate reduction | + | + | + | + | - | + | + | + | + | + |
| Arginine dihydrolase | + | + | - | + | - | - | + | + | + | - |
| Indole-3-acetic acid | - | - | + | - | - | - | - | - | + | - |
| Starch hydrolysis | + | + | NA | + | + | NA | + | - | + | - |
| Caesin hydrolysis | + | + | + | + | + | + | + | + | + | + |
| VP test | + | + | + | + | + | + | + | - | + | + |
| Citrate utilization | - | - | - | - | - | - | - | + | - | - |
| Methyl red | + | + | + | + | + | + | + | - | + | + |

D = Dull, C = Circular, S = Shiny, Sr = Serrated, F = Flat, W = White, R = Raised, E = Entire, Fil = Filamentous, Y = Yellow, O = Orange, L = Lobate, Un = Undulate, Um = Umbonate, Ir = Irregular, Sm = Smooth, NA = Not assessed.

level belonging to the genus *Bacillus* (Fig. 1 and Table 5). The predominant fatty acids shown by the isolates evaluated in the current study are in conformity with many previous reports [8, 12, 18, 31].

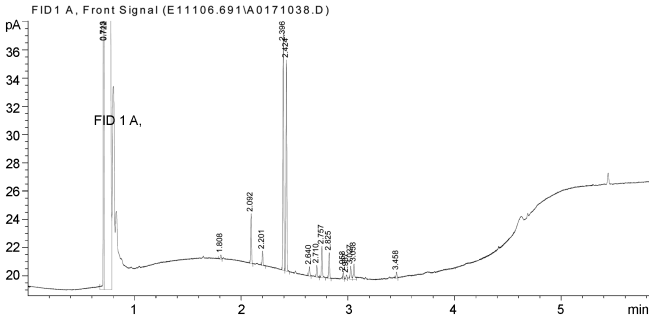


**Fig. 1.** Chromatograph showing a peak profile of the FAMEs in the KHBD-2-1A isolate [name of fatty acids at each RT: 0.723: solvent peak; 1.81: iso-$C_{13:0}$ (0.87%); 2.09: iso-$C_{14:0}$ (7.80%); 2.21: $C_{14:0}$ (2.38%); 2.396: iso-$C_{15:0}$ (36.19%); 2.42: anteiso-$C_{15:0}$ (31.68%); 2.64: ω7c alcohol- $C_{16:1}$ (1.57%); 2.71: iso-$C_{15:0}$ (1.91%); 2.76: ω11c-$C_{16:1}$ (5.14%); 2.86: $C_{16:0}$ (3.90%); 2.96: iso-ω10c-$C_{17:1}$ (1.00%); 2.99: sum of feature 4 (0.83%); 3.03: iso-$C_{17:0}$ (2.55%); 3.06: anteiso-$C_{17:1}$ (3.12%); 3.46: $C_{18:0}$ (1.05%)].

**Zinc Assimilation in Soybean Seeds upon Inoculation**

Inoculation with *Bacillus* isolates KHBD-6 (*Bacillus firmus*), KHBAR-1 (*Bacillus amyloliquefaciens*), BDN-5 (*Bacillus* sp.), and ATCC 13061 (*Bacillus cereus*) significantly increased zinc assimilation in soybean seeds as compared with the other isolates and uninoculated control under microcosm conditions (Table 3). The zinc concentration in seeds of inoculated crop ranged from 48.10 to 57.34 µg/g as compared with uninoculated control (47.17 µg/g) (Table 3). Roesti *et al*. [19] and Mader *et al*. [14] had reported that inoculation of *Pseudomonas synxantha* HHRE81 (R81) and *P. jessenii* LHRE62 (R62) increased zinc concentration in seeds of wheat and black gram. Biofortification of seeds of cereals and legumes with zinc is of immense importance in mitigating zinc malnutrition in the human population. In another study, Tariq *et al*. [35] demonstrated the efficiency of a commercial PGPR consortium acting as zinc solubilizer that increased Zn concentration to an extent of 157%. The increase in zinc concentration in soybean seeds observed in the present study with inoculation of promising *Bacillus* isolates assumes significance considering the fact that zinc deficiency is ranked as the most important risk factor

**Table 5.** Cellular fatty acid methyl ester profiles of zinc-solubilizing *Bacillus* isolates.

| Types of fatty acids | Fatty acids (%) in isolates | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | KHBD-2-1A | BDKH-3 | BD-3-1B | BDSD-2-2C | BDN-5 | KHTH-4-1 | KHBAR-1 | KHBD-6 | KDMR-1-1 |
| $C_{14:0}$ | 2.38 | 1.2 | 2.9 | 1.5 | 1.05 | 0.9 | 1.43 | 1.21 | 2.03 |
| Iso-$C_{14:0}$ | 7.80 | 2.3 | 4.0 | 1.3 | 1.3 | 0.88 | 0.19 | 3.03 | 0.8 |
| Iso-$C_{15:0}$ | 36.19 | 15.2 | 14.2 | 38.1 | 18.95 | 13.42 | 25.89 | 42.6 | 54.2 |
| Anteiso-$C_{15:0}$ | 31.68 | 37.3 | 55.1 | 23.5 | 30.42 | 48.1 | 35.13 | 25.54 | 25.63 |
| $C_{16:0}$ | 3.90 | 11.7 | 2.7 | 4.34 | 1.11 | 2.9 | 10.45 | 1.4 | 1.5 |
| Iso-$C_{16:0}$ | 1.91 | 3.4 | 5.2 | 3.46 | 0.65 | 2.3 | 1.20 | 2.81 | 0.2 |
| Iso-$C_{17:0}$ | 2.55 | 8.1 | 1.9 | 7.03 | 10.43 | 6.57 | 10.53 | 3.02 | 4.5 |
| Anteiso-$C_{17:0}$ | 3.12 | 10.2 | 8.3 | 15.01 | 12.87 | 14.7 | 8.15 | 4.65 | 4.1 |
| Identified as | *Bacillus megaterium* | *Bacillus subtilis* | *Bacillus circulans* | *Bacillus licheniformis* | *Bacillus* sp. | *Bacillus atropheus* | *Bacillus amyloliquefaciens* | *Bacillus firmus* | *Bacillus pumilus* |
| Sim index | 0.550 | 0.724 | 0.458 | 0.534 | 0.424 | 0.456 | 0.765 | 0.675 | 0.521 |

responsible for illness and death in the developing world [39]. Recently, Ramesh *et al*. [17] reported a decrease in phytic-P in soybean seeds upon inoculation with zinc-solubilizing *Bacillus*. Phytic acid P is not bio-available to either livestock or human consumption. Phytic acid also binds nutritionally important minerals, particularly zinc, making them unavailable to humans and to non-ruminant livestock [36]. Therefore, a decrease in phytic acid content may help in increasing zinc bio-availability in soybean seeds.

In the current study, it appears that rhizosphere soil-dwelling bacilli such as isolates KHBD-6, KHBAR-1, BDSD-2-2C, KHBD-2-1A, and KHTH-4-1 are promising for the solubilization of zinc from insoluble zinc compounds *in vitro*. Microcosm studies also revealed that *Bacillus* isolates KHBD-6 (*Bacillus firmus*) and KHBAR-1 (*Bacillus amyloliquefaciens*) increased the zinc concentration in soybean seeds. Field evaluation of these promising isolates is necessary to confirm the zinc solubilization potential in soil of Nimar region and Zn uptake by soybean, with a view to exploit these bacilli for commercialization.

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