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Bioremediation potential of soil microbes for nutrient management in calcareous soil

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Abstract : Soil fertility is a serious concern and a major threat in the process of increasing the crop productivity to feed the growing population. Calcareous soils are sick soil suffering from nutrient deficiency and high calcium carbonate which makes the soil more infertile. It is the need of the hour to incorporate more land under agriculture to increase crop productivity. Chemical amelioration proving to be insufficient and an integrated approach are required to remediate these sick soils. Microbial bioremediation can be a suitable amendment for calcareous soil having severe nutrient deficiencies. Soil sample collected from different arid region were enumerated for native microbes. Two alkali tolerant isolates were obtained and were able to solubilise major nutrients at high alkaline pH. Both the isolates show prominent capacity to solubilise calcium carbonate and other insoluble compounds like zinc oxide, magnesium carbonate, tri-calcium phosphate etc. The isolates were identified as *Burkholderia stabilis and Burkholderia anthina*, show prominent growth at pH 7.0-11.0.The maximum growth for both the isolates was recorded as 11.3×10^9 and 13×10^9 at pH 7.0 to6.2×10^9 and 4.33×10^9 at pH 11.0 respectively after 48 h of incubation. The isolates prove to be a potent source of bioremediation of nutrient deficient calcareous soil.
Key Words: Calcareous soil, Micronutrient Deficiency, Microbial Nutrient Management, Bioremediation.

Introduction

Soil fertility and nutrient management has always been a concern for crop cultivation in arid and semi-arid regions. These regions are rich in calcium carbonate with high alkaline pH. Nutrient deficiencies are most common problem in these soils due to relatively low solubility. Micronutrient deficiencies have become one of the major constraints in sustaining crop production in calcareous soil[18]. Nutrient management in calcareous soils differs from other soils type due to the effect of soil pH on soil nutrient availability.Calcareous soils have free calcium carbonate ($CaCO_3$) in the profile. The carbonates, due to their relatively high solubility, reactivity and alkaline character, buffer the pH of most calcareous soils within the range of 7.5 to 8.5[8].

Iron, Zinc, Manganese and Copper deficiencies are most common in soils that have a high $CaCO_3$ due to reduced solubility at alkaline pH values[8]. These deficiencies appeared most common in several parts of Punjab, Haryana and Rajasthan. Analysis of more than 15,000 soil samples from different districts of Punjab have shown that available Zn, Fe, and Mn content of Punjab soils ranged from 0.02 to 10.4, 0.5 to 176, and 0.8 to 120 mg/kg soil with mean values of 0.95, 10.7, and 11.3 mg/kg soil, respectively[12].In Punjab, Fe is considered to be the second most limiting micronutrient in crop production after Zn. Poor availability of Fe in

the soil, insufficient uptake and Fe inactivation within the plants are reported to be the main causes of Fe chlorosis in crops grown on such soils[13].

The analysis of 5673 soil samples collected across the Haryana state showed a wide variability in status of available micronutrient deficiency in soils. The current status of Zn, Fe, Mn, Cu and B varied from 1.11 to 36.5, 0.0-55.0, 0.0- 48.6, 0.0-13.0 and 0.0-13.7%, respectively with an average deficiency of 15.3, 21.6, 6.1 5.2 and 3.3 %[21]. Factor driving the nutrient deficiency in northern part of India other than calcareous soil is primarily due to the fast adoption of new agricultural technology, including: cultivation of high yielding crop varieties, increase in cropping intensity, expansion of irrigation facilities, increased use of huge quantities of fertilisersand poor quality irrigation water[12].

Chemical fertilizer management in calcareous soils has not been very useful due to the physicochemical characteristics of the soil which differ from that of other nutrient deficient soils. Soluble fertilizers hence applied get locked and are sparingly available to plant[4]. Due to unawareness, improper and heavy dose of chemical are applied to get better results which eventually deteriorate the inherent properties of soil[10]. Chemical inputs hence are not recommended for calcareous soil due to chemical reactions that affect the loss or fixation applied nutrient[8]. The presence of CaCO3 directly or indirectly affects the chemistry and availability of nitrogen (N) Phosphorus (P), Magnesium (Mg), Potassium (K), Manganese (Mn), Zinc (Zn) and iron (Fe). The availability of copper (Cu) also is affected[22].

Application of acid forming fertilizers such as ammonium sulphate and urea fertilizers, sulphur compounds cannot be a sustainable source of amendment due to its repeated application and high cost[11]. Even leaching of these sulphur and urea can cause serious ground water contamination.

Microbial bioremediation can only be a suitable amendment for calcareous soil having severe nutrient deficiencies. Microorganism in the form of Biofertilizers can act on insoluble compounds present in the soil and can increase the nutrient uptake potential for plants[20].Microorganisms resides in rhizosphere affects the growth of plants in several beneficial ways[13]. In the present study an effort shall be made to enumerate native microbial flora from calcareous soil and evaluating its role in soil nutrient management. The study will also focus on utilizing the isolates for solubilisation of insoluble compounds considering the chemistry of calcareous soil.

Experimental

Soil Sample collection

Soil samples were collected from 10 different locations of agricultural fieldfrom south western Punjab of tehsil Bhatindawhich was under paddy cultivation (30^o 13´ 48´´ N, 74^o 57´ 7´´ E). The soil physicochemical properties were also analysed. Approximately 50 g of soil sample was taken from the upper 30 cm of the soil aseptically.

Fig 1: Soil sample collection from the arid location from paddy field of Bhatinda, Punjab

Isolation of alkali tolerant zinc solubilising bacteria

The soil samples were serially diluted using sterile water blanks and plated on modified Pikovskaya's Agar medium[15]supplemented with 0.5 % insoluble Zinc Oxide. The pH of the media was adjusted to pH 9.0 by adding 4N NaOH. The inoculated plates were incubated at 28 ± 2^oC for 3-5 days. After incubation the plates

were enumerated for zinc solubilising bacteria based on the zone of clearing around the colonies. The bacterial isolates were screened on the basis of zone of solubilisation at alkaline pH. Isolates thus obtained were purified by repeated subculturing and maintained on Nutrient Agar slants at 4^{o}C. Screened bacterial isolates were genetically characterized and identified through 16S rDNA sequence.

Study of growth of alkali tolerant bacterial isolates at pH 7.0, 8.0, 9.0, 10.0, and 11.0.

The zinc solubilising isolates were further studied and screened for alkali tolerance and zinc solubilisation at increasing pH 7.0-11.0. The isolates were all inoculated at pH 7.0, 8.0, 9.0, 10.0 and 11.0. The studies were conducted in modified Pikovskaya's broth and agar[15]supplemented with insoluble zinc oxide. pH was adjusted by adding appropriate quantity of 4N NaOH. The medium were inoculated and were incubated at 28 ± 2^{o}C. The growth was documented after 48hrs of incubation. Zone of solubilisation were observed at 4 days, 7 days from agar plate. Further the solubilisation efficiency (SE) of the isolates was calculated and determined as follows[16].

SE = Diameter of solubilisation halo zone / diameter of colony × 100

Study of isolated bacterial strain for qualitative estimation of solubilisation of insoluble micro and macro nutrients.

The alkali tolerant zinc solubilising isolates thus obtained were further studied for their ability to solubilise different insoluble micro and macro nutrients at alkaline pH 9.0. Insoluble compound were enriched in modified Pikovskaya agar and were point inoculated with the isolates. The growth was observed after 4 and 7 days of incubation. The isolates were grown in enriched medium with 1.0 % supplement of insoluble compounds viz. Tri-Calcium Phosphate (TCP), zinc oxide, mica, ferric phosphate, calcium carbonate, manganese carbonate.

Statistical Analysis:

The collected data were statistically computed using Graph Pad Prism 6 software. Data were subjected to analyses of variance and treatment means were compared at significant at $P < 0.05$.

Results

Soil sample collection and isolation of alkali tolerant zinc solubilising bacteria.

Table 1:Soil samples collected were analysed and are summarized as below:

pH	CaCO3 (g kg^{-1})	CEC cmol (+) kg^{-1}	Exchangeable cations cmol (+) kg^{-1}				Particle size distribution of < 2 mm (g kg^{-1})		
			Ca	Mg	Na	K	Sand (2000 – 50 µm)	Silt (50 – 2 µm)	Clay (< 2 µm)
8.78 ± 0.32	15 ± 0.12	9.1± 0.14	4.2+0.03	2.1+0.02	0.2 +0.01	0.2 +0.02	320 +1.12	303 + 1.03	186 + 0.83

*Result expressed as mean of five replicates± SD.

Two bacterial isolates were able to solubilise insoluble zinc oxide at pH 9.0. These isolates were purified, designated and were maintained at 4^{o}C for further studies and screening for alkali tolerant. The isolates were initially isolated from Paddy growing soil and cotton growing soil in Bhatinda Punjab respectively. The isolates were designated as IPL/KB/B6 (NSB -01) and IPL/KB/B9 (NSB -02). Both the isolates were morphologically characterized as gram negative short rod bacteria. The isolates IPL/KB/B6 (NSB -01) and IPL/KB/B9 (NSB -02) were identifiedthrough 16sRNA as *Burkholderia stabilis* and *Burkholderia anthina* respectively.

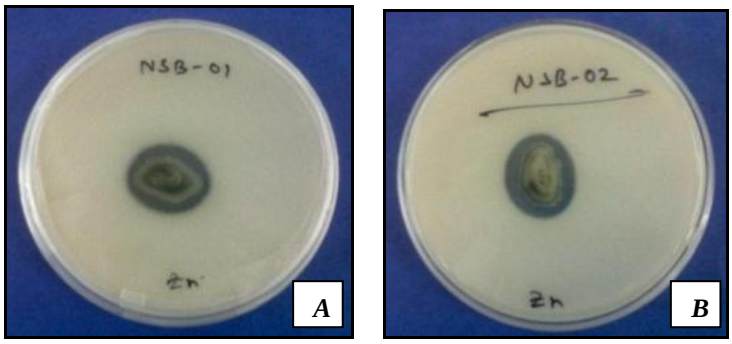

Fig 02: Isolated alkali tolerant bacterial strain IPL/KB/B6 (NSB -01) and IPL/KB/B9 (NSB -02)produce zone of solubilisation.

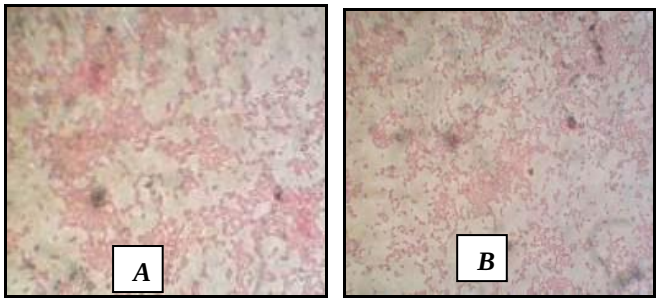

Fig 03: Microscopic view of the isolated bacterial strain (A) NSB 01 & (B) NSB 02 after Gram staining.

Study of growth of alkali tolerant bacterial isolates at pH 7.0, 8.0, 9.0, 10.0, and 11.0.

In-vitro study of acid producing bacterial isolates for alkali tolerance was studied in plate and shake flask. Increase in cell counts in terms of CFU/ ml was observed at pH 7.0, 8.0, 9.0, 10.0, 11.0 was recorded within 48 hrs. The growth was determined on the basis of CFU count. Plate assay technique was also conducted to check the growth of the bacterial isolates in alkaline pH.

Table 02: Study of Alkali tolerance of bacterial isolates IPL/KB/B6 (NSB -01) and IPL/KB/B9 (NSB -02) in enriched media under different pH in Shake flask conditions 48 h.

Isolate	Growth (log_9 CFU/ml) at different media pH				
	7.0	**8.0**	**9.0**	**10.0**	**11.0**
IPL/KB/B6 (NSB-01)	11.3 ± 0.83	9.2 ± 0.83	8.4 ± 0.54	7.8 ± 0.82	6.2 ± 0.82
IPL/KB/B9 (NSB-02)	13.0±0.70	9.6 ± 0.81	8.3 ± 0.70	7.66 ± 0.89	4.33± 0.83

*Result expressed as mean of five replicates± SD.

From the data analysed, it was observed that both the isolates were able to grow at pH from 7.0 to 11.0. The growth was recorded in terms of CFU/ ml value after 48 hrs of incubation. The initial CFU count (0 h) for both isolates IPL/KB/B6 &IPL/KB/B9 were 2×10^5 and 1×10^5 respectively.The maximum growth for both the isolates IPL/KB/B6 (NSB-01) and IPL/KB/B9 (NSB-02) was recorded 11.3×10^9 and 13×10^9at pH 7.0 to6.2×10^9 and 4.33×10^9 at pH 11.0 after 48 h respectively. There is a significant increase in growth as expressed as CFU/ml from 0hr to 48 hr.The bacterial isolates shows prominent growth rate at pH 8.0, 9.0 and 10.0 while there is a slight drop in growth at pH 11.0. More interesting observation was the complete disappearance of insoluble zinc oxide from the medium.

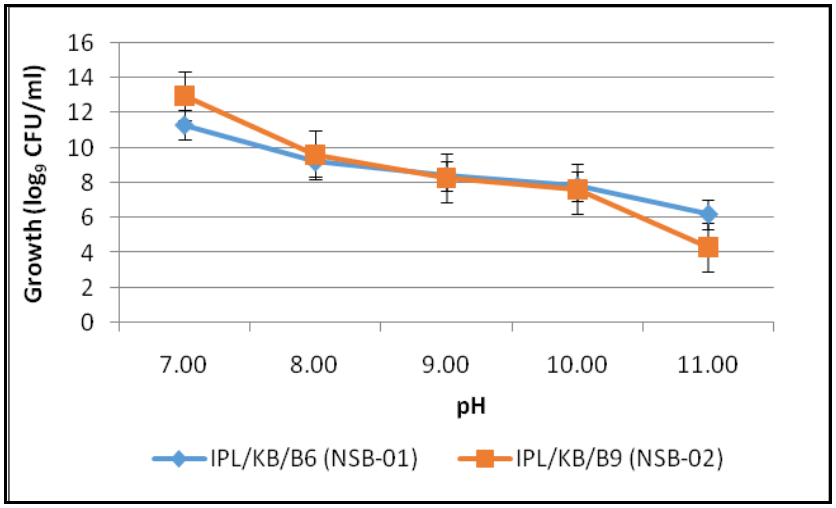

Figure 4: Growth of bacterial isolates at different media pH

Table 03: Study of Alkali tolerance of bacterial isolates IPL/KB/B6 (NSB -01) and IPL/KB/B9 (NSB -02) in enriched agar media under different pH with zone of solubility in Agar Plate Method:

Initial pH of the medium	Zone after 4 days incubation (mm)		Zone after 7 days incubation (mm)	
	NSB 01	NSB 02	NSB 01	NSB 02
7.0	22.6 ± 1.3	22.4 ± 1.14	28.4 ± 1.02	28.2 ± 1.13
8.0	20.2 ± 0.83	19.8 ± 1.09	25.2 ± 0.83	24.2 ± 0.66
9.0	14.6 ± 0.45	14.2 ± 0.86	19.2 ± 0.76	18 ± 0.82
10.0	9.2 ± 0.81	9.1 ± 0.62	13.4 ± 0.54	13 ± 0.54
11.0	2.0 ± 0.47	2.2 ± 0.44	5.2 ± 0.41	4.2 ± 0.36

*Result expressed as mean of five replicates± SD.

Form the result obtained, it was concluded that both the isolates were able to solubilise insoluble zinc oxide at pH 7.0 to 10.0. While at pH 11.0 the solubilisation decreases drastically for both the isolates IPL/KB/B6 (NSB -01) and IPL/KB/B9 (NSB -02) to 2.0 ± 0.47 and 2.2 ± 0.44 mm respectively which is significantly low compared to other pH level. Maximum solubilisation was observed for both isolates at pH 7.0 to 9.0. In this study the solubilisation potential for the bacterial isolates were correlated with the halo zone produced in the plates. Data was compared for 4 days and 7 days of incubation for each pH and it was observed that there is a considerable solubilising potential of the isolates at alkaline pH.

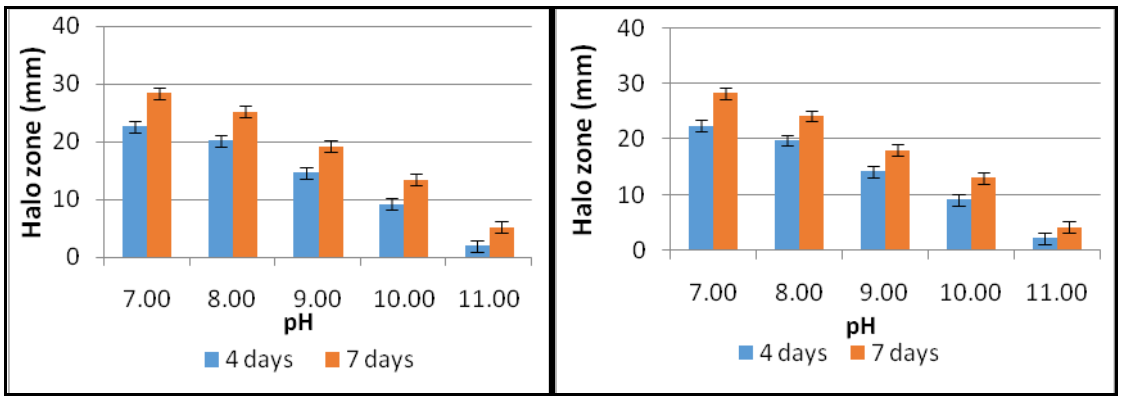

Fig 5: Comparison chart for growth of two isolates at pH range 7.0 to 11.0

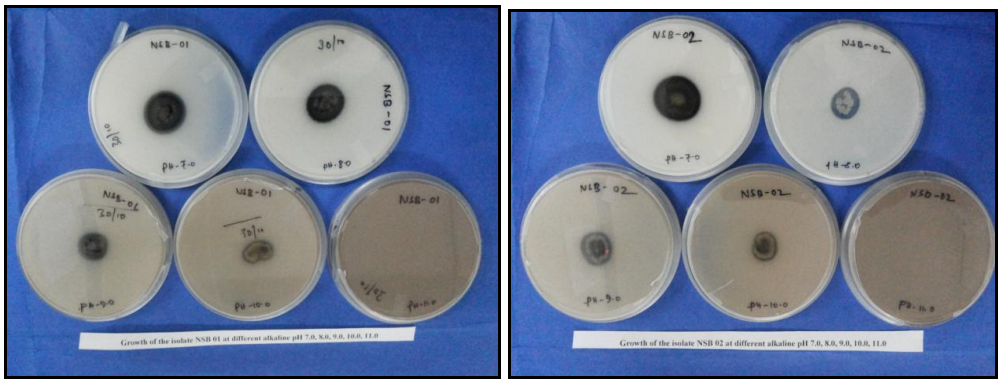

Fig 6: Isolates NSB -01 & NSB-02in enriched agar media under different pH with zone of solubility in Agar Plate Method

2. In-vitro study of isolated bacterial strain for solubilization of insoluble micro and macro nutrients.

The isolated bacterial isolates*B. stabilis* and *B. anthina*were studied for their nutrient solubility. The isolates were point inoculated at the centre of the enriched plate with different insoluble compounds. The plates were incubated to check the zone of solubility around the colony.

Table 04: Details of the result of the solubilising of minerals by NSB - 01 & NSB-02 in lab study:

Insoluble Nutrients	Zone of solubilisation after 4 days of incubation (mm)		Zone of solubilisation after 7 days of incubation (mm)	
	NSB - 01	NSB – 02	NSB - 01	NSB – 02
Mica	0	0	0	0
Zinc oxide	20.4 ± 1.14	22.2 ± 8.3	25.2 ± 0.83	28.2 ± 0.96
Calcium carbonate	21 ± 1.2	20.4 ± 0.67	24.2 ± 1.14	26.4 ± 1.04
Tri-calcium phosphate	7.2 ± 0.83	6.2 ± 0.45	8.4 ± 0.57	7.2 ± 0.81
Manganese carbonate	14.8 ± 0.91	20.4 ± 1.02	20.2 ± 1.12	24.4 ± 0.57
Ferric phosphate	0	15.2 ± 0.71	0	20.4 ± 1.11

*Result expressed as mean of five replicates± SD.

From the data obtained from plate assay it was confirmed that both the bacteria*B. stabilis* and *B. anthina* were able to solubilise insoluble compounds added in the media. *B. stabilis* (NSB 01) shows significant solubility for zinc, calcium carbonate and manganese compounds with the production of halozone. There was no solubilisation for mica and iron. Insoluble Phosphate was solubilised in very low percentage. Whereas, *B. anthina* (NSB-02)solubilises all the insoluble compounds except mica. Both the isolates shows tremendous amount of solubilising capacity of insoluble compounds at alkaline pH.*Bacillus stabilis* (NSB 01) solubilises insoluble zinc oxide and produces halozone upto 25.2 ± 0.83 mm, where as it solubilises Calcium carbonate, tri-calcium carbonate, manganese carbonate and produces halozone 24.2 ± 1.14, 8.4 ± 0.57, 20.2 ± 1.12 mm respectively. *Burkholderia anthina* (NSB 02) solubilizes zinc oxide, Calcium carbonate, tri- calcium carbonate, manganese carbonate and ferric phosphate and produces halozone28.2 ± 0.96, 26.4 ± 1.04, 7.2 ± 0.81, 24.4 ± 0.57, 20.4 ± 1.11 mm respectively.

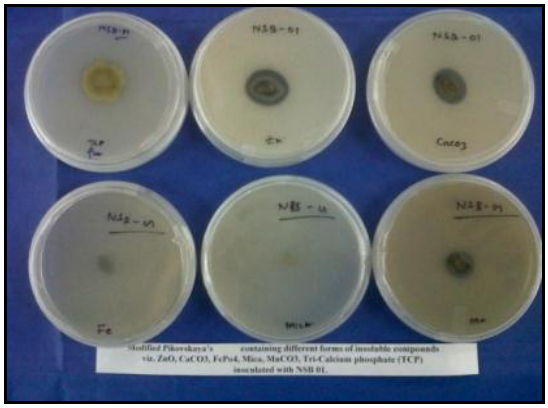

Fig 07: *Burkholderia stabilis* inoculated in modified agar medium containing insoluble compounds produces zone of solubility

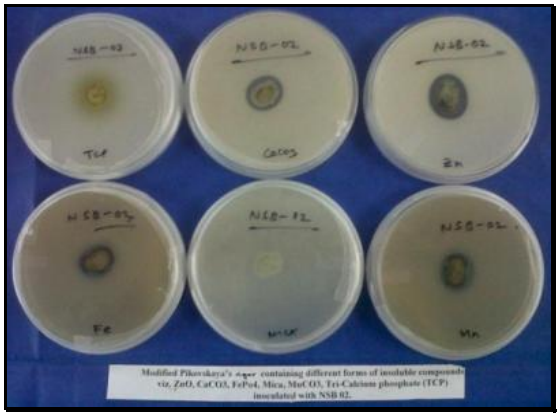

Fig 08: *Burkholderia anthina* inoculated in modified agar medium containing insoluble compounds produces zone of solubility.

Discussion

Soil contains several beneficial microbes having inherent capacity to perform numerous activities to benefit plant growth. These microbes required nutrient and other favourable condition to play their desired role.Microbial population and colonization is a major aspect to be considered in such a concept. In the present study an attempt was made to harness this native microbial flora and understand their role in bioremediation of problematic calcareous soil. Soil sample collected from arid region of Punjab with high pH and calcium carbonate content is not exactly rich in microbes. Two bacteria were isolated capable of solubilising zinc at alkaline pH. Zinc deficiency is a severe issue for crop cultivation in calcareous soil[5].Low availability of Zn in calcareous soils is one of the common abiotic stresses in many parts of the world particularly in Turkey, Australia, China and India[5]. To minimize the deficiency, ZnSO4 is applied in the field to meet the zinc requirement for crops. This practice is widely followed in states like Punjab and Haryana. However, this is neither economical nor environment -friendly in the long run, as only 20% of the applied Zn is available for plant uptake while the remainder gets adsorbed on soil colloids and is therefore rendered immobile[5]. The fact is almost similar for every nutrient which gets locked and immobile in soil. Bioremediation is a novel approach of restoring this soil fertility and eventually increases crop production. In the present study, both the isolated bacterial strains *Burkholderia stabilis* and *Burkholderia anthina* were able to grow at alkaline pH upto 10.0. Both the isolates were able to solubilise insoluble zinc oxide at pH 7.0 to 10.0. Maximum solubilisation was observed for both isolates at pH 7.0 to 9.0. Microorganism has already been reported for solubilisation of insoluble zinc compounds[7]. It was earlier reported that production of gluconic acid and 2 keto gluconic acids helped in the solubilization of the zinc salts[9]. Thus, the development of an elite culture or a consortium of strains capable of utilizing different unavailable insoluble forms of zinc and tolerant to higher zinc levels may be useful to make zinc available in the soil system[19].*B. stabilis* and *B. anthina* were able to solubilise insoluble compounds added in the media. *B. stabilis* (NSB 01) shows significant solubility for zinc, calcium carbonate

and manganese compounds with the production of halozone. There was no solubilisation for mica and iron. Insoluble Phosphate was solubilised in very low percentage. Whereas, *B. anthina* (NSB-02) solubilises all the insoluble compounds except mica. Both the isolates shows tremendous amount of solubilising capacity of insoluble compounds at alkaline pH. Microbial production of organic acids and other metabolites that eventually chelates inorganic compounds into available forms. Dissolution of the zinc oxide and other insoluble compounds may be due to production of organic acids, like gluconic acids. Gluconic acid, and its 2- and 2, 5-keto-derivatives, are produced by bacteria belonging to *Pseudomonas* or related genera as a result of an external oxidative pathway effective on glucose and other aldose sugars[2, 23,24]. Chelation of Mn, Zn, Cu and other micronutrients increases availability on calcareous soils[17]. These alkali tolerant bacterial isolates can prove to be a stable way of restoring soil nutrient content in calcareous soil. Several researches also suggest that there is a great need to assimilate the nutritional requirements forcrop varieties grown in calcareous soil to implement balanced fertilizing program[14]. Efficient plant nutrition management should ensure both enhanced and sustainable agricultural production and safeguard the environment. Developing a suitable nutrient management system that integrates use of microbial inoculants[1] can be a noble approach for crop cultivation in calcareous soil.

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