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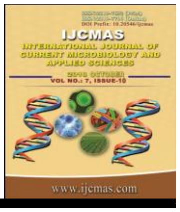

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Organic Acids Production by Zinc Solubilizing Bacterial Isolates

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ABSTRACT

Zinc (Zn) is the most effective micronutrient and Zn solubilization is triggered by the production of organic acids for the optimum growth of plants. An investigation was carried out on organic acid production profiling during zinc solubilization by zinc solubilizing bacterial (ZSB) strains (*B. aryabhattai*, *Pseudomonas taiwenensis* and *Bacillus* sp. PAN-TM1) which were obtained from the Culture Collection Centre, Microbiology Laboratory, SSAC, ICAR-IIHR, Bengaluru, India. Eleven organic acids profile was estimated by HPLC in different zinc sources *viz*., zinc oxide (ZnO), zinc carbonate ($ZnCO_3$) and zinc phosphate [$Zn_3(PO_4)_2$] by the three ZSB isolates which revealed that lactic acid (9128µg.ml^{-1}, in $ZnCO_3$-15days), malonic acid [9456µg.ml^{-1}, in $Zn_3(PO_4)_2$-10days], malic acid (6949µg.ml^{-1}, in $Zn_3(PO_4)_2$-10days), citric acid [8887µg.ml^{-1}, in $Zn_3(PO_4)_2$-5days] and succinic acid [9005µg.ml^{-1}, in $ZnCO_3$-10days] are the major organic acids produced by the isolates used in the study. Among the ZSB strain, *B. aryabhattai* produced all most all the organic acids during zinc solubilization as compared to *P. taiwenensis* and *Bacillus* sp. PAN-TM1. The above findings clearly indicated that the organic acids secretions by *Bacillus* and *Pseudomonas* will vary depend on the substrate of Zn minerals.

Keywords

ZSB, *B. aryabhattai*, Organic acids, HPLC

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Introduction

Zinc (Zn) is an essential micronutrient required for plants, animals, and humans for their normal growth and reproduction (Frassinetti *et al.*, 2006).

In plants, zinc plays a key role as a structural constituent or regulatory co-factor of a wide range of different enzymes and proteins in many important biochemical pathways (Ghosh *et al.*, 2014). Total and available Zn content in Indian soils ranged between 7-2960 mg kg^{-1} and 0.1-24.6mg kg^{-1} respectively with an average deficiency of 12 to 87%. According to the FAO, about 30% of the cultivable soils of the world contain low levels of plant available Zn (Sillanpaa, 1990).

Many Indian soils exhibit the deficiency of Zn with the content much below the critical level of 1.5ppm (Tiwari and Dwivedi, 1994). Thus Zn deficiency has become a serious problem affecting nearly half of the world's population (Cakmak, 2009). To overcome this constraint external addition of soluble Zn to alleviate deficiency results in the transformation of about 96-99% to various fractions of unavailable forms and about 1- 49% is left as available fraction in the soil. Therefore, efficient and economical methods to correct Zn deficiency have to be devised. Recently a bacterial based approach was devised to solve these micronutrient deficiency problems (Anthoni, 2002).

The rhizosphere microorganisms play a pivotal role in the enhancement of crop production by the solubilization of unavailable form of metal into available form. This mineral solubilization was due to the production of organic acids and pH drop by organisms (Alexander, 1997).

Plants take up Zn as (Zn^{2+}) divalent cation. The organic acids released sequester the cations and acidify the micro environment near root is thought to be a major mechanism of Zn solubilization.

In addition, the anions can chelate Zn and increase Zn solubility, which results in the enhanced available form of $Zn^{2+}$to plants (Jones and Darrah, 1994). Hence, it is plausible that the exploitation of native zinc mineralizing and solubilizing bacteria may aid in overcoming zinc deficiency and increased the availability of zinc to crops. In the present study, the ability of different rhizobacteria to solubilize inorganic Zn compounds *in-vitro* was tested and also identified the organic acids aiding in zinc solubilization.

Materials and Methods

Bacterial strains

The three zinc solubilizing bacterial strains (*B. aryabhattai*, *P. taiwenensis* and *Bacillus* sp. (PAN-TM1) included in the present study were procured from the Culture Collection Centre, Microbiology Laboratory, Division of Soil Science and Agricultural Chemistry, ICAR-IIHR, Hesaraghatta, Bengaluru, India.

Organic acids analysis by High Performance Liquid Chromatography (HPLC)

HPLC reverse-phase chromatography was used for the analysis of organic acids produced by bacterial isolates in broth culture. One ml of bacterial cultures were inoculated to the sterilized liquid basal medium containing 0.1% different zinc sources such as ZnO, $ZnCO_3$ and $Zn_3(PO_4)_2$. The samples were withdrawn at 5, 10 and 15 days intervals, centrifuged to remove the debris and cells. The supernatant was collected and filtered through 0.2µm poly vinylidenedifluoride (PVDF) syringe filters and estimated the different organic acids by HPLC (Model: Prominence, Shimadzu, Japan) technique (Tahir and Shakeel, 2013). The organic acids detected were identified by comparing their retention time and the peak areas of their chromatograms with the standard organic acids.

Results and Discussion

HPLC analysis of the culture filtrates was done to identify and quantify the organic acids produced during solubilization of insoluble zinc sources (ZnO, Zn_3 $(PO_4)_2$ and $ZnCO_3$).Totally eleven different organic acids were estimated. Among the eleven organic acids estimated, *B. aryabhattai* has produced (Table 1) a maximum amount of malic acid (6244µg.ml^{-1}), malonic acid (3757µg.ml^{-1}), succinic acid (662µg.ml^{-1}), citric acid (413µg.ml^{-1}), propionic acid (240µg.ml^{-1}), keto-D-gluterate (166µg.ml^{-1}) and gluconic acid (6.56µg.ml^{-1}) in broth supplemented with zinc oxide. More production of malic was recorded after 5 days of incubation; remaining organic acids production was high at 15 days after incubation. When zinc carbonate was used, the maximum production of lactic acid (9128µg.ml^{-1}), succinic acid (9005µg.ml^{-1}), malic acid (2982µg.ml^{-1}), malonic acid

(2070µg.ml^{-1}), propionic acid (234µg.ml^{-1}), keto-D-gluterate (192µg.ml^{-1}), formic acid (34µg.ml^{-1}), gluconic acid (2.99µg.ml^{-1}) and oxalic acid (0.18µg.ml^{-1}) was recorded. But, the maximum production of lactic acid was found 10 days after incubation compared to all other organic acids. When zinc phosphate was used, the highest production of malonic acid (9456µg.ml^{-1}), malic acid (6949µg.ml^{-1}), tartaric acid (1261µg.ml^{-1}), keto-D-gluterrate (335µg.ml^{-1}), propionic acid (258µg.ml^{-1}) and formic acid (133µg.ml^{-1}) was observed after 10 days of inoculation. Whereas the highest production of lactic acid (4111µg.ml^{-1}), citric acid (8887µg.ml^{-1}) and succinic acid (1237µg.ml^{-1}) were observed after 5 days of incubation. Out of eleven organic acids estimated *B. aryabhattai* could produce all most all the organic acids except tartaric acid and lactic acid in medium supplemented with zinc oxide.

P. taiwenensis could produce (Table 2) malonic acid (1077µg.ml^{-1}), keto-D-gluterate (212µg.ml^{-1}), citric acid (61µg.ml^{-1}), propionic acid (235µg.ml^{-1}), gluconic acid (3.45µg.ml^{-1}) and oxalic acid (0.62µg.ml^{-1}) while zinc oxide as a source.

Whereas in the case of zinc carbonate is used, all the eleven organic acid productions were recorded. In this study, the maximum production of malic acid (8830µg.ml^{-1}) followed by malonic acid (4992µg.ml^{-1}) and succinic acid (2829µg.ml^{-1}) was recorded after 10 days of incubation. All the eleven organic acids was recorded in medium supplemented with zinc phosphate, however the following organic acids *viz*., malic acid (5649µg.ml^{-1})followed by malonic acid (3722µg.ml^{-1}), lactic acid (3939µg.ml^{-1}), citric acid (1023µg.ml^{-1}) and succinic acid (1080µg.ml^{-1}) were found to be maximum. *P. taiwenensis* was unable toproduce malic acid, tartaric acid, lactic acid and succinic acid in medium supplemented with zinc oxide source.

The organic acids estimated in *Bacillus* sp. (PAN-TM1) (Table 3) inoculated to the medium supplemented with zinc oxide, the maximum production of lactic acid (2609µg.ml^{-1}), followed by malic acid (7580µg.ml^{-1}), malonic acid (6062µg.ml^{-1}), succinic acid (2119µg.ml^{-1}) and citric acid (9909µg.ml^{-1}) was observed after 10 days of incubation.

Wherein when zinc carbonate used, the malic acid (9104µg.ml^{-1}) after 5 days followed by malonic acid (7103µg.ml^{-1}), citric acid (4225µg.ml^{-1}) and succinic acid (1069µg.ml^{-1}) was found maximum after 10 days of incubation. In case of zinc phosphate as a source, the lactic acid (2250µg.ml^{-1}) was recorded maximum after 15 days of incubation followed by malic acid (7828µg.ml^{-1}), malonic acid (7808µg.ml^{-1}), succinic acid (1468µg. ml^{-1}) and citric acid (1280µg.ml^{-1}).

There was a restricted production of keto-D-glutaric acid, lactic acid and oxalic acid by *Bacillus* sp. (PAN-TM1) when zinc carbonate and zinc phosphate was incorporated into the medium.

In the terrestrial environment, mobilization of insoluble metal compounds is important for the release of essential minerals (Mn, Fe and Zn) as well as associated anionic nutrients, e.g. phosphate, into biogeochemical cycles (Gadd, 1999). This depends mainly on the excretion of various metabolites, including organic acids and protons (Sayer *et al.*, 1995).

The organic acid production (especially gluconic acid) is the primary mode of action for Zn dissolution, these low-molecular-weight acids can non-specifically solubilize zinc, phosphorus, potassium, calcium, and manganese from their respective minerals or from insoluble precipitates depending upon the physico-chemical properties of the soil (Uroz *et al.*, 2009).

Table.1 Organic acids production of *B. aryabhattai* in nutrient broth supplemented with 0.1% different Zn sources at different intervals

Organic Acids	**Organic acid production (μg ml^{-1})**								
	ZnO			**$ZnCO_3$**			**$Zn_3(PO_4)_2$**		
	5days	**10days**	**15days**	**5days**	**10days**	**15days**	**5days**	**10days**	**15days**
Keto-D-Gluterate	160	166	104	192	138	120	233	335	304
Tartaric acid	0.00	0.00	0.00	25	0	0	1138	1261	974
Formic acid	32	28	34	0	34	21	104	133	55
Malic acid	6244	3605	680	0	0	2982	5651	6949	3256
Malonic acid	3757	2686	3547	687	2070	1631	6968	9456	2422
Lactic acid	0	0.00	0.00	0	1244	9128	4111	0	0
Citric acid	142	47	413	39	550	741	8887	4742	175
Succinic acid	0.00	0.00	662	0	9005	7066	1237	1222	0
Propionic acid	234	234	240	234	0	234	253	258	234
Gluconic acid	0.69	6.56	4.23	2.99	0.51	1.91	1.71	19.52	34.69
Oxalic acid	0.00	0.45	0.08	0.18	0.00	0.11	0.05	0.47	0.634

Table.2 Organic acids production of *P. taiwenensis* in nutrient broth supplemented with 0.1% different Zinc sources at different intervals

Organic acids	**Organic acid production (µg.ml^{-1})**								
	ZnO			**$ZnCO_3$**			**$Zn_3(PO_4)_2$**		
	5days	**10days**	**15days**	**5days**	**10days**	**15days**	**5days**	**10days**	**15days**
Keto-D-Gluterate	0	101	212	244	354	91	111	204	867
Tartaric acid	0	0	0	1078	1864	0	0	1349	28
Formic acid	610	6	0	20	126	4	46	173	0
Malic acid	0	0	0	4497	8830	2093	0	5649	0
Malonic acid	0	1077	7.97	4951	4992	1258	6713	3722	439
Lactic acid	0	0	0	8294	1283	1832	1498	3939	392
Citric acid	0	50	61	3444	3146	99	724	1023	133
Succinic acid	0	0	0	2178	2829	546	1469	1080	0
Propionic acid	235	234	234	259	303	234	249	256	237
Gluconic acid	0.64	0.65	3.45	33.3	29.11	18.61	6.7	14.14	31.05
Oxalic acid	0.62	0.06	0.32	0.89	0.63	1.05	0.23	0.30	0.12

Table.3 Organic acids production of *Bacillus* sp. (PAN-TM1) in nutrient broth supplemented with 0.1% different zinc sources at different intervals

Organic acids	Organic acid production (μg.ml^{-1})								
	ZnO			$ZnCO_3$			$Zn_3(PO_4)_2$		
	5days	10days	15days	5days	10days	15days	5days	10days	15days
Keto-D-Gluterate	0	0	0	408	264	237	432	453	444
Tartaric acid	2055	0	0	3025	1605	0	0	1751	1869
Formic acid	304	341	245	259	283	528	65	150	17
Malic acid	3495	7580	5419	9104	5324	5636	4606	3124	7828
Malonic acid	5905	6062	5537	2737	7103	5662	1241	7808	0
Lactic acid	1853	2609	2333	0	0	0	1513	0	2250
Citric acid	5442	9909	7841	3993	4225	2996	3888	2061	1280
Succinic acid	1460	2119	2028	1429	1069	1047	1468	1540	1168
Propionic acid	238	269	243	269	260	251	252	275	291
Gluconic acid	6.42	1.81	1.78	13.43	1.52	4.94	2.03	4.21	5.08
Oxalic acid	0.82	0.09	0.18	0.204	0.06	0.08	0	0	0

The organic acids can adhere to the mineral surface and extract the nutrients non-specifically from the mineral particles through electron transfer; break the oxygen links in the minerals and release the nutrients and chelate ions present in the solution through carboxyl and hydroxyl groups and thereby indirectly accelerating the dissolution rate of minerals (Welch *et al.,* 2002). Organic acids provide both sources of protons for mobilization and metal chelating anion to complex the metal cations (Devevre *et al.,* 1996). In the present study profiles of organic acids produced in different zinc sources by the bacteria revealed that lactic acid, malonic acid, malic acid and succinic acid was the major and maximum produced organic acids by the isolates. The other organic acid produced includes keto-D-gluterate, citric acid, propionic acid, oxalic acid, tartaric acid and gluconic acid. The production of organic acids by zinc solubilizing bacteria has been reported, where *P. fluorescens* produced gluconic acid and 2-keto-gluconic acids in the culture broth during zinc phosphate solubilization (DiSimine *et al.,* 1998).

The release of organic acids that sequester cations and acidify the micro environment is thought to be a major mechanism of Zn solubilization. A number of organic acids such as citric, acetic, propionic, lactic, oxalic, glycolic, gluconic acid etc., have been considered due to its effect in pH lowering by microorganisms (Cunningham *et al.,* 1992).

From the present study, it is concluded that the production of eleven different organic acids by these zinc solubilizing bacterial strains play an important role in the solubilization of unavailable forms of zinc. Organic acids also responsible for lowering the pH of the medium or rhizosphere. Overall, these mechanisms convert unavailable form of zinc to available form as it is essential for the plant for its growth and development.

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