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Isolation and characterization of nitrogen fixing bacteria from the rhizospheric soil of Custard apple (*Annona squamosa* L.)

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Abstract

A study was conducted to evaluate the nitrogen-fixing efficiency of free-living *Azotobacter* bacteria in the rhizospheric soil of custard apple (*Annona squamosa* L.) across Purandar Tehsil, Pune. Chemical profiling of 21 soil samples revealed neutral pH, low salinity and systemic deficiency in available nitrogen below 280 kg/ha. Isolation on Jensen's agar obtained 18 distinct bacterial isolates, all confirmed as Gram-negative, motile, cyst-forming, and positive for standard morphological and cultural tests. Based on colony pigmentation, the strains were identified as *Azotobacter* spp., *Azotobacter chroococcum*, and *Azotobacter vinelandii*. Micro-Kjeldahl quantification showed nitrogen fixation capacities ranging from 1.12 mg N/ml to 3.50 mg N/ml. The superior isolate obtained NFB-PA from Pangare (3.50 mg N/ml) followed by NFB-AM from Ambodi (3.08 mg N/ml) significantly at par with standard reference strains. Further, effective PGPR isolates were tested biochemically viz Strach hydrolysis test, Catalase test, Indole formation test, Nitrate reduction test and Oxidase test.

Keywords: *Azotobacter*, rhizosphere, PGPR, Custard apple, isolates, nitrogen

Introduction

Custard apple (*Annona squamosa* L.), is a delectable and significant minor fruit crop belonging to the *Annonaceae* family. It can be called as delicacy of dry region due to its very sweet delicate flesh, Originally introduced from Tropical America, it has adapted remarkably well to Indian conditions and can now be found growing wild across many regions of the country, Only five of the 100 *Annona* species custard apple, cherimoya, sour sop, bullock's heart and atemoya are commercially significant (Jain *et al.* 2019) ^[6].

Plant growth promoting rhizobacteria (PGPR) are the group diverse bacteria that colonize the plant roots and surrounding root zone (rhizosphere), offering a multiple of benefits to plant health and growth. It plays a key role in promoting organic and sustainable agriculture, offering small and marginal farmers to enhance crop yields (Moorthy and Malliga, 2012) ^[11]. PGPR belong to various genera including *Agrobacterium*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Flavobacterium*, *Pseudomonas* and *Rhizobium* etc. *Azotobacter* is a non-symbiotic bacterium that has the ability to fixation of nitrogen and active colonizes to increasing growth and crop production because of the ability to produce growth hormones such as auxin, cytokinin, and gibberellin (Leveau and Lindow, 2005) ^[9]. The purpose of this research was to isolate, characterize and analyze the capability of rhizosphere *Azotobacter* spp. isolate origin from Custard apple root zone.

Method and Material

Collection of soil samples: Total 21 rhizosphere soil samples were collected from different locations from Purandar tehsil in Pune District to isolate Plant Growth Promoting Rhizobacteria (PGPR), soil samples were randomly collected from 10-15 cm and record the fertilizer application status of respected plot (Table 1).

Chemical analysis of properties of the Soil: Soil samples were collected from custard apple orchard after harvesting of the Custard apple for analysis of nutrient status of the soil, The collected soil samples were air dried and sieved through 2 mm sieve.

Then the soil samples were analysed for their chemical properties viz available nitrogen, phosphorus and potassium along with pH and electrical conductivity (Table 2) (Jackson, 1973)^[5].

Isolation of nitrogen fixing bacteria

Isolation of bacteria was done using serial dilution method. Soil samples were weighed as much as 1gm and added into 10 ml of sterile water, then dilution was carried out from 10^{-1} to 10^{-8} . 0.1 ml of the suspension of each dilution was cultured on selective media. Colonies that grew on selective media indicated as a colony isolate of *Azotobacter* spp. Visually after 2-3 days of incubation white colonies were obtained.

Characterization of PGPR

Morphological and cultural characterization

All the isolates were checked for their purity and then studied for the colony morphology, The cell shape and gram reaction were also recorded as per the standard procedures.

Biochemical characterization of PGPR

Different biochemical tests were performed as per the procedure described by Aneja (2001)^[11] and Cappuccino and Sherman (1992)^[12]. The protocols followed are briefly outlined below. The tests were conducted Starch hydrolysis, Catalase test, Indole production, Oxidase test and Nitrate reduction.

Catalase test (Rangaswami and Bhagyaraj 1993)^[15]

About 1 ml of 3% hydrogen peroxide (H_2O_2) will be poured over bacterial culture on microscopic slide or in broth. Intensive bubbling will be noted as catalase positive, slight bubbling as weak positive and no gas production as catalase negative

Oxidase test (Collins and Lyne, 1970)^[13]

Small pieces of filter papers soaked in 1% aqueous tetramethyl-p-phenylenediamine will be placed in Petri plate. Fresh young cultures to be tested will be scraped with a glass rod and will be rubbed on the moistened filter paper. Development of a deep violet colour after 10 seconds will be treated as oxidase positive test, whereas development of a light violet or no colour will be treated as oxidase negative test.

Nitrate reduction test (Prathyush et al. 2022)^[13]

Nitrate broth was inoculated with a loopful of selected bacterial isolate and incubated at 28°C for 7 days. Uninoculated nitrate broth was kept as a control. Control was also run without inoculation. After incubation, two drops of sulfanilic acid followed by two drops of naphthylamine solution were added. The presence of nitrate was indicated by a pink, red or orange colour and the absence of colour change was considered nitrite negative.

Indole formation test (Gordon and Weber, 1951)^[4]

The indole test will be performed by inoculating the bacterial isolates into Tryptone broth. The indole production during the growth will be detected by adding Kovac's reagent (P-dimethyl amino benzaldehyde), which will produce cherry red colour for positive reaction.

Starch hydrolysis test (Seeley and Van Demark, 1981)^[16]

The isolates will be streaked on nutrient agar plates

containing 2 per cent insoluble starch and will be incubated at room temperature for 48 h. Hydrolysis of starch will be tested by flooding with Gram's iodine solution and the plates will be observed for the presence a clear zones surrounding the colonies, which is a positive indicator for starch hydrolysis.

Evaluation of rhizobacteria isolates for their 'Nitrogen' fixing ability

The nitrogen fixing ability of *Azotobacter* isolates were estimated by Micro-Kjeldhal method in terms of the amount of total nitrogen fixed in 25 ml broth culture in 7 days under controlled condition. For that purpose, nitrogen free broth of Jensen's broth was used. After pouring 25 ml broth in 150 ml conical flask and plugged with cotton plugs then flask was autoclaved. After cooling, each flask was inoculated by 0.1 ml broth culture of each strain and the flask were kept on rotary shaker for continuous shaking of culture for 7 days at $28^\circ C \pm 2^\circ C$. From each flask 10 ml culture was taken for estimation of total nitrogen by Micro-Kjeldhal method as described.

Estimation of Phosphate Solubilizing Ability of PSB isolates

100 ml of Pikovaskaya's broth was dispensed in 250 ml conical flasks 100 mg P_2O_5 as tri-calcium phosphate (TCP) was added separately to each flask and the contents were sterilized at $121^\circ C$ for 15 min. The flasks were inoculated with 100 ml suspension of overnight grown culture and incubated at $28 \pm 2^\circ C$ for 15 days. Presence of yellow colour after addition of ammonium molybdate and ammonium vanadate in equal ratio to culture supernatant confirmed phosphate solubilizing activity. The yellow colour intensity of the solution was measured at 420 nm after 25 minutes incubation for quantitative estimation of phosphate solubilisation (Jackson, 1973)^[5].

Estimation of Potassium mobilizing ability of KMB isolates

Potassium solubilization of the bacterial isolate was studied on GYC agar media by a standard spot Plate assay inoculated with a loop-full overnight grown culture and incubated at $28^\circ C$. Clear zones around the colonies confirm positive potassium solubilization. The diameter of clear zones deduced using Khandeparkar's selection ratio (Prajapati, 2012)^[12].

Results and Discussion

Collection of rhizospheric soil samples from selected Custard apple orchard

Total 21 rhizospheric soil samples were collected from selected custard apple orchard for the isolation of Plant Growth Promoting Rhizobacteria and The primary data on GPS location, use of chemical fertilizers, organic manures and bio-fertilizers and pest and diseases status of same orchard is present in (Table.1). According to McPherson *et al.* (2018)^[10], the field site's position (latitude, longitude, and altitude) was recorded during collection of soil samples.

Chemical analysis of collected soil samples

The present study evaluated the chemical soil properties across 21 distinct locations within the custard apple (*Annona squamosa*) growing belt of the Purandar tehsil viz. available nitrogen, phosphorus, potassium, pH and Ec as per standard procedure. (Table 2).

Isolation and characterization of Nitrogen fixing bacteria *Azotobacter* spp.

Out of twenty-one rhizospheric soil samples of custard apple, 18 isolates of NFB (*Azotobacter*) were obtained by The isolation was carried out by serial dilution and pour plate method using Jensen's, media and designated as per their location initial (Table 3).

All the nitrogen fixing bacterial isolates were Gram negative, cyst former, motile and pellicle forming. Colony colour of NFB isolates were creamy white in case of NFB-Z NFB-KL NFB-C, NFB-RW, NFB-PA, NFB-SH, NFB-KH, NFB-SI, NFB-W, NFB-RJ, NFB-Ref 1 and 2 where as it was creamy black NFB-B, NFB-KM, NFB-AM and NFB-V showing probable isolate might be *Azotobacter chroococcum*. Three NFB isolates viz, NFB-SO, NFB-AD and NFB-KW showed creamy yellow colour colony similar to *Azotobacter vinelandii*. The shape of NFB isolate was oval to spherical. Remaining NFB isolates were *Azotobacter* spp. (Table. 4) isolates were similar with the results of Upadhyay *et al.* (2015)^[17]. All of the isolates of NFB were subjected to the biochemical tests for starch hydrolysis, catalase test, indole synthesis, oxidase test, and nitrate

reduction. Results of these tests are presented in (Table.5) Nitrogen fixing bacterial isolates (*Azotobacter*) showed positive results for all five tests. As per isolates findings were reported by Rashmi *et al.* (2017).

Evaluation of Nitrogen fixing ability of NFB isolates

Overall, the nitrogen fixation potential varied from minimum of 0.98 mg N/ml to a maximum of 3.50 mg N/ml (Table. 6) Isolate NFB-PA from Pangare recorded significantly the highest overall nitrogen fixation capacity (3.50 mg N/ml). However, it was at par with isolates NFB-AM from Amboli (3.08 mg N/ml), NFB-C from Chinchvale (2.66 mg N/ml), NFB-AD from Adachiwadi (2.66 mg N/ml), NFB-RW from Rautwadi (2.52 mg N/ml), NFB-Ref 2 from BNF Pune scheme (2.52 mg N/ml), NFB-KH from Khalad (2.52 mg N/ml), and NFB-SI from Singapur (2.52 mg N/ml). These results were align with findings of Kaviyarasan *et al.* (2020)^[7]. isolated seven isolates of *Azotobacter* from the ten soil samples collected from agricultural fields and reported that the NR2R fixation was ranged from 6.58 mg N/ml to 14.86 mg N/ml.

Table 1: Soil samples were collected from different location of Punrandar tehsil with GPS coordinates, application of chemical fertilizers, organic fertilizers and biological fertilizers and insect disease incidence

Sr. No.	Name of Location	GPS Coordinates	Chemical Fertilizer	Organic Fertilizer	Biological fertilizer	Insect attack	Disease incidence
1	Jadhavwadi	18°23'53.8"N 74°00'11.1"E	+	+	-	Mealy bug, Tea mosquito bug	Fruit rot
2	Dhumewadi	18°23'26.1"N 74°00'46.0"E	+	+	-	Tea mosquito bug, Aphids	Fruit rot
3	Zendewadi	18°25'14.7"N 74°01'25.4"E	+	+	+	Mealy bug, Tea mosquito bug	Fruit rot
4	Kalewadi	18°24'23.0"N 74°01'22.8"E	+	+	-	Mealy bug, Tea mosquito bug	Fruit rot
5	Dive	18°22'42.4"N 74°01'04.0"E	+	+	+	Mealy bug, Tea mosquito bug	-
6	Chinchvale	18°23'10.9"N 73°59'58.5"E	+	+	+	Mealy bug, Tea mosquito bug	Fruit rot
7	Rautwadi	18°23'14.4"N 74°01'59.9"E	+	+	+	Mealy bug, Tea mosquito bug	Fruit rot
8	Sonori	18°24'10.8"N 74°03'26.4"E	-	+	-	Mealy bug (traces)	-
9	Khenat mala	18°18'56.2"N 74°02'05.2"E	+	+	-	Mealy bug and Aphids (Less)	Less infestation
10	Pimpale	18°17'41.1"N 74°02'39.1"E	+	+	+	Mealy bug, Tea mosquito bug	Fruit rot
11	Pangare	18°16'18.6"N 74°03'38.5"E	+	+	+	Mealy bug (traces)	Less infestation
12	Adachiwadi	18°12'28.4"N 74°08'02.0"E	-	+	+	Mealy bug (traces)	Less infestation
13	Shivari	18°17'32.7"N 74°05'16.0"E	-	+	+	Mealy bug, Tea mosquito bug	Fruit rot
14	Khalad	18°19'31.8"N 74°04'02.2"E	+	+	-	Normal incidence	Normal infestation
15	Amboli	18°21'23.2"N 74°02'48.5"E	+	-	-	Tea mosquito bug (traces)	Fruit rot
16	Vanpuri	18°22'17.3"N 74°03'52.0"E	+	+	-	Tea mosquito bug (traces)	trace infestation
17	Singapur	18°22'44.2"N 74°06'31.1"E	-	+	+	Less incidence	Less infestation
18	Waghapur	18°23'37.5"N 74°06'55.2"E	-	+	+	Less incidence	Normal infestation
19	Rajewadi	18°23'30.7"N 74°08'20.4"E	-	+	+	Less incidence	Less infestation
20	Khanwadi	18°19'47.1"N 74°06'19.6"E	+	+	-	Less incidence	Less infestation
21	AICRP Center	18°23'37.5"N 74°00'22.4"E	+	+	+	Normal incidence	Normal infestation

(+ve) = Incorporated, (-ve) = Not incorporated

Table 2: Chemical analysis of collected soil samples from Custard apple orchard in different location of Purandar tehsil

Name of Location	Available Nitrogen (kg/ha)			Available Phosphorous (kg/ha)			Available Potassium (kg/ha)			pH	Ec
Jadhavwadi	1.20	125.44	150.53	0.089	255.36	22.73	18.60	11.2	208.32	7.40	0.23
Dhumewadi	1.00	125.44	125.44	0.096	255.36	24.51	16.30	11.2	182.56	6.85	0.15
Zendewadi	1.70	125.44	213.25	0.091	255.36	23.24	25.21	11.2	282.35	7.33	0.21
Kalewadi	1.30	125.44	163.07	0.103	255.36	26.30	33.48	11.2	374.98	7.23	0.29
Dive	1.40	125.44	175.62	0.114	255.36	29.11	31.58	11.2	353.70	7.80	0.17
Chinchvale	1.90	125.44	238.34	0.099	255.36	25.28	24.63	11.2	275.86	6.98	0.15
Rautwadi	1.50	125.44	188.16	0.107	255.36	27.32	34.45	11.2	385.84	7.48	0.20
Sonori	0.80	125.44	100.35	0.077	255.36	19.66	13.43	11.2	150.42	7.70	0.16
Khenat mala	1.10	125.44	137.98	0.094	255.36	24.00	23.36	11.2	261.63	7.35	0.13
Pimpale	1.00	125.44	125.44	0.101	255.36	25.79	21.77	11.2	243.82	7.70	0.14
Pangare	2.00	125.44	250.88	0.109	255.36	27.83	24.00	11.2	268.80	6.95	0.23
Adachiwadi	1.40	125.44	175.62	0.097	255.36	24.77	21.69	11.2	242.93	7.16	0.21
Shivari	0.90	125.44	112.90	0.076	255.36	19.41	16.94	11.2	189.73	7.23	0.26
Khalad	1.20	125.44	150.53	0.082	255.36	20.94	19.86	11.2	222.43	7.16	0.20
Amboli	1.60	125.44	200.70	0.063	255.36	16.09	18.59	11.2	208.21	7.24	0.23
Vanpuri	1.30	125.44	163.07	0.078	255.36	19.92	21.88	11.2	245.06	7.18	0.09
Singapur	1.00	125.44	125.44	0.088	255.36	22.47	18.48	11.2	206.98	6.92	0.13
Waghapur	1.00	125.44	125.44	0.082	255.36	20.94	20.17	11.2	225.90	7.43	0.09
Rajewadi	0.80	125.44	100.35	0.077	255.36	19.66	22.57	11.2	252.78	7.22	0.08
Khanwadi	1.20	125.44	150.53	0.094	255.36	24.00	25.99	11.2	291.09	7.45	0.15
AICRP Center	1.10	125.44	137.98	0.064	255.36	16.34	18.48	11.2	206.98	7.24	0.23

Table 3: Plant growth promoting rhizobacterial isolates in Custard apple orchard from Purandar tehsil

Sr.no	Name of Location	Nitrogen fixing bacteria	Designation	Phosphorus solubilizing bacteria	Designation	Potassium mobilizing bacteria	Designation
1	Jadhavwadi	-	-	+	PSB-JW	+	KMB-JW
2	Dhumewadi	-	-	-	-	-	-
3	Zendewadi	+	NFB-Z	+	PSB-Z	-	-
4	Kalewadi	+	NFB-KL	+	PSB-KL	-	-
5	Dive	+	NFB-D	+	PSB-D	+	KMB-D
6	Chinchvale	+	NFB-C	-	-	+	KMB-C
7	Rautwadi	+	NFB-RW	+	PSB-RW	+	KMB-RW
8	Sonori	+	NFB-SO	+	PSB-SO	+	KMB-SO
9	Khenat mala	+	NFB-KM	-	-	+	KMB-KM
10	Pimpale	-	-	+	PSB-PI	-	-
11	Pangare	+	NFB-PA	+	PSB-PA	+	KMB-PA
12	Adachiwadi	+	NFB-AD	+	PSB-AD	-	-
13	Shivari	+	NFB-SH	+	PSB-SH	+	KMB-SH
14	Khalad	+	NFB-KH	-	-	+	KMB-KH
15	Amboli	+	NFB-AM	-	-	-	-
16	Vanpuri	+	NFB-V	+	PSB-V	-	-
17	Singapur	+	NFB-SI	+	PSB-SI	+	KMB-SI
18	Waghapur	+	NFB-W	+	PSB-W	-	-
19	Rajewadi	+	NFB-RJ	+	PSB-RJ	-	KMB-RJ
20	Khanwadi	+	NFB-KW	-	-	+	-
21	AICRP Center	+	NFB-Ref 1	-	-	+	KMB-Ref 1

(+) = PGPR present, (-) = PGPR absent, NFB = Nitrogen fixing bacteria, PSB = Phosphorus solubilizing bacteria, KMB = Potassium mobilizing bacteria.

Table 4: Morphological and cultural characterization on Nitrogen fixing bacteria (NFB)

Sr. no.	Name of village	Isolates	Colony morphology		Cell morphology					Probable isolates
			Shape	Colour	Gram staining	Shape	Cyst formation	Mobility	Pellicle formation	
1	Zendewadi	NFB-Z	Irregular	Creamy white	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter spp.</i>
2	Kalewadi	NFB-KL	Irregular	Creamy white	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter spp.</i>
3	Dive	NFB-D	Irregular	Creamy black	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter chroococcum</i>
4	Chinchvale	NFB-C	Irregular	Creamy white	Negative	Oval	+ve	+ve	+ve	<i>Azotobacter spp.</i>
5	Rautwadi	NFB-RW	Irregular	Creamy white	Negative	Oval	+ve	+ve	+ve	<i>Azotobacter spp.</i>
6	Sonori	NFB-SO	Irregular	Creamy yellow	Negative	Oval	+ve	+ve	+ve	<i>Azotobacter vinelandii</i>
7	Khenat mala	NFB-KM	Irregular	Creamy black	Negative	Oval	+ve	+ve	+ve	<i>Azotobacter chroococcum</i>
8	Pangare	NFB-PA	Irregular	Creamy white	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter spp.</i>
9	Adachiwadi	NFB-AD	Irregular	Creamy yellow	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter vinelandii</i>
10	Shivari	NFB-SH	Irregular	Creamy white	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter spp.</i>
11	Khalad	NFB-KH	Irregular	Creamy white	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter spp.</i>
12	Amboli	NFB-AM	Irregular	Creamy black	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter chroococcum</i>
13	Vanpuri	NFB-V	Irregular	Creamy black	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter chroococcum</i>
14	Singapur	NFB-SI	Irregular	Creamy white	Negative	Oval	+ve	+ve	+ve	<i>Azotobacter spp.</i>
15	Waghapur	NFB-W	Irregular	Creamy white	Negative	Oval	+ve	+ve	+ve	<i>Azotobacter spp.</i>
16	Rajewadi	NFB-RJ	Irregular	Creamy white	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter spp.</i>
17	Khanwadi	NFB-KW	Irregular	Creamy yellow	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter vinelandii</i>
18	AICRP Center	NFB-Ref 1	Irregular	Creamy white	Negative	Spherical	+ve	+ve	+ve	<i>Azotobacter spp.</i>
19	BNF Scheme Pune	NFB- Ref 2	Irregular	Creamy white	Negative	Oval	+ve	+ve	+ve	<i>Azotobacter spp.</i>

(+ve) = Positive, (-ve) = Negative, NFB = Nitrogen fixing bacteria.

Table 5: Biochemical characterization of Nitrogen fixing bacterial isolates

Sr.no.	Name of village	Isolate	Starch hydrolysis	Catalase test	Indole production	Nitrate reduction	Oxidase test
1	Zendewadi	NFB-Z	+ve	+ve	+ve	+ve	+ve
2	Kalewadi	NFB-KL	+ve	+ve	+ve	+ve	+ve
3	Dive	NFB-D	+ve	+ve	+ve	+ve	+ve
4	Chinchvale	NFB-C	+ve	+ve	+ve	+ve	+ve
5	Rautwadi	NFB-RW	+ve	+ve	+ve	+ve	+ve
6	Sonori	NFB-SO	+ve	+ve	+ve	+ve	+ve
7	Khenat mala	NFB-KM	+ve	+ve	+ve	+ve	+ve
8	Pangare	NFB-PA	+ve	+ve	+ve	+ve	+ve
9	Adachiwadi	NFB-AD	-ve	+ve	+ve	+ve	+ve
10	Shivari	NFB-SH	+ve	+ve	+ve	+ve	+ve
11	Khalad	NFB-KH	+ve	+ve	+ve	+ve	+ve
12	Amboli	NFB-AM	-ve	-ve	+ve	+ve	+ve
13	Vanpuri	NFB-V	+ve	+ve	+ve	+ve	+ve
14	Singapur	NFB-SI	+ve	+ve	+ve	+ve	+ve
15	Waghapur	NFB-W	+ve	+ve	+ve	+ve	+ve
16	Rajewadi	NFB-RJ	+ve	+ve	+ve	+ve	+ve
17	Khanwadi	NFB-KW	+ve	+ve	+ve	+ve	+ve
18	AICRP Center	NFB-Ref 1	+ve	+ve	+ve	+ve	+ve
19	BNF Scheme Pune	NFB- Ref 2	+ve	+ve	+ve	+ve	+ve

(+ve) = Positive, (-ve) = Negative, NFB = Nitrogen fixing bacteria.

Table 6: Nitrogen fixing ability of *Azotobacter* isolates from different locations of Custard apple orchard from Purandar tehsil

Sr. No.	Name of Location	Isolates	Nitrogen fixation (mg of N/ml)
1	Zendewadi	NFB-Z	2.38
2	Kalewadi	NFB-KL	1.12
3	Dive	NFB-D	1.68
4	Chinchvale	NFB-C	2.66
5	Rautwadi	NFB-RW	2.52
6	Sonori	NFB-SO	1.4
7	Khenat mala	NFB-KM	1.82
8	Pangare	NFB-PA	3.5
9	Adachiwadi	NFB-AD	2.66
10	Shivari	NFB-SH	2.38
11	Khalad	NFB-KH	2.52
12	Amboli	NFB-AM	3.08
13	Vanpuri	NFB-V	1.26
14	Singapur	NFB-SI	2.52
15	Waghapur	NFB-W	1.26
16	Rajewadi	NFB-RJ	1.96
17	Khanwadi	NFB-KW	2.24
18	AICRP Jadhavwadi Center	NFB-Ref 1	0.98
19	BNF Scheme Pune	NFB- Ref 2	2.52
		SE(m) $\pm$	0.35
		CD	1.05

(NFB= Nitrogen fixing bacteria)

Conclusion

Out of 17 obtained NFB isolates NFB-PA and NFB-AM are superior isolates with characters morphology of creamy black colony, shape oval to spherical, Gram negative and positive for all biochemical analysis respected isolates which have the potential to be used as nitrogen fixing bacteria as a microbial fertilizer.

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