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RESEARCH ARTICLE

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ISOLATION AND CHARACTERIZATION OF POTENTIAL PHOSPHATE SOLUBILIZING BACTERIA FROM SOIL SAMPLES OF AGRICULTURAL FIELDS OF DHAPA, KOLKATA, WEST BENGAL.

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Abstract

Phosphate solubilizing bacteria (PSB) helps in uptake of insoluble phosphate by plants. 'Phosphate' is one of the most essential growth promoting factor and plays a vital role in plant cellular enhancement processes like root elongation, proliferation and changes of root architecture due to its deficiency, seed development, and normal crop maturity. Therefore, the present study was targeted for isolation of potential PSBs from Dhapa agricultural soil samples and was assessed for the plant growth promoting traits. The assessments include indole acetic acid assay (IAA), ammonia, siderophore, and hydrogen cyanide (HCN) production and their effects on growth and uptake of phosphorous by chilli plants. The estimation of growth of the chilli plants were observed with a pot experiment. According to the study the IAA production was 2.9, 4.9, 1.92, and 3.17 µg/ml for the isolated organisms PSB AR1, PSB AR2, PSB AR3 and PSB AR4 respectively. Amount of phosphate solubilized were 70, 60, 67.6 and 54% by PSB AR1, PSB AR2, PSB AR3 and PSB AR4 respectively.

Introduction:-

Phosphorous is one of the growth promoting factors for plants. This is because phosphorous in included in the growth factors of plants like cell division, photosynthesis, utilization of carbohydrates in plants (Wu et al., 2005). It plays a great role in several physiological processes like root elongation, proliferation, root morphology, seed development and maturity, etc. Deficiency of phosphorous results in various problems in plant growth. The deficiency includes as browning of leaves, weak stem, and slow development. It remains in an insoluble form which makes difficult for the plants to utilize to its full extent (Mittal et al., 2008). The mineral phosphorous is solubilized by bacteria known as the "phosphate solubilizing bacteria" or PSBs. Assimilation of phosphorous takes place with the help of the enzyme called the "phosphatase". These are present in variable amount in the soil, and present in wide variety of soil microorganisms (Zaidi A, Khan MS, Amil MD, 2003). The soil microorganisms transfer the phosphorous to a soluble form of phosphate. The process includes the consumption of the tricalcium phosphate by

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the psb present in the soil. The plants consume the nutrient in the orthophosphate forms (H 2 PO 4- and HPO 4 2-). Therefore psb acts as a natural bio-fertilizer which is necessary for the plants for their growth. Phosphorous is the structural components of various co-enzymes, phospho-proteins, phospholipids, and a part of the memory "DNA". These types of bacteria are majorly found in the rhizopheric region of plants. Due to the specialty of the PSB to transfer the insoluble compounds, they are widely used in the crop yield. The psb strains are associated with the release of organic acids (Bashan, Y., Kamnev, A. A., & de-Bashan, L. E. 2013). The hydroxyl and the carboxyl groups chelate the cations which are bound to the phosphate. There are different strains of psb such as, Micobacterium laevaniformans (P7), Pseudomonas putida (P13) etc. These bacteria are renowned for the rapid utilization of the insoluble phosphate to a soluble form, so that the plant can utilize it for their growth. Phosphorus nutrition has become a global issue and 30-40% crop yield of the world's cultivation land is hampered by the P availability (Rudresh DL, Shivaprakash MK, Prasad RD, 2005). Phosphate deficient soils due to poor availability of mineral phosphorous in the soil, require more amount of psb. The free phosphate concentrations are not higher than 10uM, and the favorable pH is 6.5. This is also applicable for fertile soil.

Materials and methods:-

Study area and sample collection:-

The analysis of phosphate solubilizing bacteria from soil sample was carried out in 22^o 32' 17"N and 88^o 25' 11" E field of Dhapa, near the E.M. Bypass, Kolkata, West Bengal. The soil samples from Dhapa were collected in sterile bottles for sampling. The quality of the data is replicated by its replication.

Screening and isolation of the organism:-

The soil samples taken of 1 gram and were serially diluted up to 10-folds. 10 to 20 ul of the diluted sample was plated in NBRIP (National Botanical Research Institute of Phosphate solubilizing bacteria) agar media plates and were incubated at 30^o C for 5 days. Single colonies with clear halozone were randomly selected and pure culture was obtained by continuous re-plating in NBRIP agar media. Glycerol stocks were prepared and were stored at - 20^o C.

Morphological analysis of the isolated organism:-

Physical identification of bacteria were done by morphological and biochemical analysis. The bacterial isolates with clear halo zones formation were identified by colony morphology and by different staining procedures such as simple staining and Gram's staining.

Biochemical characterization:-

Biochemical analysis for the isolates were done to examine activities of MR-VP test, Indole test, Urease hydrolization test, Gelatin test, Citrate agar test, Motility test, Starch hydrolysis test, Catalase test, TSI test, Casein hydrolysis test. Some carbohydrate utilization test like Lactate test, Mannitol test, Sucrose and D-glucose test were also performed. The above tests were performed as per Bergey's Manual of Systematics Bacteriology

Determination of growth curve:-

Growth pattern was determined for the isolated organism. 24 hrs old cultures were taken and were inoculated in LB broth. Readings were taken in triplicates at regular intervals for 3 days at 660 nm.

Genomic DNA extraction:-

The bacterial genomic DNA was extracted by cTAB alkaline lysis method. 24 hrs old liquid culture of the organism was taken and was centrifuged at 10000 rpm for 15 mins to obtain the bacterial pellet. The pellet was then dissolved in 0.8 ml of CTAB buffer along with 50 µl of 0.1mg/ml proteinase k and was incubated at 60^0 C for 1 hr. Then 0.6ml of chloroform : isoamyl alcohol at a ratio of 24:1 was added, gently mixed and then was centrifuged at the maximum speed for 10 minutes. The resulting aqueous phase was the transferred to a fresh tube without disturbing the organic phase and about $2/3^{rd}$ volume of chilled isopropanol was added and vortexed gently by hand until thread like DNA appears. The DNA was then pelleted down by centrifugation and the supernatant was discarded and the pellet was washed with 70% chilled ethanol and centrifuged at maximum speed. The pellet or DNA was then dissolved in 50 to 100 µl of T.E. buffer depending on the amount of DNA obtained and stored at 4^oC for further use.

16s rDNA PCR amplification:-

Complete 16s rDNA amplification was done using universal Forward primer (5'-AGA GTT TGA TCC TGG CTC AG-3') and Reverse primer (5'-GGT GTT TGA TTG TTA CGA CTT-3') to know the bacterial species. Colony and

Genomic DNA PCR were performed. PCR was performed using 50-µl reaction mixture containing 1-µl of DNA extract as a template, 5 mM of each primers, 25 mM of $MgCl_2$, 2 mM of dNTPs, 1.5 U of *Taq* polymerase and buffer recommended by the manufacturer (GCC Biotech, India). After initial denaturation for 5 min at 95°C, 35 cycles of reaction was performed consisting of denaturation at 95°C for 30 secs, annealing at 48°C for 1 min 30 secs, extension at 72°C for 1 min and final extension at 72°C for 7 min. The PCR products were analyzed by using 1% (w/v) agarose gel electrophoresis (C.Edward Raja, G.S Selvam, Kiyoshi Omine).

Assay of inorganic phosphate solubilising ability:-
Bacterial strains were grown in sterilized liquid NBRIP broth medium (30 ml) at 30°C for two days with continuous shaking at 150 rpm, and were tested for phosphate solubilizing ability. Sterilized uninoculated medium served as a control. 50ml of phosphate reagent were prepared to evaluate the phosphate solubilizing ability of the organisms. 4ml of the phosphate reagent were mixed with 1ml of the bacterial culture and was incubated for 1 hour. The absorbance after the incubation was measured at 829 nm. Standard curve of the phosphate were prepared using dihydrogen sodium phosphate and were extrapolated to evaluate the amount of phosphate solubilized (Murphy and Riley, 1962).

Production of indole acetic acid:-
IAA production was determined following the method described by Gutierrez et al. (2009). Bacterial strains grown in sterilized 100 ml liquid NBRIP medium containing 1 ml of 0.2% tryptophan were incubated for 72 h with continuous shaking at 30°C. A sterilized uninoculated medium was served as the control. Treated sample and control were taken into centrifugation tube for every 24 h and centrifuged 10 min at 12000 rpm. The clear supernatant of l ml was mixed with 4 ml of the Salkowski's reagent (50 ml of 35% perchloric acid and 1 ml of 0.05 M FeCl3 solution). The mixture was incubated in the dark at 37°C for 30 min. Development of pink color indicates the IAA production and optical density was measured at 530 nm using UV spectrophotometer (Shimadzu UV-VIS).

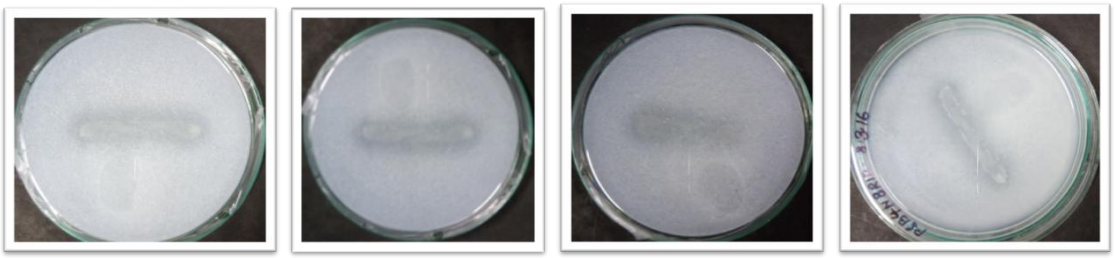
Production of hydrogen cyanide (HCN):-
HCN production was assessed by growing the bacteria in 10% tryptic soy agar (TSA) supplemented with glycine (4.4 g/L). Filter paper soaked in picric acid and Na2CO3 (0.5 and 2%, respectively) solution was fixed to the underside of the lids of plates and incubated for five days at 30°C. A change in filter paper color from yellow to orange-brown was considered to be the indication of HCN production (Donate-Correa et al., 2005).

Production of ammonia:-
The bacterial isolates were tested for the production of ammonia in peptone water. Fresh cultures were inoculated into 10 ml peptone water and incubated for 48 hrs at 30°C. Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow colour was considered to be a positive test for ammonia production (Cappucino and Sherman, 1992).

Production of siderphore:-
Siderphore production was assayed qualitatively using chrome azurol S (CAS) blue agar as described by Schwyn and Neilands (1987). The bacterial strains were inoculated on the CAS agar plates and incubated at 30°C for 24 h. Orange halos around the colonies were recorded as the measurement of siderphore production (Payne, 1994).

Results:-

Screening and isolation of the organism:-
After plating in NBRIP medium the organisms showing halo zones around the colonies were subjected for further tests and were named as PSB AR1, PSB AR2, PSB AR3 and PSB AR4.

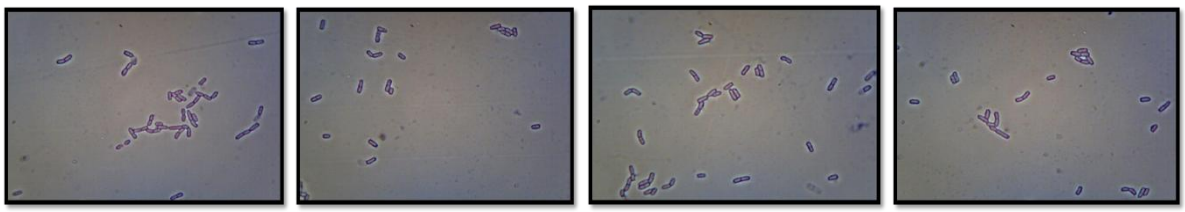


A. PSB AR1 B. PSB AR2 C. PSB AR3 D. PSB AR4

Figure 1:- Initial isolation and halozone by the organisms.

Morphological analysis of the isolated organism:-

First line of identification for the organism was done by Gram staining technique. The isolates appeared rod shaped gram positive cells when viewed under both phase contrast and 100 X oil immersion microscopy (Figure 2). The colonies were creamish white in color, round and small (Table 1).



A. PSB AR1 B. PSB AR2 C. PSB AR3 D. PSB AR4

Figure 2:- Oil immersion and Phase contrast microscopic image of the isolated PSB.

Table 1:- Morphological Characteristics of the organism

Morphological characters				
	PSB AR1	**PSB AR2**	**PSB AR3**	**PSB AR4**
Colour of colony	Creamish	Creamish	Creamish	Creamish
Cell shape	Rod	Rod	Rod	Rod
Gram staining	Positive	Positive	Positive	Positive
Motility	Motile	Motile	Motile	Motile

Biochemical characterization:-

Table 2:- Biochemical characteristics of the organisms

Biochemical tests				
	PSB AR1	**PSB AR2**	**PSB AR3**	**PSB AR4**
Urease	Negative	Negative	Negative	Negative
Catalase	Positive	Positive	Positive	Positive
MR	Positive	Positive	Positive	Positive
VP	Positive	Positive	Positive	Positive
Starch hydrolysis	Negative	Negative	Negative	Negative
Gelatin	Negative	Negative	Negative	Negative
Indole production	Negative	Negative	Negative	Negative
Citrate utilization	Positive	Positive	Positive	Positive

Table 3:- Carbohydrate test results of the organisms

Carbohydrate utilization tests				
	PSB AR1	**PSB AR2**	**PSB AR3**	**PSB AR4**
Fructose	Positive	Positive	Positive	Positive
Mannitol	Positive	Positive	Positive	Positive
Sucrose	Positive	Positive	Positive	Positive
D- Glucose	Positive	Positive	Positive	Positive

Table 4:- TSI test results of the four organisms

Results for TSI test		
PSB AR1	Positive	Yellow but = acid production; Broken but = (move upward) gas production; Black colour = H2S; acid; and gas production;
PSB AR2	Positive	Yellow but = acid production; Broken but = slight gas production;
PSB AR3	Positive	Yellow but = acid production; Broken but = (move upward) high gas production;
PSB AR4	Positive	Yellow but = acid production; Broken but = (move upward) high gas production; Black colour = H2S; acid; and gas production;

Determination of optimum growth condition:-

The isolate showed a wide range of dependence for both temperature and pH. The optimum pH for growth for the organism was found to be pH 7 (Table 5) and the optimum temperature was found out to be 30^0 C (Table 6).

Table 5:- represents the pH range of the organism and the optimum pH for the growth

	pH 4	**pH 5**	**pH 6**	**pH 7**	**pH 8**	**pH 9**	**pH 10**	**pH 11**	**pH 12**
PSB 1	-	+	+	++	+	-	-	-	-
PSB 2	-	+	+	++	+	-	-	-	-
PSB 3	-	+	+	++	+	-	-	-	-
PSB 4	-	+	+	++	+	-	-	-	-

-, Indicates no growth; +, Indicates growth; ++, Indicates optimum condition

Table 6:- shows the optimum temperature for the growth of the organism

	25 ° C	**30 ° C**	**37 ° C**	**40 ° C**
PSB 1	+	++	+	-
PSB 2	+	++	+	-
PSB 3	+	++	+	-
PSB 4	+	++	+	-

-, Indicates no growth; +, Indicates growth; ++, Indicates optimum condition

Determination of growth curve:-

After incubation the organisms showed different growth rate (Figure 12, 13, 14, 15). All the organisms showed similar growth pattern. In accordance with rate of growth the organisms can be arranged as PSB AR2 <PSB AR3 <PSB AR4< PSB AR1.

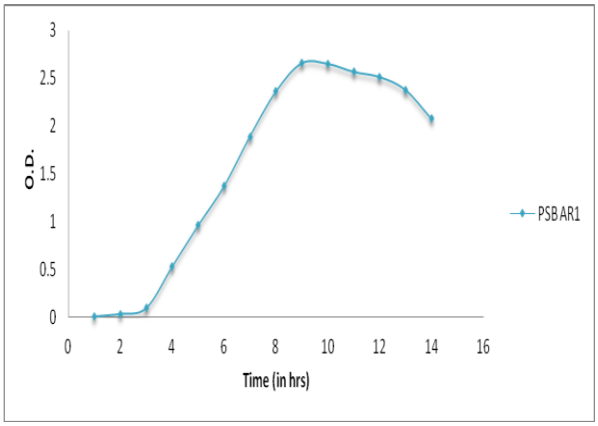


Figure 3:- Growth curve for PSB AR1

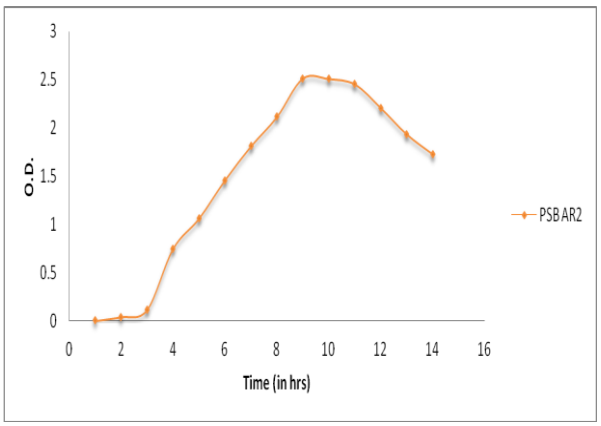


Figure 4:-Growth curve for PSB AR2

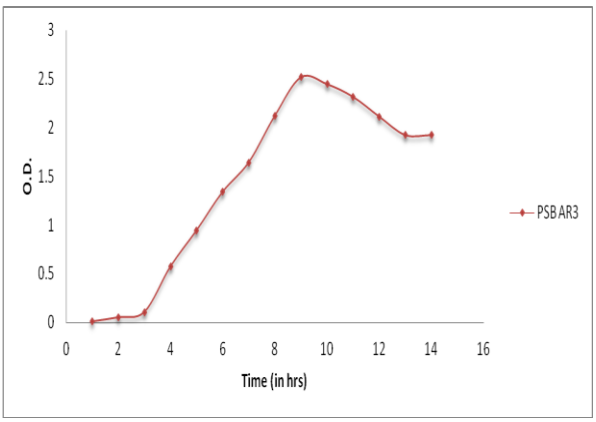


Figure 5:-Growth curve for PSB AR3

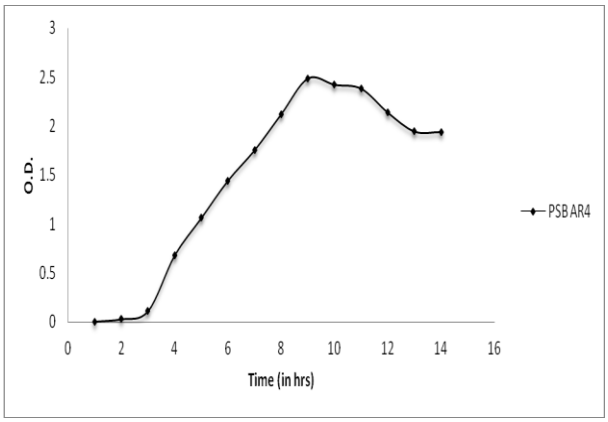


Figure 6:-Growth curve for PSB AR4

Molecular characterization:-

After 16s rDNA analysis the isolates were submitted to GenBank for acquiring accession number for the organisms. Figure 16 shows the band of genomic and Figure 17 shows the PCR band of the 16s rDNA when observed through gel imaging system after running the sample in 1% agarose gel.

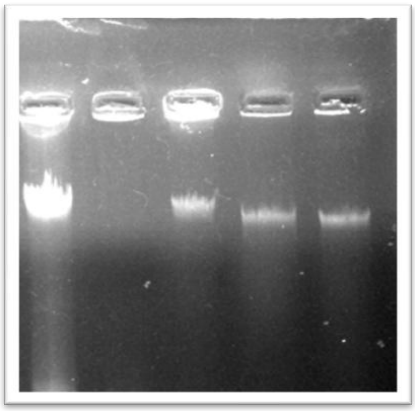


Figure 7:- Genomic DNA band

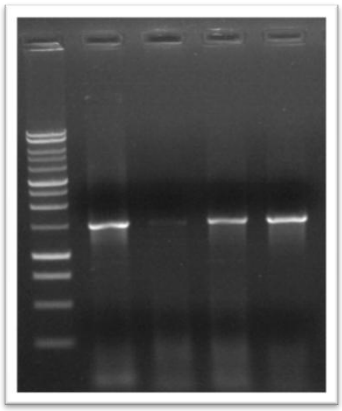


Figure 8:-PCR product band

Assay of inorganic phosphate solubilising ability:-

After incubation at 30°C for two days with continuous shaking at 150 rpm, the organisms showed a varied amount of phosphate solubilisation. Table shows the amount of phosphate solubilised by the four organisms (Figure 18). Figure shows the standard curve from which the amount of phosphate was determined.

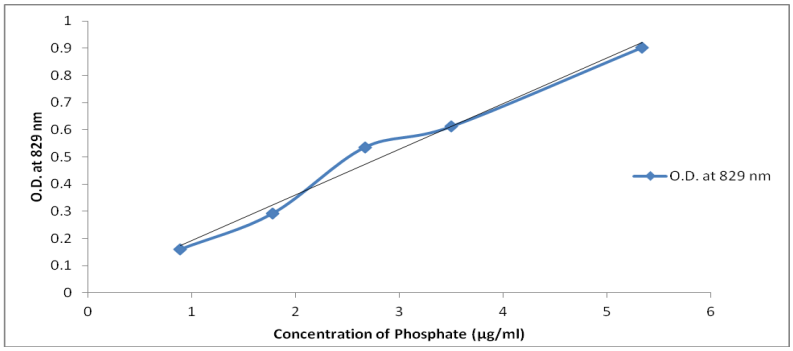


Figure 9:-Standard Curve for phosphate assay

Table 7:- shows the concentration of Phosphate solubilised by the four PSB

	Initial Conc. Of Phosphate in media (µg/ml)	Final Conc. of Phosphate in media after 48 hrs. (µg/ml)	Amount of phosphate solubilized (µg/ml)	% of phosphate solubilized
PSB AR 1	5	1.5	3.5	70
PSB AR 2	5	2	3	60
PSB AR 3	5	1.62	3.38	67.6
PSB AR 4	5	2.3	2.7	54

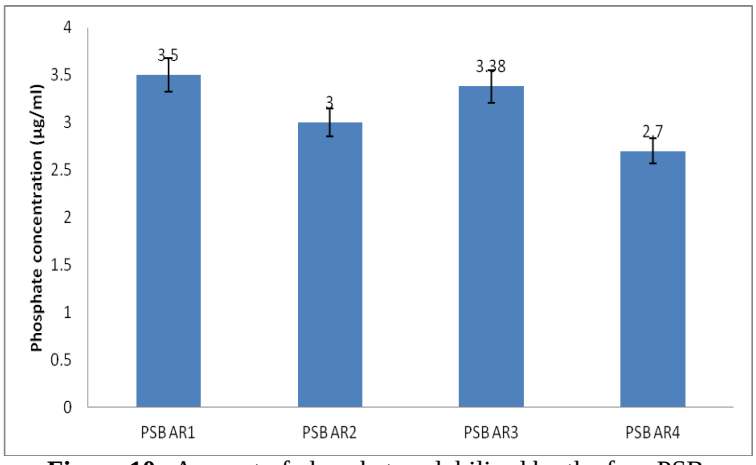


Figure 10:-Amount of phosphate solubilised by the four PSB

Production of indole acetic acid:L-

All the four PSBs were positive for production of IAA. The production of IAA (Table 8) varied with time (Figure 20). Figure shows the standard curve for estimation of IAA production.

Table 8:- shows the concentration of IAA produced by the four PSB

Concentration of IAA produced (µg/ml)				
	PSB AR1	**PSB AR2**	**PSB AR3**	**PSB AR4**
Day 1	0.04	4.9	1.8	3
Day 2	2.9	3.2	2.1	2.3
Day 3	0.6	2.89	0.2	2.98
Day 4	1.6	3.1	0.55	1.76
Day 5	1.92	4	1.92	3.17

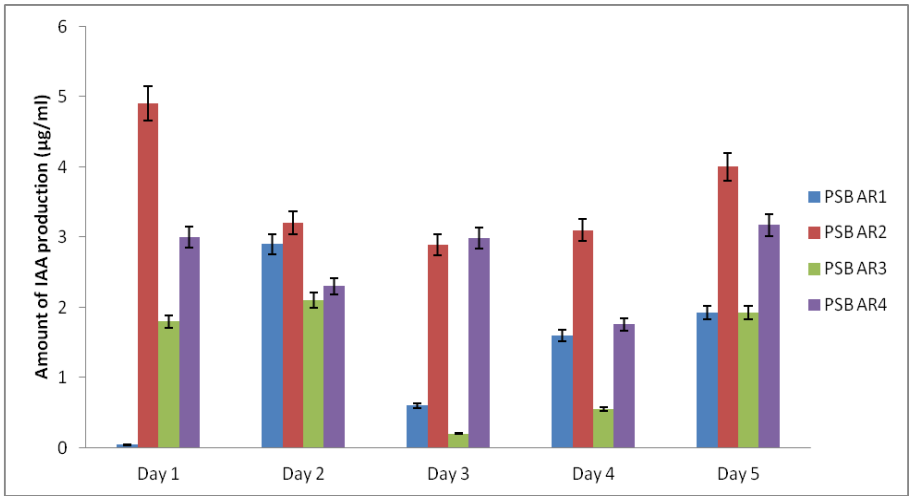
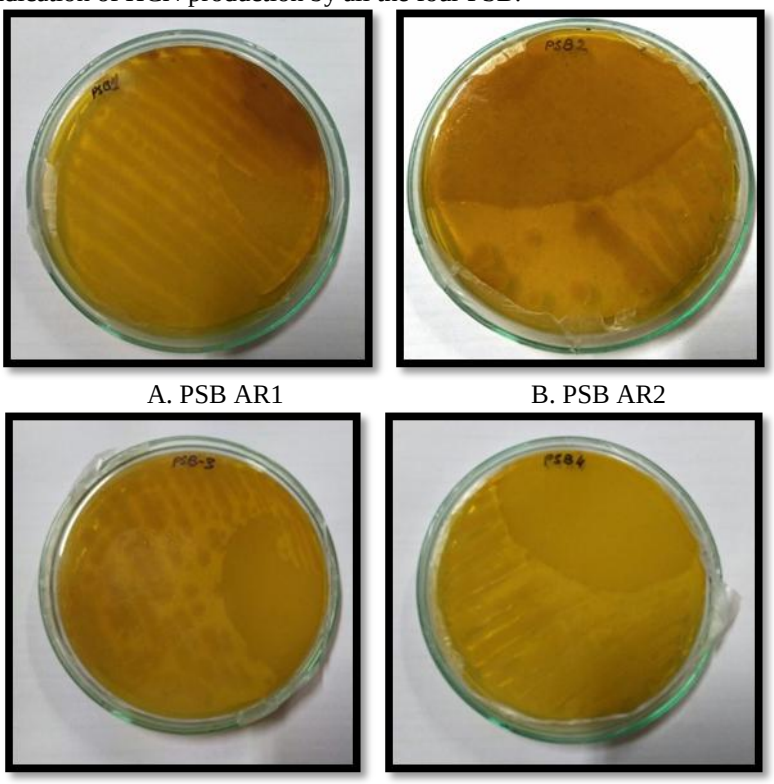


Figure 11:-Production of IAA by the four PSB

Production of hydrogen cyanide (HCN):-

After incubation for five days at 30°C the filter paper colour changes from yellow to orange-brown and was considered to be the indication of HCN production by all the four PSB.



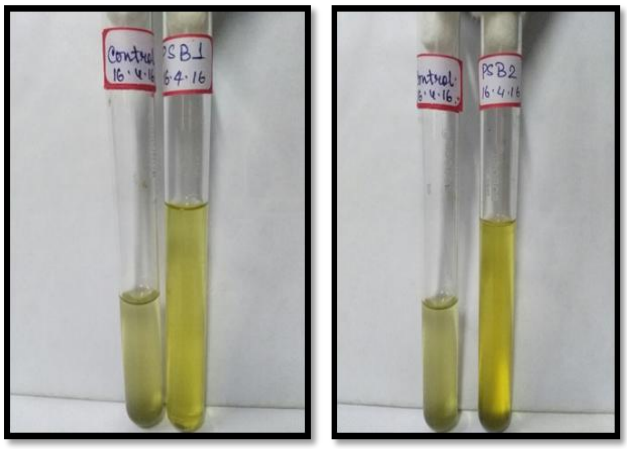
A. PSB AR1 B. PSB AR2

C. PSB AR3 D. PSB AR4

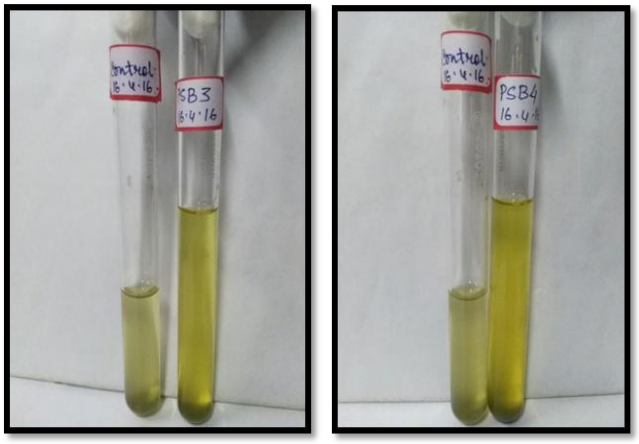
Figure 12:-HCN production results

Production of ammonia:-

All the four bacterial isolates that were tested for the production of ammonia in peptone water showed a development of yellow color after addition of Nessler's reagent, which was considered to be a positive test for ammonia production.



A B

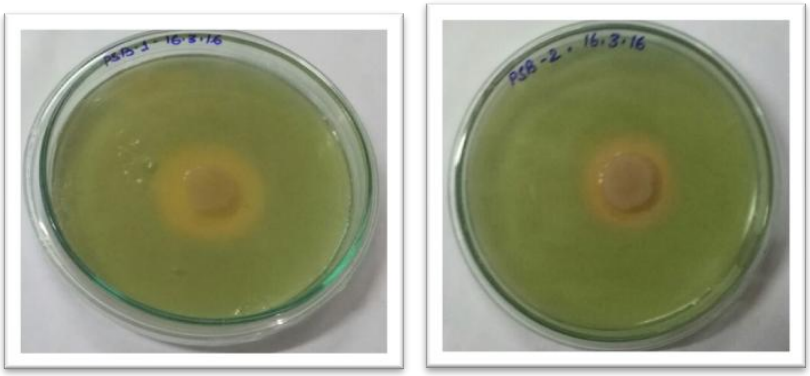


C D

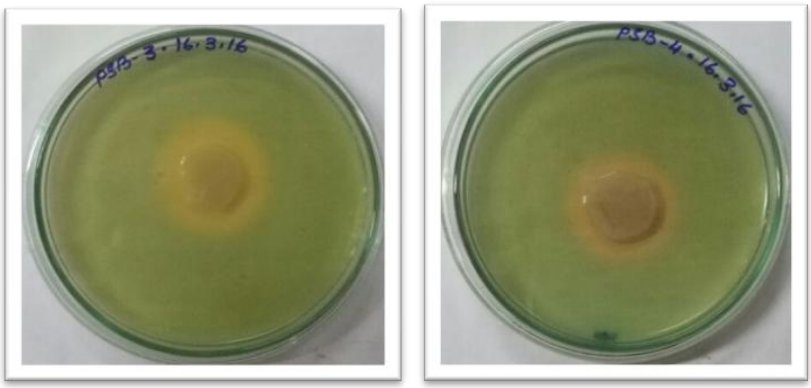
Figure 13:-Yellow color formation conferring positive result for ammonia production.

Production of siderphore:-

Siderphore production was assayed qualitatively using chrome azurol S (CAS) blue agar as described by Schwyn and Neilands (1987). The bacterial strains on the CAS agar plates showed orange halo zones around the colonies after incubation at 30°C for 24 hrs which was recorded as positive result for siderphore production.



PSB AR1 PSB AR2



PSB AR3 PSB AR4

Figure 14:-shows the orange halos around the colonies conforming siderphore production

Discussions:-

Phosphorus is an important limiting factor in agriculture production, and microbial activation seems to be an effective way to solve the solidified phosphorus in soil. The result obtained in this study shows that the isolates PSB AR1, PSB AR2, PSB AR3 and PSB AR4 has a broad growth range: it can survive at a pH range of 5.0 – 8.0and it

can solubilize both inorganic phosphorus. The optimum temperature for the growth of the organisms was all around 30^0 C.

The formation of the clear zones is concerned with the phosphate solubilization of the strains (Figure 4). It may secrete some substances into surroundings in the course of growing, which can solubilize phosphate. Phosphate solubilization result may vary depending on kinds of the metabolism, how quickly it releases, and also its spread degree on the medium. Therefore, observational method of phosphate solubilizing zone can only be used to qualitative assays (Piccini and Azcon, 1987).

The major mechanism associated with the solubilization of insoluble phosphate is the production of organic acids, accompanied by acidification of the medium (Puente et al., 2004). Reductions in releasing rate of soluble phosphorous during the later stages of the incubation might be due to the depletion of nutrients in the culture medium, in particular, carbon source needed for the production of organic acids (Kang et al., 2002; Kim et al., 2005; Chaiharn and Lumyong, 2009). However, as reported by Varsha-Narsian et al. (1994) availability of soluble phosphorus in the culture medium might also have an inhibitory effect on further phosphate solubilization.

IAA stimulates a rapid response (example increased cell elongation) as well as a long-term response (example cell division and differentiation) in plants (Ahmad et al., 2008). Furthermore, IAA stimulates lateral root formation which in turn could facilitate high root surface area for nutrient absorption from soil (Compant et al., 2010). Therefore, IAA production by microbes could have definite effect on growth of the host plant. Similar to these findings, IAA production by PSB strains such as *Achromobacter xylosoxidans* and *Klebsiella* SN 1.1 have also been reported (Jha and Kumar, 2009; Chaiharn and Lumyong, 2011). The present study also reveals that the isolated organisms produced good amount of IAA that can be of use for plant growth when applied as bio-fertilizers.

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