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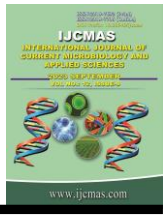

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Isolation, Characterization of *Azotobacter* and PSB from Xerophytic Plants of Maharashtra State, India

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A B S T R A C T

The present study was conducted at Department of Plant Pathology and Agricultural Microbiology, MPKV, Rahuri during year 2021 to 2023 to characterize and screen different isolates of *Azotobacter* and PSB from Different Xerophytic plants from Different districts of Maharashtra State.68 *Azotobacter* isolates were biochemically characterized and screened under *In- vitro* conditions for their plant growth promoting properties. The highly efficient *Azotobacter* isolates was characterized by its nitrogenase activity, which was 189.4 nmol C_2H_4. mg protein^{-1}.h^{-1} in Azt 2 (*Zizipus* spp) isolates, Out of sixty eight *Azotobacter* isolates, 3 isolates *viz*., Azt- 2 (*Ziziphus* spp), Azt-17 (*Aloe* spp), Azt- 8 (*Agave* spp), recorded significantly higher nitrogenase activity. The amount of Pi released from tri- calcium phosphate (TCP) by the isolates at 10 day after inoculation was ranged from 18.5 to 29.65 %. The quantitative estimation of per cent inorganic phosphorus released from Tri- calcium phosphate had recorded highest for PSB-4 (*Zizipus* spp) which was 29.65 % Thus, isolate PSB-4 (*Zizipus* spp) i.e. efficient strain isolated from ber was found to be significantly superior over rest of the isolate in solubilizing phosphate.

Introduction

The number of microorganisms at the plant root region of Xerophytic plant is very high as compared with the rest of the soil. So, it is clear that plant roots have an assortment of mineral, nutrient, and metabolite components, which are considered the principle factor for captivating microorganisms to assemble and link together. Root exudate of plants is a critical factor for microbial settlement in the rhizosphere. Shifting of microorganisms regarding the root exudates has an important role in pulling force of the microbial population to colonize the plant roots. The adaptation and survival of crop plants in any abiotic environment depend heavily on the interactions between the microbial community

and those plants. Microbes that are connected with plants can affect how plants react to environmental factors such as drought stress (Budak *et al.,* 2013) As a result, methods based on microbes are required to lessen the effect of drought stress. The ability of plant associated microbial community to boost agricultural yield and provide stress resistance has recently attracted more attention. Drought tolerance and response methods in agricultural plants may be significantly influenced by microorganisms, Exopolysaccharides synthesis and biofilm formation are two strategies used by plant growth promoting rhizobacteria (PGPR) to reduce effect of drought stresses on plant cells in response to drought through a variety of processes, including the activation of osmoprotectants and heat shock proteins. Bacterial cells under drought stress build up compatible solutes like amino acids, quaternary amines and sugars that stop deteriorationg processes and enhance cell development in unfavourable osmotic conditions (Potts, 1994). Phytohormones, such as indole acetic acid (IAA), gibberellins, ethylene, abscisic acid (ABA), and cytokinin, in addition to playing a crucial role in plant growth and development, can help plants cope with abiotic stresses. (Porcel *et al.,* 2014). Microbes connected to plants have the capacity to improve resistance under low water situations by preserving water status, ion homeostasis, and nutrient uptake to generate oxidative stress and improve stress tolerance. In dry and semi arid areas, inoculating plants with beneficial microorganisms encourages plant development and improves drought tolerance (Marulanda *et al.,* 2007) plant growth promoting (PGPR) organisms can adapt to difficult environments stresses harmful consequences (Glick *et al.,* 1997; Timmusk and Wagner, 1999 and Marulanda *et al.,* 2009)

Therefore, isolation of bacterial strains of *Azotobacter* and Phosphate Solubilizing Bacteria from dry lands for drought tolerance is a long term strategy and hence selection for drought resistance has to be either drought escape or drought tolerance and assumes greater importance. Screening of isolated *Azotobacter* and Phosphate Solubilizing Bacteria from soils of Xerophytic plants moisture tolerance has been attempted by various workers using different growth parameters, moisture content and microbial count.

The present study was conducted for isolation, characterization and morphological study of Azotobacter and PSB isolates.

Materials and Methods

Standard laboratory equipments like Autoclave, Hot air oven, BOD incubator, Laminar air flow, Research microscope, Electronic balance, Mechanical shaker, Centrifuge, Digital camera, Grinding machine, Digital pH meter, Micro pipettes, The Corning brand glass wares *viz*., Beakers, Pipettes, Test tubes, Petriplates, Conical flasks, Funnels, Measuring cylinder, Microscopic glass slides, etc. available at Department of Plant Pathology and Agricultural Microbiologywere used ever necessary.

The glasswares mentioned earlier were dipped in cleaning solution (100 g of potassium dichromate and 500 ml conc. Sulfuric acid in 1 lit. Water). rinsed thoroughly under running tap water and sterilized in hot air oven at 180°C for 1 hr. Working table tops were surface sterilized and hands were disinfected with Sodium hypochloride (minimum available Cl=4%).

Collection of soil samples from xerophytic plants of Ber, Alovera, Opuntia, Cactus, Agave etc

Soil samples were collected from root rhizospheric soil of wild xerophytic plants *viz*. Wild ber, Alovera, Agave, Opuntia, Cactus from drought and water stress areas and brought to the laboratory for isolation of rhizospheric microorganisms.

Isolation of*Azotobacter*

Azotobacter strains were isolated from the soil samples, collected from different xerophytic plants from Kolhapur, Sangli, Satara, Pune and

Ahmednagar districts of Maharashtra, by serial dilution and pour plate technique, using Jensen's Agar Medium.1000ml nitrogen free Jensen's broth was prepared and dispensed 100ml each in 250 ml conical flasks after cooling 1 gm soil samples and inoculated in each conical flasks which was then placed on incubator shaker for 7 days at 28°C temperature, provided aerobic conditions to enhance the growth of bacterial cells. After full growth in flasks a loopful of inoculum from the pellicles of each flasks was streaked on Jensen's agar plates to obtain well isolated colonies and further incubated in BOD at 28°C temperature, in inverted position for 5 days. The plates were observed for the development of typical *Azotobacter* colonies. On frequent isolation pure culture of *Azotobacter* was obtained and further used during the present studies. The *Azotobacter* isolates were labelled as Azt-(*Zizipus* spp), Azt-(*Aloe* spp), Azt- (*Cacus* spp), Azt- (*Opuntia* spp), Azt- (*Agave* spp).

Isolation of Phosphate Solubilizing Bacteria

For isolation of PSB soil, sample were collected from different xerophytic plants from Kolhapur, Sangli, Satara, Pune and Ahmednagar districts of Maharashtra, and isolated in enriched culture by serial dilution of soil and streaking technique, using Pikovskaya's Agar Medium.For the preparation of water blanks, 90 ml tap water was taken in each of five conical flasks of 150 ml capacity with the help of a graduated pipette. The flasks were properly plugged with non- absorbent cotton and wrapped with paper with the help of rubber band. In order to prepare serial dilutions, Ten gram rhizospheric soil samples was suspended in 90 ml of sterilized water blank. Serial dilutions were made from 10^{-1} to 10^{-6}. one ml aliquot of dilutions from 10^{-3} to 10^{-5} was transferred to sterilized petri plates separately.

The sterilized Pikovskaya's medium before solidification (45° temperature) was poured in each petri plate and the content in plates was mixed by rotating the plates were kept at 28±2° C in BOD incubator for 4-5 days. All the plates were observed for the appearance of bacterial colony showing clear zone of solubilisation of Tri-calcium Phosphate (TCP). Distinct colonies showing clear zone of phosphate solubilization on the plates were selected and purified by repeated culturing and maintained on PSB slants at 4°C in the refrigerator for further studies.

Purification, further multiplication of isolatesand preparation of slant

Loopful of growth from the slants were streaked again on fresh plates of Pikovskaya's agar medium incubated for independent colony development at 28°C in incubator. There after every isolate were transferred on Petriplates to record maximum zone of inhibition. The purified isolates were maintained in pure state and used for further studies. The Jensen's medium was used for the isolation of *Azotobacter*. Jensen's broth was prepared by excluding agar from the prescribed composition. All the ingredients of Jensen's agar medium were accurately weighed and dissolved in about 800 ml water by boiling in a sauce pan. Then transferred aseptically in to a measuring cylinder of 1000 ml capacity, the volume was made up to one litre by adding water.

The medium was mixed well and about 5 to 6 ml was transferred in to several test tubes of 20 ml capacity for making slants. Rest of the medium was equally distributed in to conical flasks. Tubes and conical flasks were properly plugged with cotton, wrapped with paper and rubber band. After sterilization, medium test tubes were kept in slanting position on clean air work station to solidify.

Cultural tests

The plates of nitrogen free Jensen's medium and Pikovaskaya's medium were streaked and incubated for a week.Well separated colonies were examined daily for various colony characters. The colony attributes *viz.* diameter, opacity, edge, elevation, consistency, surface appearance and zone of clearance were recorded with the descriptive defined terms by using colony chart.

Biochemical characterization of *Azotobacter* and PSB

The highly efficient nitrogen fixing *Azotobacter* and phosphate solubilising bacterialisolates was further subjected to different biochemical tests including catalase, oxidase, gelatin liquification, methyl red test, growth on carbon sources and growth at various temperatures for confirmation. The test isolates were subjected to biochemical characterization employing the standard procedures given by Experiments in Microbiology by Aneja (2003) and Cappuccino and Sherman (1987). Different biochemical tests performed are briefly outlined below.

Catalase test

Nutrient Agar Petriplates were inoculated with the overnight grown test bacterial isolate and were incubated at 37°C for 24 h. After incubation, the plates were flooded with one ml of three per cent hydrogen peroxide and observed for the formation of gas bubbles. The occurrence of gas bubbles were scored positive for catalase production.

Oxidase test

Overnight grown cultures of the test isolates were spotted to the nutrient plates and the plates were incubated for 24 h at 37°C. After incubation, two to three drops of Tetramethyl-Phenylene Diamine Dihydro Chloride was added on the surface of the growth of test isolate. The colour change to dark blue was taken as oxidase positive.

Gelatin liquefaction

The gelatin liquefaction ability of the bacterial isolates was examined. Tubes of gelatin agar in triplicates were inoculated with test cultures. These test tubes were incubated at 28±2°C for 24 h. Following this, the tubes were kept in a refrigerator at 4°C for 30 minutes. The tubes with cultures that remained liquefied were taken as positive and those that solidified on refrigeration were taken as negative for the test.

Starch hydrolysis

Starch agar medium was poured in Petriplates. A single streak inoculation of organism was done into the centre of plate and incubated at 28+2°C for 24-48 hrs. Upon incubation, the plates were flooded with 10 ml of iodine solution. Hydrolyzed starch appeared as clear zone around microbial colony because of ß-amylase activity. Reddish brown zone around the colony indicated partial hydrolysis of starch as a result of a- amylase activity. The unhydrolyzed starch formed a blue colour with iodine.

H_2S production

Large test tubes having Sulfide Indole Motility (SIM) Agar, containing Fe $(NH_4)SO_4$ that behave as the H_2S indicator adjusted to pH 7.3 were used and inoculated with each isolate. Ferrous ammonium sulphate in the medium serves as an indicator by combining with the gas forming an insoluble black ferrous sulphide precipitate that was seen along the line of stab inoculation and is indicative of H_2S production. Observation for blackening in tubes was taken after three days of incubation.

Gram staining

The efficient bacterial isolates were characterized for their Gram reaction. A thin bacterial cell suspension of one day old culture was taken with the help of inoculating loop and it was uniformly spread on the centre of a clean microscopic slide, The bacterial cells were fixed by heating by passing it repeatedly for a few seconds over a flame. They were stained with a drop of crystal violet solution (30 seconds), iodine (30 seconds) followed by ethyl alcohol and safranin (1 to 1.5 minutes) solution. After staining, the cells were washed under tap water jet and examined under compound microscope using oil immersion lensand gram staining was performed for all the bacterial isolates by standard procedures given by Cappuccino and Sherman (1999). The isolates which retained dark blue or violet colour of primary stain (crystal violet) were

recognised as a Gram positive, whereas those that lost the crystal violet and got counter stained (safranin) appeared red were referred as Gram negative (Gram, 1884).

Screening of *Azotobacter* and PSB isolates for nitrogen fixation and P solubilization

The isolates were screened to appraise phosphate solubilizing activity by allowing the isolates to grow in selective media Pikovskaya's agar medium and nitrogen free medium for 4 days at 25°C.The amount of nitrogen fixed by the *Azotobacter* isolates during incubation in broth culture were estimated by micro- Kjeldhals method, and Phosphorous estimated by Olsen's method.

Nitrogen fixing ability of free-living bacteria

The Acetylene Reduction Assay (ARA) is an indirect method widely used for measuring N_2-fixation at a time point (Hardy *et al.,* 1968). The technique involves incubation of nitrogenase containing system (free-living bacteria) in a known atmosphere of acetylene (10% C_2H_2) in the gas phase, and after an optimum time of incubation (24hrs), ethylene (C_2H_4) produced was measured by a gas chromatograph using flame ionization detector (FID). The slants were prepared by taking 5 ml Pikovskayas medium in each of 15 ml test tubes for Phosphate Solubilizing Bacteria. The Jensen's Agar Medium was used for preparing slants for *Azotobacter* and Pikovskayas Agar Medium for PSB. A loopful culture of each isolate of respective organism in three replicates was streaked on the slants and incubated at 28+2°C for 8 days.

Cotton plugs were replaced with air tight rubber serum stoppers. Then 10% of air in test tube was removed and replaced with equal volume of acetylene gas using gas-tight syringe. The tubes were incubated at 28+2°C for 24 hrs. After completion of incubation period, 1 ml of gas sample from test tube was injected into the pre-conditioned gas chromatograph for ethylene estimation and recorded the output data as peaks using a strip-chart recorder and noted the attenuation. The volume of assay tube gas phase was recorded. Total protein of each sample tube was estimated by collecting bacterial cells in 2 ml of 2N NaOH. Keep the cell suspension in boiling water for 10 min. Cool it, neutralize with 2 ml of 2N HCI and protein was estimated by Lowry's method using Folin-Phenol reagent (Lowry *et al.,* 1951).

$$\text{nmole } C_2H_4 \text{ produced.mg protein.hr}^{1} = \frac{Ce \times Ps \times Va \times As \times 60}{Pstd \times Vs \times Astd \times T \times P}$$

Phosphate solubilizing ability of the bacterial isolates

The ability of the bacterial isolates to solubilize insoluble inorganic phosphate was tested by spotting 10 µl overnight cultures on Pikovskaya's agarplates and incubating at 28-30°C for 2-3 days. The isolates which showed clear zone of solubilization of tricalcium phosphate (TCP) around the colony were noted as phosphate solubilizers. The diameter of the zone of TCP solubilization was measured and expressed in millimeters.

The bacterial isolates positive for P solubilization on Pikovskaya's agar medium were subjected to quantification of Pi released from TCP in broth medium. The Erlenmeyer flasks containing 50 ml Pikovskaya's broth (Pikovskaya, 1948) were inoculated with 500 µl overnight culture of each isolate in two replicates and incubated for 10 days at 28 ± 2°C. The TCP broth cultures were spun at 10,000 rpm for 10 minutes to separate the cells and insoluble phosphate and the available P content of the supernatant was estimated by using phosphomolybdic blue colour method (Jackson, 1973)

Procedure

One ml of the TCP broth culture supernatant was dispensed in 50 ml volumetric flask To this, 10 mL of chloromolybdic acid was added, mixed

thoroughly and the volume was made to approximately 3/4" with distilled water. Chlorostannous acid (0.25 ml.) was added and the final volume was made to 50 ml with distilled water and mixed thoroughly. The flasks were kept 15 minutes for color development and the blue color developed was read in a spectrophotometer at 610 nm using a reagent blank.

Results and Discussion

Collection of soil samples

A total sixty eight rhizosphere soil samples of *Azotobacter* and 32 rhizosphere soil samples of Phosphate Solubilizing Bacteria were collected during August 2021 to October 2021 from rhizospheric soils of xerophytic plants such as Ber, Alovera, Cactus, Opuntia and Agave, from different villages of five districts of Maharashtra State *viz*., Kolhapur, Sangli, Satara, Pune and Ahmednagar in accordance with GPS location. So as to study of isolates and characterize different microorganisms (*Azotobacter*, Phosphate Solubilizing Bacteria).

A total of Sixty eight isolates of *Azotobacter* were obtained from these 125 collected soil samples, by serial dilution and pour plate technique, using Jensen's medium.Out of all *Azotobacter* samples, five samples were found efficient *viz,* Azt-2 (*Ziziphus spp*) isolated from *ziziphus mauritiana*, Azt-12 (*Aloe spp*) isolated from *Aloe barbadensis*, Azt-16 (*Cactus spp*) isolated from *Cactus spp*, Azt-10 (*Opunita spp*) isolated from *Opuntia ficus* and Azt-8 (*Agave spp*) isolated from *Agave americana*.

The significantly highest *Azotobacter* population (9.28 X 10-4 CFU gm^{-1}) was recorded by Azt-2 (*Zizipus* spp) isolate obtained from (Shenoli) Satara district of Maharashtra state distributed at 17°18'97" North latitude and 74°26'12" East longitude followed by Azt-8 (*Agave* spp) was 8.80 X 10^{-4} CFU gm^{-1} from (Kadegaon) Sangli district of Maharashtra State distributed at 17° 29' 64" North latitude74° 33' 15"East longitude. A total of 32 isolates of Phosphate solubilizing bacteria were obtained from the collected soil samples, by serial dilution and pour plate technique, using Pikovskaya's medium. Only three samples out of all PSB isolates were found efficient *viz,* PSB-4 (*Ziziphus* spp) isolated from *Ziziphus mauritiana*, PSB-1(*Cactus* spp) isolated from *Cactus* spp, and PSB-2(*Agave* spp) isolated from *Agave americana*.

The Phosphate Solubilizing Bacteria isolated from rhizosphere soils of different regions of Maharashtra revealed significant variation 2.25 X $10^{-4}$to 6.48 X 10^{-4}.CFU gm^{-1}soil in microbial population among the different isolates. The maximum values of population recorded by PSB-4 (*Ziziphus spp*) isolates which was 6.48 X 10^{-4} CFU gm^{-1}obtained from (Rethare Bk) Sangli district of Maharashtra state distributed at 17°16'31" North latitude and 74°22'38" East longitude followed byPSB-1(*Cactus spp*) isolates obtained from (Supane) Satara district of Maharashtra state distributed at 17°29'75 North latitude and 74°12'03" East longitude.

Among sixty eight *Azotobacter* isolates and thirty two Phosphate solubilizing bacteria, efficient strains was identified as *Azotobacter chrocoocum*, *Pseudomonas striata* and *Bacillus megaterium* based on morphological characters. The well separated individual colonies were picked up with sterile loop and transferred to Jensen's media and Pikovskaya's media plates and maintained as pure culture.

Characterization of *Azotobacter* and PSB isolates

Out of all, 43 *Azotobacter* isolates were found rod shape while 25 were oval shape. The cells of all 68 *Azotobacter* isolates were observed to be motile. Based on cell arrangement they were grouped in : 36 isolates having cells in pair and 32 were single celled.The growth of 22 isolates were found abundant; 25 isolates growth to be moderate while 21 isolates as light growth. On the basis of colony elevation, isolates were grouped into three different groups. The first group of 22 isolates showed raised in shape, the second group of 25 isolates to be flat and third group of 19 isolates was convex shape.

Table.1 Evaluation of Nitrogenase activity of *Azotobacter* isolates by Acetylene Reduction Assay

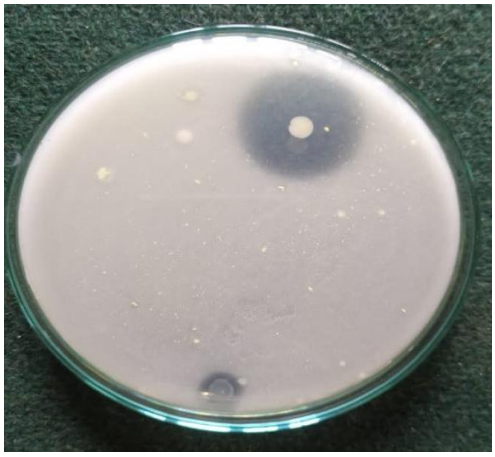
Sr. No	Isolates	Nitrogenase activity (nmol C_2H_4. mg protein^{-1}.h^{-1})	Sr. No.	Isolates	Nitrogenase activity (nmol C_2H_4. mg protein^{-1}.h^{-1})
1	Azt -1 (*Zizipus* spp)	70.89	35	Azt -10 (*Cactus* spp)	135.28
2	Azt -2 (*Zizipus* spp*)*	189.4	36	Azt -11 (*Cactus* spp)	105.6
3	Azt -3 (*Zizipus* spp)	78.6	37	Azt -12 (*Cactus* spp)	142.35
4	Azt -4 (*Zizipus* spp)	111.85	38	Azt -13 (*Cactus* spp)	35.64
5	Azt -5 (*Zizipus* spp)	97.8	39	Azt -14 (*Cactus* spp)	31.8
6	Azt - 6 (*Zizipus* spp)	45.8	40	Azt -15 (*Cactus* spp)	104.5
7	Azt -7 (*Zizipus* spp)	35.64	41	Azt -16 (*Cactus* spp)	37.8
8	Azt -8 (*Zizipus* spp)	76.9	42	Azt-1 (*Opuntia* spp)	55.8
9	Azt- 1 *(Aloe* spp)	102.85	43	Azt-2 (*Opuntia* spp)	68.4
10	Azt- 2 ((*Aloe* spp)	107.64	44	Azt-3 (*Opuntia* spp)	105.8
11	Azt- 3 ((*Aloe* spp)	95.69	45	Azt-4 (*Opuntia* spp)	111.4
12	Azt- 4 ((*Aloe* spp)	80.64	46	Azt-5 (*Opuntia* spp)	94.6
13	Azt- 5 ((*Aloe* spp)	74.6	47	Azt-6 (*Opuntia* spp)	59.4
14	Azt- 6 ((*Aloe* spp)	36.8	48	Azt-7 (*Opuntia* spp)	23.6
15	Azt- 7 ((*Aloe* spp)	153.9	49	Azt-8 (*Opuntia* spp)	55.74
16	Azt- 8 ((*Aloe* spp)	105.3	50	Azt-9 (*Opuntia* spp)	102.7
17	Azt- 9 ((*Aloe* spp)	65.2	51	Azt-10 (Opuntia spp)	155.25
18	Azt- 10 ((*Aloe* spp)	37.68	52	Azt-11 (*Opuntia* spp)	115.64
19	Azt- 11 ((*Aloe* spp)	49.68	53	Azt-12 (Opuntia spp)	127.36
20	Azt- 12 ((*Aloe* spp)	109.48	54	Azt-13 (*Opuntia* spp)	66.2
21	Azt- 13 ((*Aloe* spp)	115.6	55	Azt- 1 (*Agave spp*)	43.8
22	Azt- 14 ((*Aloe* spp)	82.45	56	Azt- 2 (*Agave* spp)	45.25
23	Azt- 15 ((*Aloe* spp)	93.0	57	Azt- 3 (*Agave* spp)	58.61
24	Azt- 16 ((*Aloe* spp)	86.54	58	Azt- 4 (*Agave* spp)	120.3
25	Azt- 17 ((*Aloe* spp)	176.64	59	Azt- 5 (*Agave* spp)	64.31
26	Azt -1 (*Cactus* spp)	77.64	60	Azt- 6 (*Agave* spp)	97.35
27	Azt -2 (*Cactus* spp)	84.6	61	Azt- 7 (*Agave* spp)	116.7
28	Azt -3 (*Cactus* spp)	98.7	62	Azt- 8 (*Agave* spp)	174.6
29	Azt -4 (*Cactus* spp)	114.3	63	Azt- 9 (*Agave* spp)	105.6
30	Azt -5 (*Cactus* spp)	102.4	64	Azt- 10 (*Agave* spp)	100.4
31	Azt -6 (*Cactus* spp)	115.6	65	Azt- 11(*Agave* spp)	108
32	Azt -7 (*Cactus* spp)	78.64	66	Azt- 12(*Agave* spp)	98.5
33	Azt -8 (*Cactus* spp)	94.2	67	Azt- 13(*Agave* spp)	75.6
34	Azt -9 (*Cactus* spp)	36.4	68	Azt- 14(*Agave* spp)	94.65
				S.E. $\pm$	5.32
				C.D. at 5 %	15.95

Table.2 Zone of Phosphate solubilization on Pikovskaya's agar medium and per cent Pi released from Tri-calcium phosphate (TCP) broth by the PSB isolates

Sr. No	Isolates	Zone of P solubilization on Tri-calcium Phosphate (mm)	Per cent released from TCP after 10 days
1	PSB-1 (*Zizipus* spp)	9.0	20.50
2	PSB-2 (*Zizipus* spp)	8.0	22.36
3	PSB-3 (*Zizipus* spp)	10.0	24.54
4	PSB-4 (*Zizipus* spp)	12.0	29.65
5	PSB-5 (*Zizipus* spp)	6.0	25.90
6	PSB-6 (*Zizipus* spp)	6.0	20.48
7	PSB - 1 (*Aloe* spp)	6.0	19.65
8	PSB - 2 (*Aloe* spp)	5.0	20.48
9	PSB - 3 (*Aloe* spp)	6.0	23.98
10	PSB - 4 (*Aloe* spp)	4.0	22.35
11	PSB - 5 (*Aloe* spp)	6.0	21.35
12	PSB - 6 (*Aloe* spp)	3.0	19.84
13	PSB - 7 (*Aloe* spp)	7.0	21.43
14	PSB-1 (*Cactus* spp)	11.0	28.22
15	PSB-2 (*Cactus* spp)	6.0	25.60
16	PSB-3 (*Cactus* spp)	5.0	24.85
17	PSB-4 (*Cactus* spp)	7.0	22.32
18	PSB-5 (*Cactus* spp)	10.0	21.48
19	PSB-1 (*Opuntia* spp)	9.0	19.89
20	PSB-2 (*Opuntia* spp)	5.0	20.48
21	PSB-3 (*Opuntia* spp)	5.0	21.48
22	PSB-4 (*Opuntia* spp)	9.0	21.64
23	PSB-5 (*Opuntia* spp)	6.0	22.35
24	PSB-6 (*Opuntia* spp)	9.0	18.5
25	PSB -1 (*Agave* spp)	7.0	20.5
26	PSB -2 (*Agave* spp)	11.0	28.4
27	PSB -3 (*Agave* spp)	8.0	21.65
28	PSB -4 (*Agave* spp)	5.0	22.48
29	PSB -5 (*Agave* spp)	7.0	25.98
30	PSB -6 (*Agave* spp)	8.0	23.65
31	PSB -7 (*Agave* spp)	6.0	21.47
32	PSB -8 (*Agave* spp)	9.0	20.85

Fig.1 Efficient strain of *Azotobacter* Isolates

Fig.2 Efficient strain of PSB Isolates



On the basis of appearance, out of 68 isolates, 26 isolates recorded opaque, 18 isolates were mucoid, 18 isolates were milky in appearance and 18 isolates were slimy. On the basis of pigmentation 14 isolates recorded light brown, while 31 isolates produced grey brown pigmentation, further pale colour pigment was also observed to be produced by the 23 *Azotobacter* isolates.

The biochemical characteristics of all the sixty eight *Azotobacter* along with MPKV is referance strains were studied by performing various biochemical tests like starch hydrolysis, catalase tests, casein test, H_2S production, Gelatin liquification and Methyl red test etc. The interpretation were found positive for catalase, starch hydrolysis and H_2S production tests whereas, in Gelatin liquification and Methyl red tests were found negative.

All PSB isolates collected were observed having rod shape and motility. Based on cell arrangement they were grouped in: 19 isolates having cells in chain and 13 isolates were single celled. On the basis of appearance they were grouped as, 16 as whitish while 16 isolates having creamy appearance. The colony margin of all 32 isolates were circular form while 15 isolates were with entire margin and remaining 17 isolates recorded as lobate margin. According to colony elevation PSB isolates were divided into three groups, first group of 10 isolates were found flat, second group of 12 isolates, as raised and third group of 10 isolates as convex in elevation.

All PSB isolates showed positive reaction for Starch hydrolysis, Gelatin liquification, Catalase tests and Methyl red tests while they shown negative reaction for H_2S production.

Nitrogen fixing ability of Bacterial Isolates

The nitrogenase activity of the *Azotobacter* isolates were determined by acetylene reduction assay. There was a wide variation in nitrogenase activity among the 68 different *Azotobacter* isolates tested from 23.6 to 189.4 nmol C_2H_4. mg protein^{-1}.h^{-1}. The nitrogenase activity of all isolates were compared with MPKV is Referance strain. The highly efficient *Azotobacter* isolates was characterized by its nitrogenase activity, which was 189.4 nmol C_2H_4. mg protein^{-1}.h^{-1} in Azt -2 (*Zizipus* spp) isolates, followed by Azt-17 (*Aloe* spp), Azt- 8 (*Agave* spp), Azt- 10 (*Opuntia* spp), Azt- 7 (*Aloe* spp) Azt-12 (*Cactus* spp) isolates with nitrogenase activity 176.64, 174.6, 155.25, 153.9 and 142.35 nmol C_2H_4. mg protein^{-1}.h^{-1} respectively while Azt-7 (*Opuntia* spp) found less efficient (23.6nmol C_2H_4. mg protein^{-1}.h^{-1}). The MPKV is Reference strain had nitrogenase activity of 160.62, 171.94 and 164.37 nmol C_2H_4. mg protein^{-1}.h^{-1}.

Out of sixty eight *Azotobacter* isolates, 3 isolates *viz*., Azt- 2 (*Ziziphus* spp), Azt-17 (*Aloe* spp), Azt- 8 (*Agave* spp), recorded significantly higher nitrogenase activity than the MPKV Referance strain.

Phosphate solubilizing ability of PSB isolates

The ability of all 32 PSB isolates for solubilizing insoluble phosphate both quantitatively and qualitatively were evaluated on Pikovskaya's Agar Medium by incubating at 28° to 30°C for 2 to 3 days. Results pertaining to solubilization index were due to formation of halo zone around different isolates of phosphate solubilizing bacteria and per cent Pi released were obtained from TCP after 10 days.

All the 32 isolates were able to form zone of P-solubilization on the medium. The diameter of zone of solubilization indicated the wide variations among the isolates. The diameter of the zone of P-solubilization ranged from 3 to 12 mm in different *Azotobacter* isolates. The zone of P- solubilization was noticed maximum in PSB-4 (*Zizipus* spp) followed by PSB-1 (*Cactus* spp.) and PSB -2 (*Agave* spp) isolates which was 12 mm, 11 mm and 11 mm respectively.

The amount of Pi released from tri- calcium phosphate by the PSB isolates on pikovskaya's medium was estimated at 10 days after inoculation. The amount of Pi released from tri- calcium phosphate (TCP) by the isolates at 10 day after inoculation was ranged from 18.5 to 29.65 %. The per cent Pi released from TCP of all 32 PSB isolates were compared with standard MPKV is Referance strain. The quantitative estimation of per cent inorganic phosphorus released from Tri- calcium phosphate had recorded highest for PSB-4 (*Zizipus* spp) which was 29.65 % while the lowest percentage of inorganic phosphorus for PSB-6 (*Opuntia* spp) recorded as 18.5%. Thus, isolate PSB-4 (*Zizipus* spp) i.e. efficient strain isolated from ber was found to be significantly superior over rest of the isolate in solubilizing phosphate.

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