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Isolation and Characterization of Potential Zn Solubilizing Rhizobacteria from Cumin (*Cuminum cyminum*)

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A B S T R A C T

The present investigation was carried out during the year 2017-18 in Laboratory of Microbiology, Division of Crop Production, ICAR-National Research Centre for Seed spices Ajmer, Rajasthan. The aim of study was to isolate potential Zn solubilizing plant growth promoting rhizobacteria from rhizosphere soil and plants of cumin. For this investigation 155 soil and plant samples were collected from Ajmer, Nagaur Jodhpur, Jaisalmer, Barmer, Jalore districts of Rajasthan. From 155 samples, 153 isolates were collected and out of 153, 23 isolates were Zn solubilizers. However 23 Zn solubilizing rhizobacteria were selected for further studies viz., Qualitative and quantitative estimation of Zn *in vitro* and characterized them against biochemical identification and evaluate for plant growth promoting traits. In qualitative study of Zn estimation, highest solubilization zone was 26 mm due to isolate DCU-451 followed by 21, 20 and 19 mm associated with DCU-453, DCU-188 and DCU-184, respectively, and least solubilization zone was 12 mm due to isolate DCU-460. Similarly highest solubilization index was found 3.7 due to isolate 451 followed by 3.4, 3.3 and 3.2 were associated with rhizobacterial isolates DCU-453, 184 and 188 respectively, and lowest SI was 2.2 due to isolate DCU-169. In quantitative assay at 5 days highest amount of released Zn was found 152 µg followed by 144, 141 µg /ml due to isolate DCU-453, DCU-451 and DCU-188, respectively, and least was 81µg /ml in DCU-176. At 10 days highest amount of released Zn was found 268 µg followed by 253, 238 µg /ml due to isolate DCU-451, DCU-453 and DCU-172, respectively, and least was 117µg /ml in DCU-165. Similar results were found at 15 days also, that were highest amount of released Zn was found 419 µg followed by 391, 389 µg /ml associated with DCU-451, DCU-188 and DCU-453, respectively, and least was 184µg /ml in DCU-198. However rhizobacterial isolate DCU-451 was found superior in both qualitatively and quantitatively.

Introduction

Cumin (*Cuminum cyminum*) is a member of Umbelliferae (Apiaceae) family and an annual plant, which is widely cultivated in arid and semi-arid regions. Cumin has along history of use as food flavours, perfumes and medicine. In addition to its common use as spice in our daily life, recent studies have indicated its pharmaceutical and medicinal importance. Total area, total production and yield of cumin in India is 966170 hectare, 688660 tonnes and

713 kg per hectare, respectively, (Directorate of arecanut and spices development, Calicut, 2017-18). Among seed spices, cumin is an important crop of western Rajasthan and is mainly grown in the districts of Jaisalmer, Jalore, Pali, Barmer, Ajmer, Nagaur, Tonk and Jodhpur. In fact, the extreme susceptibility to disease like wilt, powdery mildew and blight and also to aphids and lack of knowledge of suitable agricultural practices are the reasons of poor productivity in this crop. There is no doubt that this crop has tremendous scope and the availability of suitable improved practices will result in increase in area as well as production by solving the above constraints.

Cumin (*Cuminum cyminum* L.) is an aromatic plant from the Apicaceae family and its seeds has been used as a spice from ancient times, being mentioned also in the Bible (Sahana *et al.,* 2011). The seeds are used in cooking, while volatile oil it is useful in flavouring foods but also in cosmetics and perfume industries (Dubey *et al.,* 2017). Cumin is the second most important spice in the world, after black pepper (*Pepper nigrum*), according to (Sowbhagya, 2013; Mnif *et al.,* 2015).

Cumin known as zeera, could be cultivated in the plains areas, semi-arid regions, but also in the hills, being resistant to water deficit and having a seeds production of about 12000 tons per year (RezaeiChiyaneh *et al.,* 2018). Apicaceae family is one of the biggest plant families, including 455 genera and over 3500 species (Bhmankar *et al.,* 2018). As reported by the literature, *Cuminum cyminum* may have positive effects on metabolic disorders like hyperglycemia, dyslipidemia, on weight reduction and could ameliorate insulin function, preventing the progression of diabetes (Jafari *et al.,* 2018 and Rostami *et al.,* 2018) described that cumin could have beneficial effect on digestion, flatulence, diarrhea and possess antioxidant and antispasmodic properties.

Bacteria are known to immobilize metal by precipitation and adsorption. The ability to dissolve immobilized zinc viz. zinc phosphate, zinc oxide and zinc carbonate in appreciable quantity is not common feature amongst the cultivable bacteria. Few Zn solubilizing bacterial genera viz., *Thiobacillus thioxidans*, *Thiobacillus ferroxidans, Acinetobacter, Bacillus, Gluconacetobacter,* Pseudomonas and facultative thermophilic iron oxidizers have been reported as zinc solubilizers (Saravanan *et al.,* 2007). 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. However, only some bacterial species of the genera *Acinetobacter, Bacillus, Gluconacetobacter*, and *Pseudomonas* have been reported (Gadd, 2007).

Materials and Methods

The present investigation was carried out in Microbiology laboratory Division of Crop production, ICAR-NRCSS, Tabiji, Ajmer for isolation and characterization of zinc solubilizing bacteria from cumin rhizosphere and plant itself.

Collection of samples

A total of 155 soil and plant samples were collected from cumin grown agricultural fields of Ajmer, Barmer, Jalore, Nagaur, Jaisalmer and Jodhpur Districts of Rajasthan (India). All rhizospheric soil samples were collected from 0-15 cm depth by carefully uprooting the plants and for endophytes healthy cumin roots were collected. The samples were properly tagged, sealed and stored. Collected soil samples were preserved in a polythene bag for physico-chemical properties and microbial analysis.

Isolation of rhizobacteria

The isolation of rhizobacteria from cumin roots as well as rhizosphere soil of cumin was done using six different media viz. Nutrient agar (NA) and Zinc solubilizing agar medium for Zn solubilizing microbes. All the media were prepared and autoclaved at 15 psi and 121°C for 20 minutes.

Isolation of rhizobacteria from rhizospheric soil

Ten grams of the fresh soil was transferred to Erlenmeyer flask (150 ml) containing 90 ml sterile distilled water (10^{-1}) and was shaken at 120 rpm for 15 min. Then, 1.0 mL of this suspension was transferred into a 9 mL blank (10^{-2}). This serial dilution was continued up to 10^{-10}, followed by pour plating on Nutrient agar (NA) and Zinc solubilizing agar medium. The petri plates were inoculated and incubated for 24-48 hrs at 28°C. Colonies which appeared to be morphologically different were isolated and subcultured.

Isolation of rhizobacteria from cumin roots

The collected plant material used for the isolation was first surface sterilized following the method of Santos *et al.*, (2003) with few modifications. Plant material was first cleaned by washing several times under running tap water then Surface sterilization was performed by sequentially rinsing the plant material with 70% ethanol (C_2H_5OH) for 30 seconds, then with 0.01% mercuric chloride ($HgCl_2$) for 5 minutes followed by 0.5% sodium hypochlorite (NaOCl) for 2-3 minutes and finally with sterile distilled water for 2-3 times. Plant roots were then dried in between the folds of sterile filter papers. After proper drying, the surface sterilized roots were cut vertically into small segments each segment was placed on different types of medium. All the plates were incubated at 28ºC to promote the growth of endophytes and were regularly monitored for any microbial growth. On observing the microbial growth, sub culturing was done. Each endophytic culture was checked for purity and transferred to freshly prepared medium plate. Appropriate controls were also set up in which no plant tissues were inoculated.

Screening for solubilizing rhizobacteria

All the bacterial isolates were tested for their ability to solubilize the insoluble form Zinc oxide, on Zinc solubilizing agar for isolate Zinc solubilizing bacteria, respectively. 23 rhizobacteria were obtained those had capacity to solubilizing Zn *in vitro* . Out of these 23, some isolates also had able to solubilize another mineral like P and K depicted in Table 1.

Biochemical characterization of rhizobacteria

Biochemical characterization of bacterial isolates was done on the basis of catalase production, nitrate reduction, starch hydrolysis and methyl red test. These were conducted as per the standard methods (Cappuccino and Sherman 1992).

Catalase production

A drop of 3% H_2O_2 was taken on a glass slide and small amount of bacterial culture was mixed with platinum inoculation loop. Rapid and sustained production of gas bubbles or effervescence constituted positive test.

Nitrate reduction tes

5ml nitrate broth was inoculated with pure culture of the test organism. It was incubated at 28^0 C for 48 hours. Equal volume (0.5ml) of both the reagents A (Sulfanilicacid 8g + Acetic acid 1000 ml) and B (5 g

Alphanaphthylamine + 5N Acetic acid 1000 ml) were added. The development of red color within 30 seconds indicated the positive test.

Hydrolysis ofstarch

Sterilized starch agar medium was poured onto petriplates. The log phase cultures were spotted on the plates and incubated at 28°C for 48 hrs.

After full growth of cultures, the petriplates were flooded with Gram's iodine. The hydrolysis of starch was observed as a colorless zone surrounding the colonies against purple background. A blue or purple zone indicated that starch was nothydrolyzed.

Methyl red (MR) test

Dye was dissolved in alcohol followed by addition of water to make 100 ml volume. It was stored at room temperature.

A tube of GPPW (5 ml) was inoculated with pure culture of the test organism. It was incubated at 28^0 C for 48 hours.

At the end of this, 5 drops of the MR reagent was added directly to the broth. The development of a stable red color indicated positive test.

HiAssorted biochemical test

Each HiAssorted™ Biochemical Test kit is a standardized colorimetric identification system utilizing seven conventional biochemical tests and five carbohydrate utilization tests. The tests are based on the principle of pH change and substrate utilization. On incubation organisms undergo metabolic changes which are indicated by a colour change in the media that can be either interpreted visually or after addition of the reagent.

Qualitative assay for Zn solubilization

One loop full of overnight matured cultures of Zn solubilising bacterial isolates were spotted on zinc solubilizing agar medium to observe the zone of solubilisation/clearance by the isolates.

The plates were incubated at 28 C̊ for 48 h and the zone of solubilisation/clearance was observed and expressed in mm. SI was measured using the following formula (Premono *et al.*, 1996).

SI = (Colony diameter + Halo zone formation) / (Colony diameter)

Quantitative assay for Zn solubilization

The ZSB isolates that showed halo zone formation were further tested for their ability to release inorganic Zn from insoluble ZnO using Atomic Absorption Spectrometer (Varian AAS 240 FS).

The ZSB were grown in 50.0 mL Zn solubilising broth at 28˚C for different interval of days (5^{th} day, 10^{nd} day and 10th day) with three replicates in incubator cum shaker at 120 rpm along with their controls.

After 24 h of incubation, cultures and control were withdrawn and transferred aseptically to centrifuge tubes. They were centrifuged at 8,000 rpm for 15 min at 4˚C. The supernatant was collected in test tubes. Then 1.0 mL aliquot from the supernatant was transferred to 50.0 mL standard flask and the volume was made up to 50.0 mL using distilled water.

The soluble Zn was estimated from standard curve by plotting readings drawn from standard solution against mg of Zn taken. The same procedure was followed for the remaining sampling days along with their respective controls.

Determination of pH

The pH of the ZSB culture filtrates and the uninoculated samples was determined at 5, 10 and 15 days after inoculation. The culture was filtered using Whatman No.1 filter paper. The pH was estimated using Elico pH meter.

Statistical analysis

Statistical analysis of data was carried out using online statistical analysis pack- age (OPSTAT, Computer section, CCS HAU Hisar, Haryana) for calculation of ANOVA.

Results and Discussion

Results of biochemical tests were depicted in Table 4.

Qualitatively assay of Zn solubilization *in vitro*

Bacterial isolates were tested for halo zone formation by plate assay using zinc soulubilizing agar medium containing 0.1% zinc oxide. The halo zone and colony diameters were measured after the incubation of plates at 28℃. Table 2 shows data on zinc solubilization by bacterial isolates on qualitative basis. The efficacy of P solubilization by individual isolate on agar medium was shown in Plate 4.1. Based on halo zone formed by an isolates, solubilization index (SI) was calculated. SI ranged from 2.2-3.7. Highest solubilization zone was 26 mm due to isolate DCU-451 followed by 21, 20 and 19 mm associated with DCU-453, DCU-188 and DCU-184, respectively, and least solubilization zone was 12 mm due to isolate DCU-460. Similarly highest solubilization index was found 3.7 due to isolate 451 followed by 3.4, 3.3 and 3.2 were associated with rhizobacterial isolates DCU-453, 184 and 188 respectively, and lowest SI was 2.2 due to isolate DCU-169. Variation in solubilization index by 23 isolates depicted in Table 2 and Figure 1. Solubilization zone of potential isolates DCU-451, DCU-188 and DCU-451 were showing in Plate-1.similarly, Gandhi *et al.,* (2014) isolated 240 zinc solubilizing bacterial strains from rhizosphere of rice. Similar observations noticed by Bhagwan Singh *et al.,* (2017). They reported that Zn solubilizing isolate ZnSF-1 showed maximum solubilization zone of 85 mm followed by ZnSF-2 with 34 mm for ZnO. The solubilization zone is ranged from 6 mm to 25 mm for ZnP. The isolate ZnSB-8 showed maximum solubilization zone of 25 mm for zinc phosphate. The solubilization efficiency (%) ranges from 157.14 to 500 % which was maximum for ZnSB–8 (500.0 %) and least for ZnSB- 6 (157.14 %).

Qualitatively assay of Zn solubilization *in vitro*

Zinc solubilisation by rhizobacterial isolates were studied using zinc solubilizing medium broth inoculated with respective isolates and incubated for 15 days. To study the efficiency of zinc solubilisation by the isolates, zinc solubilisation was calculated at different time interval. At 5 days highest amount of released Zn was found 152 µg followed by 144, 141 µg /ml due to isolate DCU-453, DCU-451 and DCU-188, respectively, and least was 81 µg /ml in DCU-176. At 10 days highest amount of released Zn was found 268 µg followed by 253, 238 µg /ml due to isolate DCU-451, DCU-453 and DCU-172, respectively, and least was 117 µg /ml in DCU-165. Similar results were found at 15 days also, that were highest amount of released Zn was found 419 µg followed by 391, 389 µg /ml associated with DCU-451, DCU-188 and DCU-453, respectively, and least was 184 µg /ml in DCU-198.Variation of isolate for released Zn and reduction in pH was observed in all cultures over incubation time shown in Table 3, Figure 2 and 3.

Plate-1 Solubilization of Zn on zinc solubilizing agar media by potential rhizobacteria from cumin

DCU-451 on zinc solubilizing agar media

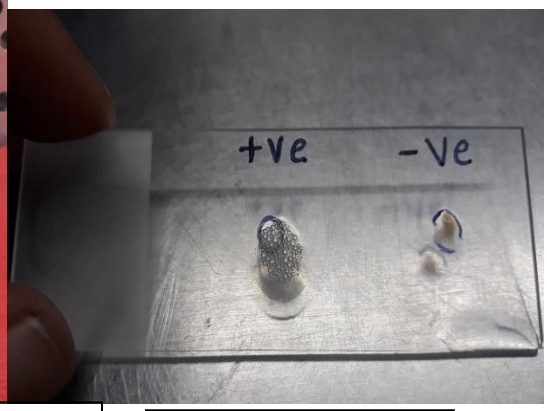
DCU-451 on zinc solubilizing agar mediamedia

DCU-453 on zinc solubilizing agar media

DCU-188 on zinc solubilizing Agar mediamedia

Plate-2 Biochemical test and quantitatively zinc estimation

Zn solubilization by DCU-451 in broth @ 15 DAI



Catalase activity test

Biochemical tests of DCU-451 usingHiAssorted Biochemical test kit

Table.1 Beneficial traits of isolates against mineral solubilization

S.N.	Isolates	PSolubilizers	K Solubilizers	Zn Solubilizers
1	DCU-112	+	-	+
2	DCU-121	+	+	+
3	DCU-154	-	-	+
4	DCU-157	-	-	+
5	DCU-159	-	-	+
6	DCU-162	-	-	+
7	DCU-165	-	-	+
8	DCU-169	-	-	+
9	DCU-172	-	-	+
10	DCU-176	-	-	+
11	DCU-181	-	-	+++
12	DCU-183	-	-	+
13	DCU-184	+	-	+++
14	DCU-187	-	-	+
15	DCU-188	+	-	++
16	DCU-195	-	-	+
17	DCU-197	-	-	+
18	DCU-198	-	-	+
19	DCU-251	+	+	+
20	DCU-451	+	+	+++
21	DCU-453	+	+	+++
22	DCU-460	-	-	+
23	DCU-651	+	+	+

Table.2 Qualitatively estimation of soluble Zn by potential PGPR isolates

S.N.	Isolates	Solubilization zone (mm)	Solubilization Index
1.	DCU-460	12	2.6
2.	DCU-181	17	3
3.	DCU-184	19	3.3
4.	DCU-188	20	3.2
5.	DCU-251	18	3.0
6.	DCU-112	15	2.7
7.	DCU-121	18	2.5
8.	DCU-154	14	2.8
9.	DCU-157	15	2.9
10.	DCU-159	16	2.8
11.	DCU-162	15	2.6
12.	DCU-165	14	2.5
13.	DCU-169	17	2.2
14.	DCU-172	16	2.4
15.	DCU-176	15	2.8
16.	DCU-183	16	2.6
17.	DCU-187	18	2.7
18.	DCU-195	15	3.2
19.	DCU-197	17	2.5
20.	DCU-198	16	2.4
21.	DCU-451	26	3.7
22.	DCU-453	21	3.4
23.	DCU-651	13	2.6
	SEm	0.983	0.112
	CD	2.106	0.223
	CV	4.212	3.734

Table.3 Quantitatively estimation of Zn released by rhizobacterial isolates in zinc solubilizing medium broth

S.N.	Isolates	5 Days		10 Days		15 Days	
		Soluble Zn (µg /ml)	pH	Soluble Zn (µg /ml)	pH	Soluble Zn (µg /ml)	pH
1.	DCU-460	118	6.45	198	6.12	362	6.0
2.	DCU-181	121	6.78	219	5.65	363	5.54
3.	DCU-184	138	6.35	223	5.41	384	5.30
4.	DCU-188	141	6.67	253	5.82	391	5.53
5.	DCU-251	130	6.45	226	6.36	358	6.34
6.	DCU-112	96	6.84	187	6.31	259	5.98
7.	DCU-121	82	6.65	145	6.42	257	5.81
8.	DCU-154	126	6.29	211	6.12	269	5.82
9.	DCU-157	94	6.94	168	6.40	297	6.14
10.	DCU-159	116	6.47	215	6.15	275	5.86
11.	DCU-162	88	6.36	184	5.84	212	5.51
12.	DCU-165	95	6.72	117	6.46	187	6.22
13.	DCU-169	133	6.81	241	6.28	352	6.12
14.	DCU-172	133	6.96	238	5.86	373	5.47
15.	DCU-176	81	6.62	126	6.11	195	5.76
16.	DCU-183	92	6.69	158	6.47	198	5.92
17.	DCU-187	125	6.46	226	6.32	258	6.11
18.	DCU-195	128	6.47	216	6.23	299	6.37
19.	DCU-197	83	6.37	183	5.98	191	5.30
20.	DCU-198	94	5.96	194	5.14	184	5.71
21.	DCU-451	144	6.10	268	5.16	419	4.81
22.	DCU-453	152	6.26	236	5.84	389	5.63
23.	DCU-651	125	6.94	223	6.72	287	6.42
	SEm	7.362		10.384		14.469	
	CD	13.422		21.351		31.685	
	CV	16.366		11.713		13.478	

Table.4 Biochemical tests of Potential Zn solubilizing bacterial isolates

S.N.	Isolates	Starch hydrolysis	Catalase production	Methyl Red test	HiAssorted Biochemical test kit											
					1	2	3	4	5	6	7	8	9	10	11	12
1.	DCU-112	+	+	+	+	-	+	-	-	+		-	-	-	-	-
2.	DCU-121	+	+	-	-	-	+	-	-	-	-	+	-	-	-	-
3.	DCU-154	-	-	+	-	+		-	-	-	+	-	-	-	+	-
4.	DCU-157	-	+	+	-	+	-	-	-	+	-	-	-	+	-	-
5.	DCU-159	-	+	-	-	-	-	+	+	-	+	-	-	-	+	-
6.	DCU-162	+	-	+	+	-	+	-	-	-	-	-	-	+	-	-
7.	DCU-165	-	+	+	-	-	-	+	-	-	+	-	-	-	+	-
8.	DCU-169	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-
9.	DCU-172	+	+	-	-	-	-	+	-	-	-	-	-	-	+	-
10.	DCU-176	-	-	+	-	+	-	+	-	-	-	-	-	-	+	-
11.	DCU-181	+	+	+	+	-	-	-	-	-	+	-	-	-	+	-
12.	DCU-183	-	+	-	-	+	-	-	+	-	-	-	+	-	-	-
13.	DCU-184	+	+	+	-	-	+	+	-	-	-	-	-	-	+	+
14.	DCU-187	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-
15.	DCU-188	-	+	-	+	-	-	+	-	-	+	-	-	-	+	-
16.	DCU-195	+	+	+	-	+	+	-	-	-	+	-	-	-	-	-
17.	DCU-197	-	-	-	-	-	+	-	+	-	-	-	+	-	+	-
18.	DCU-198	-	+	+	-	+	-	-	-	-	+	-	-	-	+	-
19.	DCU-251	+	-	+	-	+	-	-	+	-	-	-	-	-	+	-
20.	DCU-451	+	+	+	+	-	-	+	+	-	-	+	-	-	-	-
21.	DCU-453	+	+	-	+	-	+	-	-	+	-	-	-	+	-	-
22.	DCU-460	+	-	+	-	+	-	-	-	+	-	-	-	-	+	-
23.	DCU-651	+	+	-	+	-	+	+	-	+	-	-	-	+	-	+

1 Citrate utilization **2 Lysine utilization** **3 Ornithinine utilization** **4 Urease**
5 Phenylalanine deaminase **6 Nitrate reductase** **7 H_2S Production** **8 Glucose**
9 Adonitol **10 Lactose** **11 Arabinose** **12 Sorbitol**

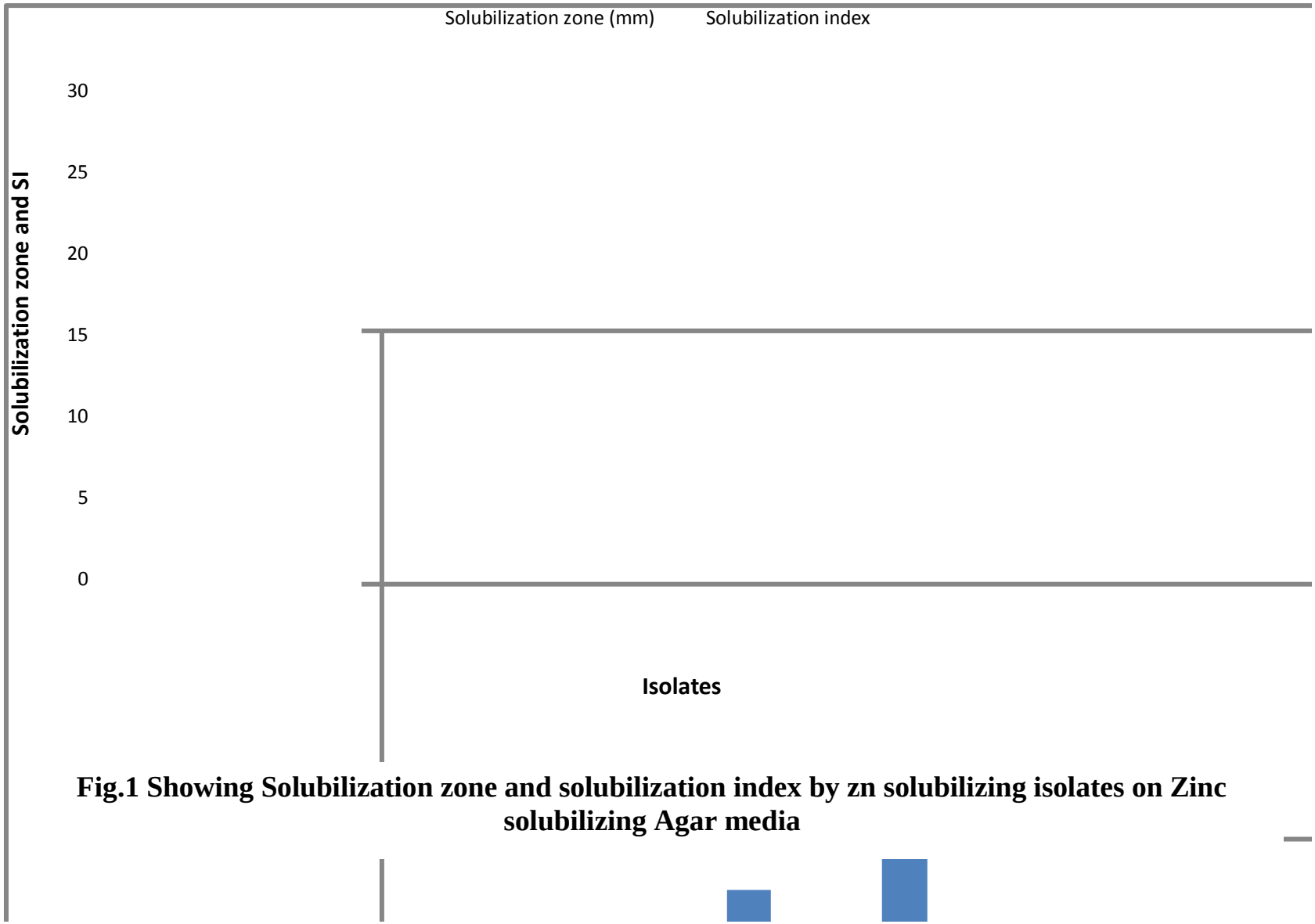


Fig.1 Showing Solubilization zone and solubilization index by zn solubilizing isolates on Zinc solubilizing Agar media

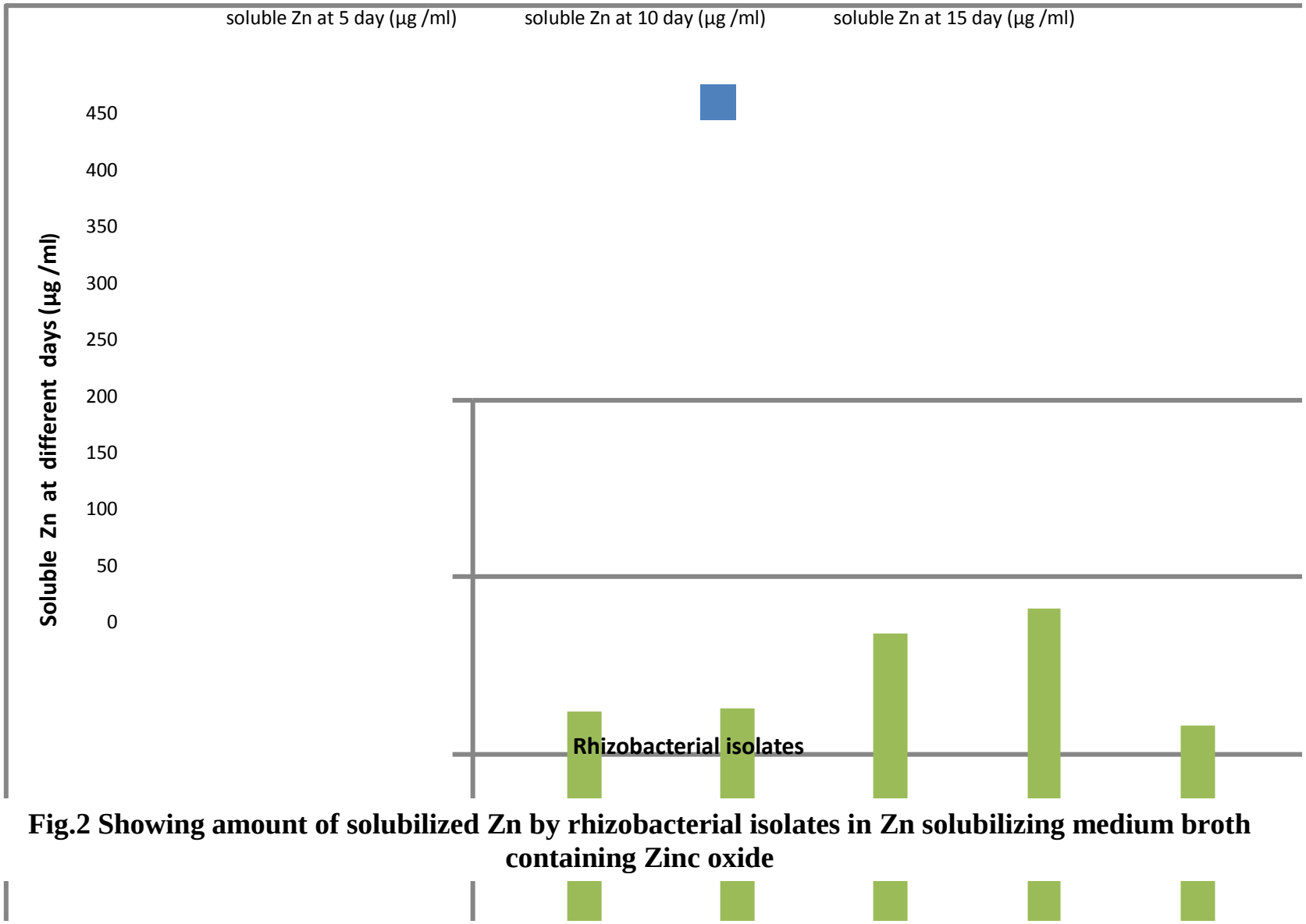


Fig.2 Showing amount of solubilized Zn by rhizobacterial isolates in Zn solubilizing medium broth containing Zinc oxide

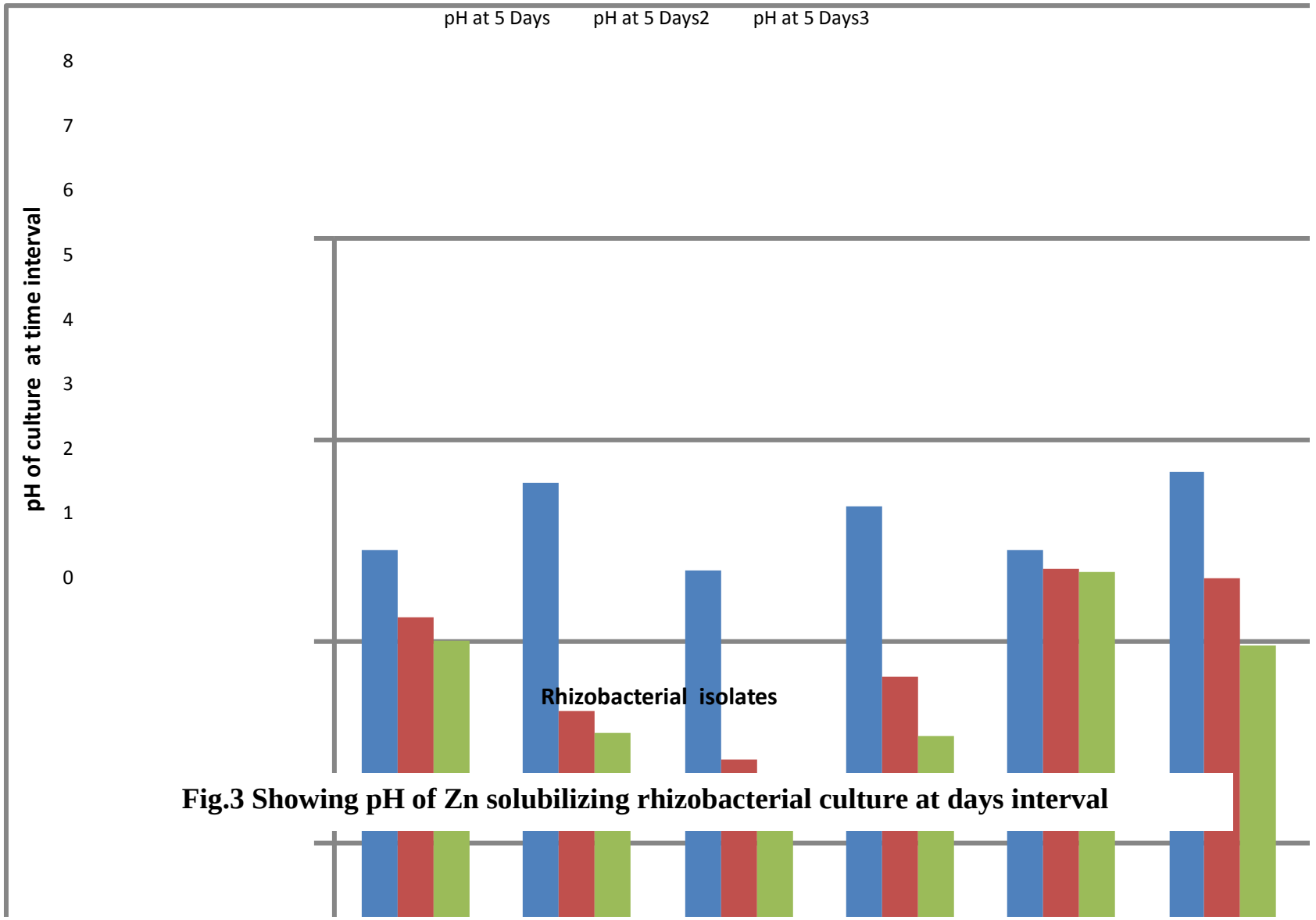


Fig.3 Showing pH of Zn solubilizing rhizobacterial culture at days interval

However the superior Zn solubilizing isolate was DCU-451, showing the strong ability to solubilize Zn *in vitro* (Plate-2). Isolates gives a clue that the solubilization could be due to production of organic acids. The more production of organic acid improves the available zinc in the culture broth. Similar observations noticed by Desai *et al.*, (2012) reported that higher availability of Zn is directly proportional to acidic pH of the culture broth.

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