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RESEARCH ARTICLE

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POTENTIAL SCREENING OF AZOTOBACTER FROM SOIL.

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Abstract

Today the world is searching for remedies from the nature. Hence, in the agricultural sector, soil is the answer. As soil is the home for microorganisms, we isolate important biofertilizers like Azotobacter from soil. This Azotobacter supplies Nitrogen and Phosphorus to important crops like wheat. Hence, it can rightly be called 'Food for the crops'. As these free-living bacterial cells divide in the soil, they fix atmospheric nitrogen for the crops. Even the phosphate minerals in the soil may be unavailable for the plants. This bacteria solubilises the phosphate so that the crop roots can easily take up the macronutrient ,for the healthy growth of plants.

Hence, this paper aims at the screening out of Azotobacter from rhizosphere soil. The study also includes large scale production of Azotobacter.

Introduction:-

Biofertilizers are microorganisms, prepared by laboratory techniques , with the aim that its multiplication in soil enhances plant growth, thereby supplementing chemical fertilizers.They are low cost renewable sources of plant nutrients which supplement chemical fertilizers. They are Bioinoculants , whose multiplied cells in the soil participates in Nitrogen-fixation , phosphate solubilisation and potassium mineralization. Thus, Biofertilizers are the ultimate solution for the farmers as these Bioinoculants supply NPK to crops , just like chemical fertilizers. These Biofertilizers are becoming popular as they aid to proper maintenance of soil health ,minimize environmental pollution and cuts down the use of chemicals.

Materials and Methods:-

Collection of sample:-

Soil sample was collected from a garden in Kolkata. After digging for 10cms,the soil adhering to the roots of grass and other plants are collected. Hence, Rhizosphere soil is collected in a packet and taken to the laboratory.

Serial Dilution:-

In order to ensure adequate sterility, the process of Serial Dilution is carried out in the presence of the HEPA filters of the Laminar Air Flow .Firstly , 1g of soil sample is weighed and diluted with 10ml of sterile distilled water. The

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tube is shaken lightly against the left thumb, to achieve better solubility in the sterile distilled water .This tube is marked as 'Stock'. 9 more tubes ,each containing 9ml sterile distilled water ,is taken . With the help of 1000µl micropipette, from 'Stock', 1ml is transferred to the adjacent tube and marked as 10^{-1} .Likewise, from 10^{-1} , 10^{-2} is prepared by transferring 1ml from 10^{-1} tube. Hence, dilutions from 10^{-1} to 10^{-9} are prepared.

Primary Isolation of Bacteria:-

1oo ml of Nutrient Agar medium is prepared and poured into 4 sterile petridishes, within the Laminar Air Flow. The media is then allowed to solidify. From each of Serial Dilution tubes marked 10^{-2},10^{-4},10^{-6} and 10^{-8} , 100µl is transferred to the sterile agar plates .Then, with the help of spreader, 100µl inoculum from the serial dilution tubes, are spreaded. The inoculated petriplates are then inverted and kept in the incubator at 37^{o}c for 24hrs.

After 24hrs of incubation, external morphology of the colonies in the petriplates are studied.

Table 1:- Shows the external morphology of colonies

Dilution	No.of Colonies	Form	Elevation	Margin	Colour	Transparency	Size
10^{-2}	12	Irregular	Flat	Curly	Milky white	Transluce	Small
10^{-4}	8	Circular	Flat	Entire	Cream	Translucent	Moderate
10^{-6}	3	Circular	Flat	Entire	Clear white	Translucent	Medium
10^{-8}	2	Circular	Flat	Curly	White	Translucent	Small

Isolation of Azotobacter from Mixed Culture:-

Colonies that are identified to be Azotobacter, after Gram's staining, are sub-cultured on Nutrient agar plates. Colonies that are identified ,are first marked and then sub-cultured. The dilution plates for eg. 10^{-2} ,10^{-4} ,10^{-6} and 10^{-8} plates are taken to the Laminar Air Flow. Nutrient agar is prepared and poured into 4 petridishes , allowed to solidify. Then, with the help of sterile inoculation loop , very small amount of the identified colony is picked and streaked on to the fresh nutrient agar plates. The inoculated plates is then kept for incubation at 37^{o}c , for overnight growth.

The pure cultures of bacterial species that are prepared after overnight incubation ,are further purified. The pin-pointed single colonies were picked up and again transferred onto fresh nutrient agar plates.

Gram Staining of Azotobacter:-

Gram staining is performed at every step to identify the colonies of Azotobacter. Gram staining is done in Laminar Air Flow. Firstly, the pure culture plates are taken to the hood and the locations are marked on the reverse side of the plate with marker. Slides are then made grease-free by washing in soap water and then alcohol-wash. Then, after air-drying the slide , a drop of sterile distilled water is added on the slide. The sterile inoculation loop is touched on the marked colony and then a smear is made on the slide. The smear is then heat-fixed by passing the slide over the flame for a number of times, till we see the dried smear on the slide.

Then, the slides are taken out of the Laminar Air Flow hood and Gram's staining is done. For Gram's staining , Crystal violet is added on the smear, kept for 1min., then washed under slow –running tap water. Next, Gram's iodine is added on the smear and kept for 1min.Then the slides are subjected to 95% ethanol wash. Then the slides are washed with distilled water and saffranin is added on the smear.
After keeping safranin for 1min. , the slides are again washed with distilled water. Then, the slides are air-dried and observed under microscope(Collee *et.al* .,1996)

First, the slides are observed under 40X and then under 100X oil immersion.

Screening of *Azotobacter*:-

After Gram's staining ,the bacterial species that were grown on nutrient agar plates, are further confirmed to be Azotobacter by their ability to grow on Ashby's Medium and Mannitol Broth and Agar Medium. As Ashby's Medium and Mannitol Agar Medium are Selective media for Azotobacter, their growth in the media confirms Azotobacter. As Azotobacter is a Nitrogen-fixing bacteria , their growth in Nitrogen-free Mannitol Agar is very much expected.

The bacterial colonies are picked from the pure-culture Nutrient Agar plates and streaked onto Ashby's Medium and Mannitol Broth and Agar medium, with the help of sterile inoculation loop. The plates are then kept for overnight incubation at 37°c .On, the next day, flat ,soft ,milky mucoid colonies of Azotobacter are observed. More pin-pointed colonies are found on Ashby's Medium than on Mannitol Agar medium.

The colonies on Ashby's medium are further transferred to fresh Ashby's Medium and Mannitol Agar Medium ,for Biochemical tests.

Confirmation of Azotobacter by Biochemical Tests:-

Citrate Utilization Test:-

Principle – Simmon's Citrate Agar medium containing sodium citrate , the sole source of carbon is used to detect whether the organism is able to use citrate or not. This agar contains indicator ,bromothymol blue which changes from green to blue when the growth of microorganism causes alkalinity. Growth on the medium and changing of the colour from green to blue indicate positive test.

Procedure – Preparation of Simmon's Citrate Agar as per the composition- Ammonium dihydrogen phosphate-0.05g and Dipotassium phosphate – 0.05g are dissolved separately. NaCl-0.25g ,Sodium citrate-0.1g and MgSO4 – 0.01g are dissolved in another flask. Then the contents of the flasks are mixed and the volume is made upto 50ml in a 100ml conical flask. Then, 1g Agar and Bromothymol blue – 0.04g are added. The flask is then autoclaved .Slants are prepared and after the media gets solidified and cooled, streaking is done from the colonies on Ashby's medium and Mannitol Agar Medium, in the Laminar Air Flow. The tubes are then kept for incubation for 48 hrs

Starch Hydrolysis Test:-

Principle – Starch is a polysaccharide made of 2 components- Amylose and Amylopectin. Amylose is not truely soluble in water but forms hydrated cells which forms a blue colour with iodine. As such, the polysaccharide chain is twisted into a helical coil.Amylase gives a characteristic blue colour with iodine due to its ability to occupy a portion at the interior of a helical coil of glucose units. This is formed when Amylase is suspended in water. Amylopectin yields a colloidal state giving violet colour with iodine.

Procedure – Preparation of Starch Agar Medium as per the composition-1 g of Starch is dissolved in a little quantity of water and stirred while heating ,till the starch dissolves. Then, Peptone-0.25g , Beef extract – 0.15g and Agar are added and the volume is made up to 50ml. The flask is then autoclaved and after cooling to 45oc, the contents are poured into petridishes and the media is allowed to solidify. Then , single inoculation from Ashby's medium and Mannitol Agar Medium , is done on the plates ,under aseptic conditions of Laminar Air Flow and kept for 48 hrs. incubation . After incubation, the plates are flooded with iodine and kept for 30 seconds. After that , the excess iodine is poured off.

Hydrogen Sulphide Utilization:-

Principle – This experiment marks the utilization of H2S by the test organism – Azotobacter ,in this case. H2S is produced by test organism through reduction of sodium thiosulphate, added in the medium. This H2S is detected by incorporating Ferrous ion in the medium.H2S, a colourless gas reacts with metal salt (Ferrous sulphate) forming visible insoluble black ferrous sulphide precipitate.

Procedure – Preparation of SIM medium as per the composition – Peptone – 1.2g , Beef extract - 0.18g , Ferrous ammonium sulphate – 0.012g , Sodium thiosulphate -0.012g and Agar are dissolved in 60ml distilled water. Autoclaving is then done and after cooling the medium to 45°c, it is poured into test tubes .The medium is further cooled to room temperature and in the Laminar Air Flow inoculation is carried out. The tubes are then kept for 48hrs. Incubation.

Glucose Fermentation:-

Principle – This experiment marks the utilization of glucose by test organism. As a result of fermentation, acid and gas are produced by the organism. Bromo-cresol purple indicator added to glucose broth, marks the acid production by turning the colour of the broth to yellow from purple colour. Gas production is marked by bubble in Durham's tube, that is inserted in glucose broth.

Procedure – Preparation of Glucose broth as per the composition – Beef extract – 3g , Tryptone – 5g and Glucose – 5g to 1000ml distilled water.1ml indicator is then added to the broth. The broth (6ml) is then distributed to test tubes and Durham's tubes are placed inverted in all the test tubes, such that no bubble remains in the Durham's tubes before inoculation. The tubes are then autoclaved and after cooling the tubes to room temperature, inoculation is done in Laminar Air Flow. Incubation is carried out and observation is done after 24hrs.

Urease Test:-

Principle – Urease is a hydrolytic enzyme which attacks the carbon and nitrogen bond amide compounds like urea with the liberation of ammonia, which ultimately rises the ph of media by forming ammonium carbonate. This alkalinity is then marked by phenol-red indicator as it turns the colour of the media from yellow to pink-red.

Reaction -

H_2N- C=O-H_2N + H_2O —Urease→ $2NH_3$ + CO_2

Urea Hydrolysis

Procedure – Preparation of Urea broth medium as per the composition – peptone-1g ,NaCl -5g and KH_2PO_4 -2g dissolved in 1000ml distilled water. The ingredients are dissolved by heating and then autoclaving is done. After autoclaving , the flask is cooled to 50^oc and then 1g glucose with 6ml phenol red indicator(0.2 %) solution is added to the molten base , steamed for 1hr and cooled to 50^oc.Then , 100ml urea (20% aqueous solution) is added to the main media. Then filtration is done in Laminar Air Flow . Then, the media is distributed to test tubes and inoculated after sufficient cooling and incubated for 48hrs.

Preparation of Starter Cultures of Azotobacter:-

After screening of the Nitrogen-fixing bacteria Azotobacter, starter cultures of Azotobacter are prepared in Production medium. The Production medium used here is Ashby's broth. This medium is chosen as the Production medium because viable cells are found profusely in this liquid medium after fermentation.

For this part of the experiment, one 100ml flask is taken and Ashby's medium is prepared. After checking the Ph , autoclaving is done. After autoclaving, the flask is taken to the Laminar Air Flow. The flask is then inoculated with the selected colonies from Ashby's plate. The flask is then kept in B.O.D Incubator for 7 days .The viable count in the Production medium or Ashby's broth is found to be 10^9 cfu/ml. As this is the Starter culture ,inoculum from this is transferred to larger flasks for large scale production of Azotobacter.

Large scale production of Azotobacter Biofertilizer:-

A 1000ml flask is taken and Ashby's broth is prepared on a large scale**. I**noculum from the Starter culture is taken and the broth is inoculated under aseptic conditions . The broth is then kept for incubation, for 7 days till it is 10^9cfu/ml. The flask is kept for continuous agitation and aeration for one week. The flask is observed from time to time for cell growth and care is taken to avoid contamination. Then the flask is kept in cool conditions.

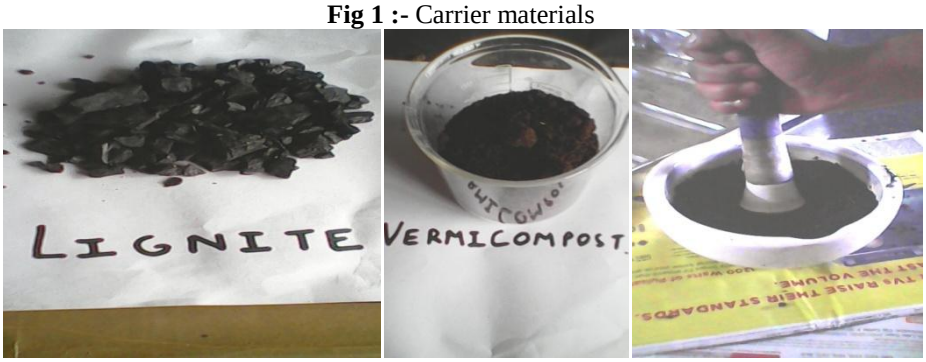
Carrier Material:-

A good carrier material possess the following properties- 1. It should have high organic matter content. 2. Water holding capacity of more than 50% 3. No toxic chemicals 4. Easy to process ,friability and vulnerability.

Here, the carrier material used are charcoal, cowdung compost and vermicompost.

As for the process, lignite is first grinded by mortar and pestle and then by mixer and grinder ,to form a powder. To this 1% CaCO3 and activated charcoal was mixed to avoid contamination. Cowdung is then crushed and powdered with the help of mixer and grinder. Then, Vermicompost is also added as carrier material. Lignite, vermicompost and cowdung is mixed in the ratio of 1:2:1. Then the carrier mixture is autoclaved in glass beakers.

Fig 1 :- Carrier materials



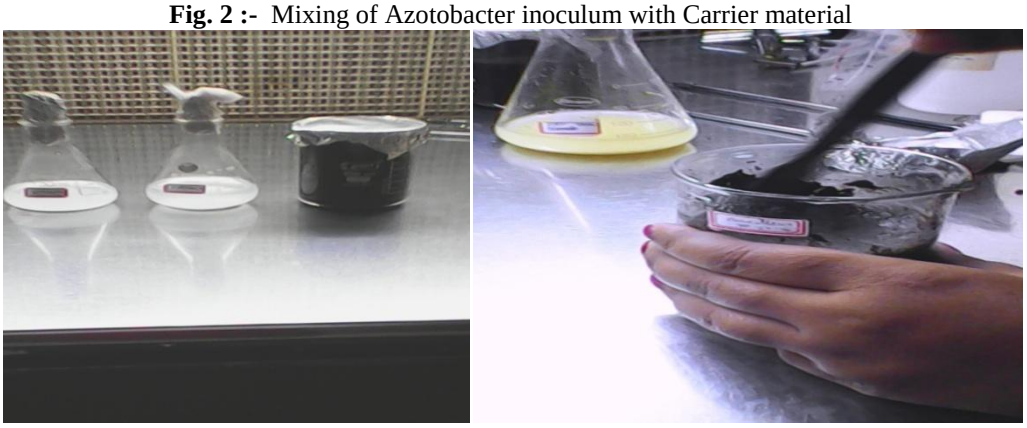
Lignite (1 part) Vermicompost (2 parts) Cowdung (1part)

Mixing Carrier material with inoculums:-

The cell culture is taken out from the refrigerator and allowed to be at room temperature. Meanwhile, the carrier materials are taken out from the autoclave and is kept in Laminar air flow. When the carrier mixture comes to room temperature, 10ml of distilled water is poured in the beaker to make it moist. Then Production medium is added to make the mixture more than 60% moist. After thorough mixing of inoculum with the carrier mixture, a thin layer of liquid medium is created above the carrier mixture to allow the Azotobacter to grow rapidly.Then the mixture in beaker is kept for overnight incubation at room temperature. Next day morning the mixture is mixed with 200gms of soil , such that the soil is just wet. This biofertilizer is left for curing in shade for about 7days.

As per the second method, the autoclaved carrier material is thoroughly mixed with the Azotobacter inoculum until the mixture is 60% moist . This biofertilizer is filled in packets and left for curing for 7days.

Fig. 2 :- Mixing of Azotobacter inoculum with Carrier material



Application of Biofertilizer:-

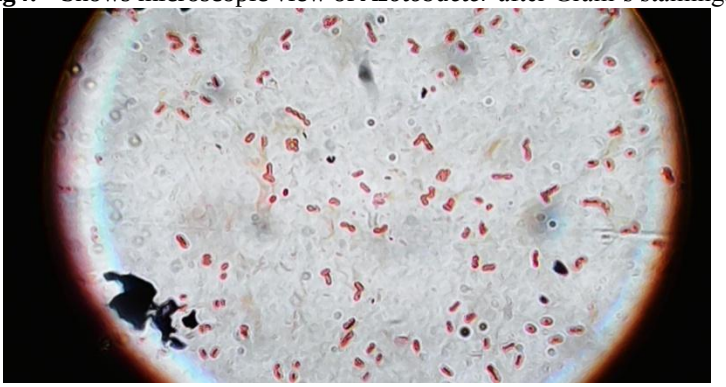
100g of biofertilizer is thoroughly mixed with 7kgs of garden soil. This mixture is done in a large tank. Here the seeds of brinjal and mustard are sown and further studies are carried out.

Result and Discussion:-

Fig3: Shows the culture plate of *Azotobacter*.

Flat ,soft, milky mucoid colonies of *Azotobacter* can be seen

Fig4:- Shows microscopic view of *Azotobacter* after Gram's staining**.**



Gram negative,fluffy rods of Azotobacter can be seen.

Azotobacter being a free-living Nitrogen-fixing bacteria, fixes atmospheric nitrogen to the roots of the plant.It also breaks down the nitrates present in the soil so that they can be easily taken up by the roots of wheat crop.It is also a phosphate-solubilizing bacteria.

Hence the addition of Azotobacter biofertilizer will definitely give a very good yield to crops.

Table 2:- Results of Biochemical Tests for *Azotobacter*

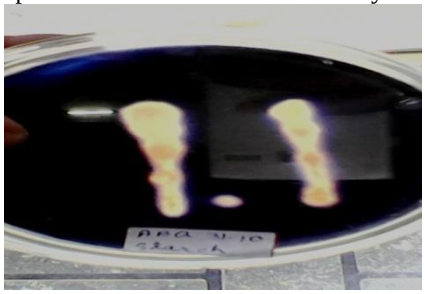
Sl.no	Tests	Results
1.	Citrate Utilization	Positive
2.	Starch Hydrolysis	Positive
3.	H_2S Utilization	Positive
4.	Glucose Fermentation	Positive
5.	Urease Test	Positive
6.	Mannitol Fermentation	Positive

Some strains have given negative results.

Fig. 5:- Response of Azotobacter to Citrate Utilization Test

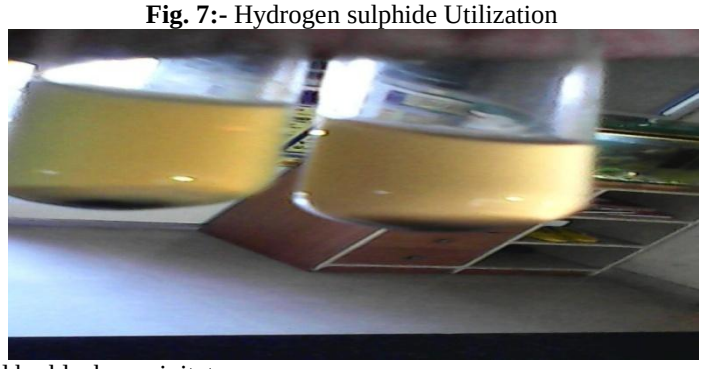
The left hand side tube shows positive result, marked by blue colouration.

Fig.6:- Response of Azotobacter to Starch Hydrolysis Test.



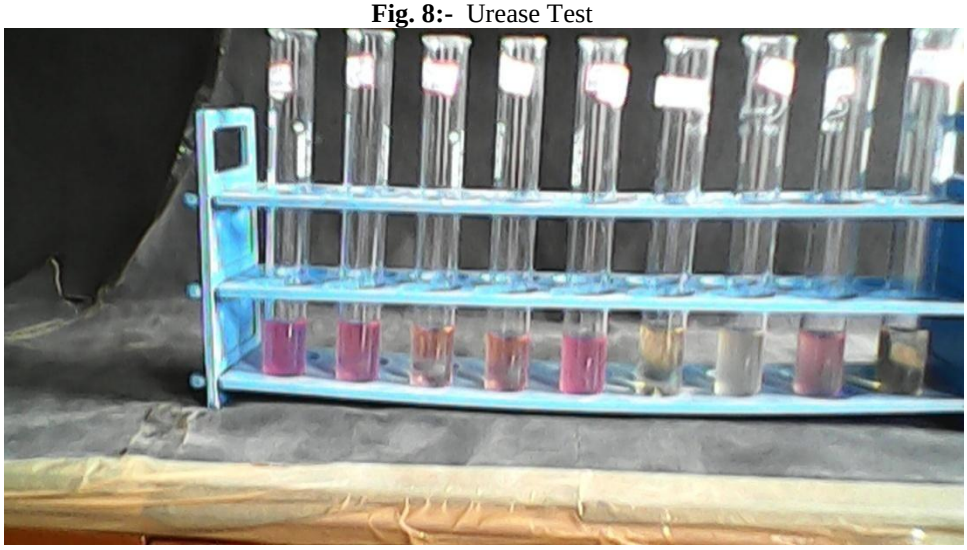
After flooding the plates with iodine,clear zone can be seen around the single-streaked inoculum.

Fig. 7:- Hydrogen sulphide Utilization



H_2S Utilization is marked by black precipitate.

Fig. 8:- Urease Test



Pink colouration in Urea broth indicates presence of Azotobacter.

Conclusion:-

After performing several Biochemical Tests, the presence of Azotobacter can be concluded. As this bacteria is having enormous potential as a biofertilizer, its application to crop fields such as wheat , is a very important piece of study.

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