“ISOLATION AND CHARACTERISATION OF NON-SYMBIOTIC NITROGEN FIXING BACTERIAL ISOLATE FROM PADDY SOIL”

A PROJECT REPORT

BY

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LABORATORYCERTIFICATE

This is to certify that **Miss. Shrutika Umesh Ayare** has satisfactorily carried out therequired project work prescribed by theVivekanand College, Kolhapur. for B.Sc. Part III (SemesterVI) Microbiology courseand herprojectwork entitled **"ISOLATION AND CHARACTERISATION OF NON-SYMBIOTICNITROGEN FIXINGBACTERIALISOLATE FROM PADDY SOIL"** represents her bonafide work in the B. Sc. Part-III (Semester VI) oftheacademicyear 2021-2022.

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Miss:ShrutikaUmeshAyare

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CHAPTER - 1

INTRODUCTION

INTRODUCTION

Soil is the outermost covering of earth which consists of earth which consists of loosely arranged materials composed of inorganic and organic constituents in different stages of organization. It is natural medium in which plant live and multiply and die and thus providing a perennial source of organic matter which could be recycled for plant nutrition. Physical property of soil type depends on in it these are known as gravels, slit and clay. Soil is medium for plants because of its chemical properties contain nutrients like C, H, O, N, P (most important basic component of cells). S, K, Ca, Mg, Fe, Mn, Zn, Cu, Mo, B, Cl etc. (Subbarao N.S. 1999)

The soil is composed of five major components, mineral matter, water, air, organic matter and living organisms. The quality of these constituents is not the same in all soils but varies with the locality of the inanimate portion the amount of mineral and organic matter is relatively fixed at single site, the proportion of air and water however fluctuates. The living portion of the soil body including various small animals and microorganisms makes up appreciably less than 1% of the total volume, yet it is undoubtedly essential for crop production and soil fertility. (Kumbhar, 2001).

Microbes can be considered as engineers of soils (Rajendhran and Gunasekaran, 2008) and many ecosystem services that are linked to terrestrial ecosystems, including plant production, safeguarding of drinking water or carbon sequestration, are closely linked to microbial activities and their functional traits (Torsvik and Ovreas, 2002). The soil matrix and physco-chemical properties of soil are the most crucial factor which determines the structure of microbial community (Lombard et al., 2011). In addition to that, the diversity of soil microbes is huge and can still be considered as a black box (Simon and Daniel,

2011). The beneficial microbes are fascinating, versatile and capable of growing on a wide range of substrates and carry out extremely useful processes that cannot be achieved by other physical and chemical means. Moreover, microorganisms are able to degrade many substances to a remarkable spectrum of products that support the growth of living organisms and fulfil numerous human requirements. These all are not a result of a single organism but of microbial communities which closely interact with each other (Aneja et al., 2006).

Nitrogen is a critical limiting element for plant growth and production. Nitrogen (N_2) is the most important element in all form of living things since many organic compounds which have an essential role in the structural and functional processes of life is derived from nitrogen. It is a major component of chlorophyll, the most important pigment needed for photosynthesis, as well as amino acids, the key building blocks of proteins. It is also found in other important biomolecules, such as ATP and nucleic acids. The process of nitrogen fixation is dependent on certain parameters like moisture conditions, amount of oxygen, the supply of organic substrates, and genotypes of microorganisms and plant (Church et al., 2008). Even though it is one of the most abundant elements (predominately in the form of nitrogen gas (N_2) in the Earth's atmosphere), plants can only utilize reduced forms of this element. Plants acquire these forms of "combined" nitrogen by: 1) the addition of ammonia and/or nitrate fertilizer (from the Haber-Bosch process) or manure to soil, 2) the release of these compounds during organic matter decomposition, 3) the conversion of atmospheric nitrogen into the compounds by natural processes, such as lightning, and 4) biological nitrogen fixation (Vance 2001).

Biological nitrogen fixation (BNF), discovered by Beijerinck in 1901 (Beijerinck 1901), is carried out by a specialized group of prokaryotes. These organisms utilize the enzyme nitrogenase to catalyze the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). Plants can readily assimilate

NH_3 to produce the aforementioned nitrogenous biomolecules. These prokaryotes include aquatic organisms, such as cyanobacteria, free-living soil bacteria, such as *Azotobacter*, bacteria that form associative relationships with plants, such as *Azospirillum*, and most importantly, bacteria, such as *Rhizobium* and *Bradyrhizobium*, that form symbioses with legumes and other plants (Postgate 1982).

There is, however, a high heterogeneity of its distribution throughout the world: some areas subjected to pollution whereas others to depleted soil, decreased crop production, and other consequences of inadequate supply. Nitrogen enters living organism via nitrogen fixation (Egamberdieva and Kucharova, 2008; Berman-Frank et al., 2003). This is accompanied by microbial processes, these microbes may be symbiotic or free-living in nature (Reghuvaran et al., 2012). Nitrogen fixing organisms are generally active in plant root zone soil. Plants that are capable of releasing exudates exhibit higher nitrogen fixation activity in soil (Egamberdieva and Kucharova, 2008). Nitrogen-fixing free-living microorganisms have frequently been reported as plant growth promoters (Requena et al., 1997; González-López et al., 2005). Biological nitrogen fixation can be an important source of nitrogen for supporting aquatic primary productivity (Affourtit et al., 2001). Nitrogen is one of the most essential elements for all forms of life; a basic material for synthesizing proteins, nucleic acids, and other organic nitrogenous compounds. Unfortunately, no plant species are able to reduce atmospheric dinitrogen into ammonia and use it directly for its growth. It appears that only a number of prokaryotic microorganisms including bacteria and cyanobacteria have been shown to possess the ability to fix dinitrogen (Nghia and Gyurján, 1987). Bacteria known collectively as the ‘‘Rhizobia’’ are famous for their ability to induce nodules on the roots (and occasionally, stems) of legume plants. Within these nodules, the differentiated, ‘‘bacteroid’’ forms fix atmospheric nitrogen and the resultant ammonia being used as a source of fixed

nitrogen. This symbiosis provides the bacteria with an exclusive niche and, in return, the plants obtain a personalized nitrogen source (Johnston et al., 2007). They occur in the so-called free-living forms, for example, aerobic Azotobacter, anaerobic Clostridia or in symbiosis with certain higher plants, for example, Rhizobia with legumes or Azolla Anabaena Azollae with Azolla. The potential for biological nitrogen fixation is increased greatly by the fact that there is a close relationship between plants and nitrogen Prokaryotes. Nitrogen-fixing prokaryotes are able to make ranges of useful associations with plants: from loose associations to intercellular symbioses. There exist associative symbioses in which nitrogen-fixing prokaryotes (for example, *Azospirillum, Azotobacter, Enterobacter* species) have been found to occur in rhizosphere of different plants such as sugarcane, maize, wheat, rice, grasses and others (Affourtit et al., 2001). The activity of nitrogen-fixing microorganisms depends greatly upon excessive amount of carbon compounds and adequately low level of combined nitrogen (Johnston et al., 2007). Although synthetic fertilizers give short sustain high yield product, it causes long-term negative impact on the farmlands (Nghia and Gyurján, 1987). In Ethiopia, most of the farmers use chemical fertilizers since they have limited knowledge about the role of nitrogen-fixing bacteria. Moreover, these microbes were not explored and their inoculants were not found in the market. Therefore, a research project has been initiated with the objectives of isolation and characterization of nitrogen-fixing bacteria native to Ethiopia from lab soil and root nodules.

The genetic determinants for nitrogenase:-

The genetic determinants for nitrogen fixation in both symbiotic and free-living system can be divided into those which specify the structure and regulation of the enzyme nitrogenase (nif gene) and those which govern the phenotypic expressions of nif genes. In *Klebsiella pneumoniae* it has been established

(through transduction and conjugation) that the nif operon is located on the chromosomes close to the genes for histidine biosynthesis.

Nitrogen fixing bacteria -

Nitrogen cycle in the atmosphere the concentration of nitrogen is about 78% it is essential for plants. Nitrogen forms a structural component of many important molecule such as DNA, RNA and other vitamins. Plants cannot absorb nitrogen from atmosphere certain forms of bacteria are able to convert atmospheric nitrogen into usable forms such as nitrate and nitrites such nitrogen fixing bacteria are commonly found in the roots of legumes inside special structure called root nodule. These usable forms of nitrogen are absorbed by plants to produce many compounds such as amino acid, which in turn form protein. When an animal feed on plants, nitrogen enters its body when plants and animal die. they start decomposing after sometime during this process protein are converted into nitrate and nitrites by the action of decomposing bacteria certain other forms of bacteria convert nitrates and nitrites into elemental nitrogen. Thus, nitrogen flows between the various components of the biosphere in a cyclic manner.

Animal secure nitrogen and all other compound from plants or animal on which they fed. Microorganisms have a central role in almost all aspect of this nitrogen availability and thus life support on earth Nitrogen s required in large amount as an essential component of nucleic acid. Protein and other cellular constituents. Biologically nitrogen fixing is significant process of entry of nitrogen into an ecosystem and many symbiotic microbial species play a major role in this. In an aquatic ecosystem the major region where this activity occurs is benthic and litoral zone (Oreland RS.1990). The nitrogen fixing bacteria can divided into 2 groups

A. The free-living bacteria:

The free-living bacteria having the ability to fix molecular nitrogen can be distinguished into-

1. Obligate aerobic-:

Azotobacter, Acromobacter, Arthrobacter, Mycobacterium etc

2. Facultative anaerobic-:

Clostridium, Chromatium, Rhodomicrobium, Desulfovibria, Rhodospirillum Rhodopseudomonas etc. *Rhodopeudomonas* fixes atmospheric nitrogen by photosynthetic process while *Desulfovibrio* fixes nitrogen by reducing sulphate.

B. Symbiotic nitrogen fixers:

Rhizobium associated with leguminous plants and spirillum lipoferum associated with cereal grass. The symbiotic nitrogen fixing bacteria invade the root hairs of host plants. where they multiply and stimulate formation of root nodules, enlargement of plant cells and bacteria in intimate association. Within the nodules the bacteria convert free nitrogen to nitrates which the host plant utilizes for its development. To insure sufficient nodule formation and optimum growth of legumes. e.g., soyabean seeds are usually inoculated with commercial culture of appropriate *Rhizobium spp* especially in soil poor or lacking in the required bacteria see also nitrogen cycle.

Nitrogen fixation method-:

1. During lightning and thunder, the higher temperature and pressure in the air convert atmospheric nitrogen into oxides of nitrogen that can dissolve in water to produce nitric acid and nitrous acid these fall along with rain.

2. Certain forms of bacteria are able to convert atmospheric nitrogen into usable forms such as nitrates and nitrites, such nitrogen fixing bacteria are commonly found in the roots of legumes [plant and pules] inside special structure called root nodules.

The enzyme responsible for atmospheric nitrogen fixation is nitrogenase This nitrogenase has been isolated from genera *Clostridium*, *Bacillus, Klebsiella* etc. The enzyme contains the two protein fraction Fe, MO containing protein (M w 2,20,000-2,70.000d and the Fe containing protein M wt. 55,000- 66,800d]

Mechanism of N_2 fixation:

Nitrogenation reaction has 2 steps

1. Electron activation by suitable donor [ADP]

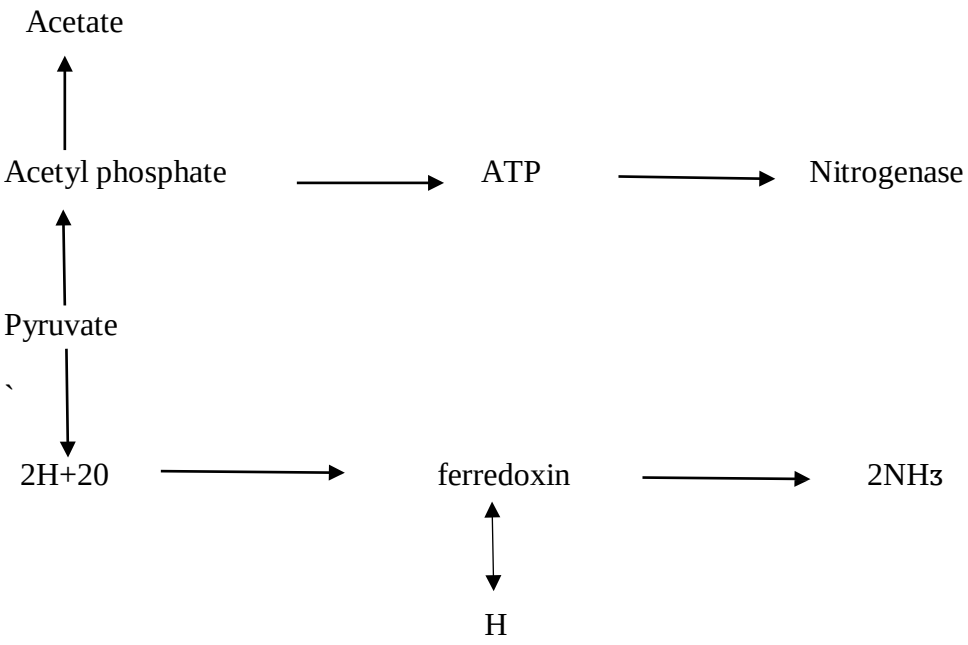
2 Substrate reduction.

These reaction takes place at different sites on the N_2 molecule but are independent. Purified preparation of nitrogenase is highly sensitive to O_2, especially the Fe protein part of enzyme. Energy requirements for nitrogenation reaction comes from the cellular metabolic cycles in the form of ATP. Pyruvate functions both as an e^- donor and as energy source in the phosphoroclastic reaction, pyruvate forms acetyl phosphate which in the presence of adenosine diphosphate gives rise to ATP. The reductants are the strongly reducing naturally occurring electron carrier proteins ferredoxin and flavodoxin, dithionate and certain dyes can also serve as artificial extra cellular sources of electron donors since all nitrogen fixing microorganisms contain hydrogenase, this enzyme

system in cells catalyse the transfer of e from pyruvate or H_2 to ferredoxin or flavodoxin.

Ferredoxins are naturally occurring iron-sulphur electron carrier protein capable of undergoing reversible oxidation and reduction. They have been isolated from plants blue-green algae and bacteria such as *Clostridium, Azotobacter Rhizobium, Desulfovibrio spp.* Ferredoxins are differ in molecular weight, iron and sulphite contents.

Flavodoxin is a flavoprotein the role of pyruvate and ferredoxin in nitrogenase reaction can be illustrated as follows,



Nitrogenase in addition to reducing atmospheric N_2 to NH_3 can also reduce certain other compounds.

According to Hardy et al. The active site of the enzyme for substrate reduction is believed to be composed of an Mo-Fe dinuclear site bridged by sulphur, having the proper size and electron characteristics to provide Mo-Fe

distance of about $3.8A^0$ this distance is specific so as to accommodate various nitrogenase substrates including N_2 and to exclude other.

The first reaction in nitrogen reduction is formation of a linear complex of N_2 with the Fe of nitrogenase this is followed by transfer of e^- from Mo which is end point of the electrons activating system, resulting in the formation of diamide which is stabilized by hydrogen bonding from the protein as well as the metal nitrogen bonds: Successive addition of e^- produce hydrazine followed by cleavage of NN bond to yield 2 moles of NH_3. The increase in the NN bond length during reduction is accompanied by compensating changes in MNN angle so that Mo-Fe distance remains constant [Subba Rao NS1982].

CHAPTER 2

AIMS AND OBJECTIVES

AIM AND OBJECTIVES

Aim and objectives of our present research work as follows-

Aim- To carry out characterization of the nonsymbiotic nitrogen fixing bacterial isolates from rhizospheric soil.

Objectives-

The objective of present study would be:

1.Isolation of nonsymbiotic nitrogen fixing bacteria from soil sample.

2. Morphological, Biochemical and Physical characteristics of obtained isolates.

3. Enzymatic and salt tolerance studies of isolates.

CHAPTER 3

MATERIAL AND METHODS

MATERIALS AND METHODS

Cultivation of Nitrogen fixers -

Nonsymbiotic nitrogen fixers from paddy field of Saidapur, Enumeration of non- symbiotic N_2 fixing bacteria was carried out by soil dilution plate technique, were cultivated on modified Ashby's nitrogen free mannitol medium. Four nitrogen fixing bacteria were used for the present study. That was cultivated at 30°C for 48-72 hours. The cultures were preserved on slope of same medium used for cultivation and maintained under refrigeration temperature. Fresh transfer was given after every month. Study on colonial characteristics and morphological characters of isolates were done.

Isolates were studied for its colony characters, Gram's nature by Hackers and Conn (1923) modified Gram's staining method and motility by hanging drop method. Isolates were subjected for microscopic observations of spore and capsule formation standard procedures.

Studies of biochemical characteristics of the isolates -

Purified bacterial isolates were studied for their different characteristics. The biochemical characterisation was performed in Ashby's nitrogen free mannitol broth with pH7.0.

Studies on enzymatic activities of isolates –

1.Catalase test-

Growth of isolates from Ashby's nitrogen free agar plates were picked with sterile nichrome wire loop and dipped in 10% H_2O_2 solution Evolution of gas bubble was observed as positive reaction.

2.Oxidase test

Growth of isolates were transferred by a sterile glass rod on to a Whatman's filter paper strip moistened with freshly prepared 1% aqueous solution of oxidase reagent (N, N, N, N'tetramethyl P- phenylene diamine dihydrochloride) and observed for appearance of purple colour on strip within 10-15 seconds.

3.Starch hydrolysis test-

A loopful culture of each isolate were spot inoculated on sterile Ashby's media containing starch. Plates were incubated at 37 °C for 48-72 hours. Ability to hydrolyse starch was detected by flooding iodine solution on medium plates.

4.Carbohydrate utilization test-

A loopful of each isolate were inoculated in Ashby's nitrogen free mannitol broth with pH10.5, NaCl 1% with phenolphthalein as an indicator and 1% solution of carbohydrates glucose, mannitol, sucrose, fructose, maltose, lactose, xylose. A control was also run during each experiment. Tubes were incubated at 37°C for 48-72 hours The tubes were observed for acid and gas production indicated by change in colour of medium and bubble formation in Durham's tube.

5.Nitrate reduction test -

A loopful of each isolate were inoculated in tube containing 10 ml of sterile peptone nitrate broth with pH 10.5. Tubes were incubated at 37°C for 48 hours.

The reduction of nitrate to nitrite was detected by adding few drops of sulphanilic acid followed by naphthyl amine. Development of red colour indicates positive test.

6. Casein hydrolysis test-

A loopful of each isolate were spot inoculated on milk agar medium with pH 10.5. The plates were incubated overnight at 37°C and observed for zone of hydrolysis around growth.

7. Gelatinase activity -

Nutrient agar containing 0.4% gelatine was sterilized and plates were prepared. They were then spot inoculated with each isolate and incubated at room temperature for 48 hours. After incubation the ability of the strains to hydrolyse concentration gelatine was detected by flooding the plates with gelatine precipitating Frazier's reagent.

8. IMVIC Test -

a) Indol Test:

A loopful suspension of each isolate was inoculated in sterile peptone broth and incubated at room temperature for 24 hours. After incubation 3-4 drops of xylene was added in broth and shaked after that 1 ml of Kovac's reagent was added and formation of pink coloured ring was taken as positive test.

b) Methyl red Test:

A loopful of suspension of each isolate was inoculated in sterile glucose phosphate broth and at room temperature for 24 hours after incubation methyl red indicator was added formation of red colour was taken as positive test.

e) Vogus Proskauer Test

A loopful suspension of each isolate was inoculated in sterile glucose phosphate broth and incubated at room temperature for 24 hours after incubation 40% KOH and alpha naphthol was added formation of red color was taken as positive test.

d) Citrate utilization Test:

Test culture was inoculated in sterile Koser's citrate broth and incubated at room temperature for 24 hours. After incubation turbidity was taken as positive Citrate utilizationtest.

9. Urea Hydrolysis Test-

Sterile slants of Christensen's urea agar were inoculated with test cultures and were incubated at room temperature for 48 hours. After incubation change in colour from orange to magenta red indicated a positive test.

Studies on physiological characteristics of isolate-

A. Effect of temperatures on growth of isolates -

A loopful of suspension of each isolates were inoculated in Ashby's nitrogen free mannitol broth and incubated at different temperature 0°C, 9°C,18 °C, 32 °C,38 °C. After incubation turbidity of each broth tube were checked.

B. Effect of NaCl concentrations on growth of isolates -

A loopful of suspension of each isolates were inoculated in separate Ashby's nitrogen free mannitol broth with different concentrations of salts 0.5%, 1%, 2.5%, 3%, 3.5%. After incubation the turbidity of each broth tube were checked.

C. Effect of pH on growth of isolate –

A loopful of suspension of each isolate were inoculated in separate Ashby's nitrogen free mannitol broth tubes with different pH concentrations 5, 6.5, 8, 9,10. After incubation the turbidity of each broth tube were checked.

CHAPTER 4

RESULTS AND DISCUSSION

RESULT AND DISCUSSION

1) Collection of sample :

Photograph 1- Sample collection

2) Isolation of non-symbiotic nitrogen fixing bacteria :

Nitrogen fixing bacteria were isolated by Ashbey's nitrogen free mannitol agar. Four different colonies were selected for further studies. These were I-1, I-2, I-3, I-4.

RESULTS AND DISCUSSION

The total four Isolates were cultivated on modified Ashby's nitrogen free mannitol agar. Isolates were obtained from the laboratory of Yashwantrao Chavan College of Science, Karad. The isolates were appropriately coded as I1, I2, I3, I4.

Results and colony characteristics of isolates.

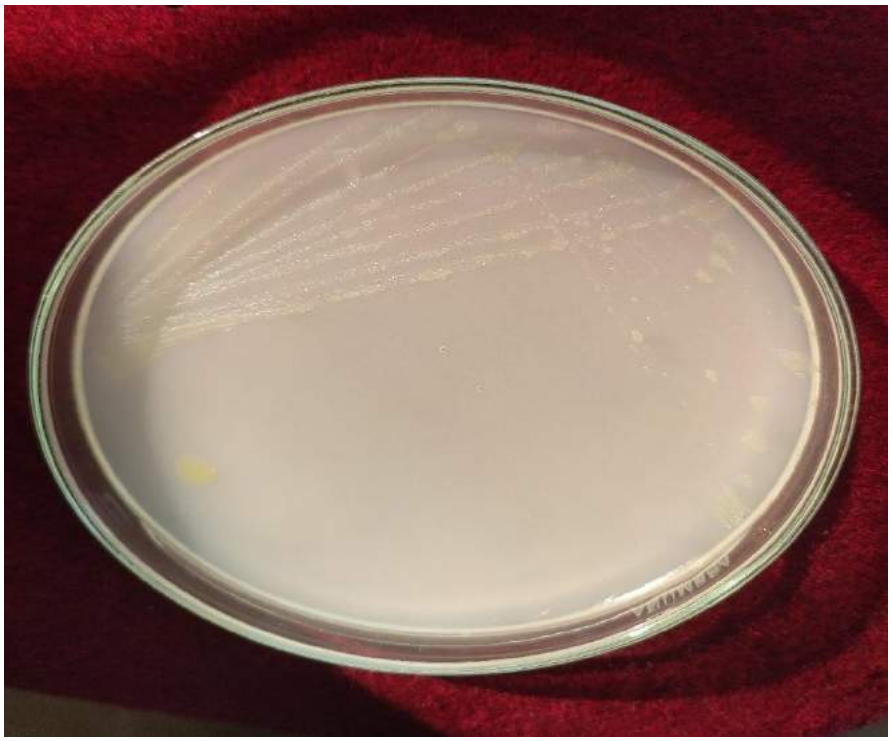
Colony Characters of isolates were studied and showed in table no.

Table No.1 Colony Characteristics of the isolates on Ashby's N_2 free mannitol agar medium.

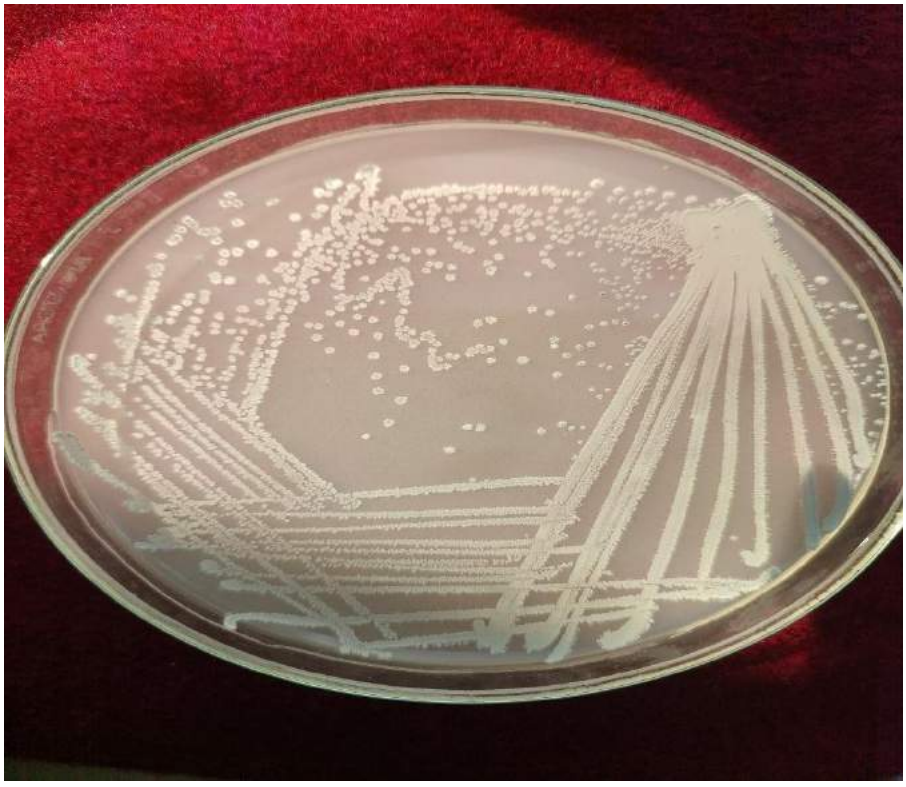
Isolate code	Size	Shape	Color	Margin	elevation	Opacity	consistency
I1	4mm	Regular	White	Entire	Convex	Opaque	Mucoid
I2	3mm	Regular	Colorless	Entire	Convex	Transparent	Mucoid
I3	1mm	Regular	Colorless	Entire	Convex	Mucoid	Translucent
I4	5mm	Irregular	Green	Filamentous	Raised	Opaque	Powdery

Photograph 2- Colony morphology of I-1 grown on Ashby's N_2 free mannitol agar medium.

Photograph 3- Colony morphology of I-2 grown on Ashby's N_2 free mannitol agar medium



Photograph 4- Colony morphology of I-3 grown on Ashby's N_2 free mannitol agar medium



Photograph 5 - Colony morphology of I-4 grown on Ashby's N_2 free mannitol agar medium

Results of morphological characteristics and microscopic observations of the isolates.

Table No.2 Results of morphological characteristics and microscopic observations of the isolates.

Isolate code	Gram nature & morphology	Motility
I1	Gram negative short rod	Non-motile
I2	Gram negative cocci	Non-motile
I3	Gram negative short rod	Non-motile
I4	Gram positive filamentous	Non-motile

Results of microscopic observation of isolates.

Some microscopic observations were studied and shown in table no. 3

Table no.3 microscopic observation of isolates.

Isolate code	Endospore staining	Capsule staining
I1	-	+
I2	-	+
I3	-	+
I4	-	-

Results of enzymatic activities of isolates.

The isolates were studied for various enzymatic activities and result were shown in table no.4.

Table no.4 enzymatic activities of isolates.

Isolate code	Catalase	Oxidase	Starch hydrolysis	Nitrate Reduction	Gelatinase	Caseinase	Urease
I1	+	+	+	+	-	-	-
I2	+	-	-	+	+	-	-
I3	+	+	+	+	+	-	-
I4	+	-	+	+	-	-	-

It was found that all four isolates were found to be catalase, nitrate reduction positive and caseinase, urease negative. only two isolates were oxidase, starch hydrolysis, gelatinase positive.

Results of various carbohydrates utilization of isolates.

The isolates were studied for their ability to utilize carbohydrates and result were shown in table no.5.

Table no.5 carbohydrates utilization of isolates.

Sugar tested	Isolates			
	I1	I2	I3	I4
Glucose	-	AG	-	-
Lactose	-	AG	-	-
Sucrose	-	AG	-	-
Sorbitol	-	AG	-	-
Maltose	-	AG	-	-
Xylose	-	AG	-	-
Mannitol	-	AG	-	-

KEY –

A – Acid

AG – Acid and gas

\- – negative test

It was found that only I2 was found to be ferment all sugars with acid and gas production. I1, I3, I4 did not ferment any sugar.

Results of effect of various temperatures on growth of isolates

The effect of different temperatures on growth of isolate were studied and results were shown in table no 6.

Table No.6-Effect of temperatures on growth of Isolates.

Isolates	Different temperature				
	9°C	18°C	32°C	0°C	38°C
I1	-	-	+	-	-
I2	-	-	+	-	-
I3	-	-	+	-	-
I4	-	+	+	-	-

Results of effect of various salt concentration on growth of isolates.

The effect of different salt conc. on growth of isolate were studied and results were shown in table no 7.

Table No.7-Effect of salt conc. on growth of Isolate

Isolates	Salt concentrations				
	0.5%	1%	2.5%	3%	3.5%
I1	+	+	+	-	+
I2	+	+	+	-	+
I3	+	+	+	+	+
I4	-	+	-	-	+

Results of effect of various pH on growth of isolates.

The effect of different pH on growth of isolates were studied and results were shown in table no 8.

Table No.8-Effect of pH on growth of Isolates.

Isolates	pH				
	5	6.5	8	9	10
I1	-	+	+	+	+
I2	+	+	-	-	-
I3	+	+	+	+	+
I4	+	+	+	+	+

CHAPTER 5

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Nitrogen is an important element for the growth of the plants. plants can only utilize reduced forms of this element.

In the course of nature, microorganisms like bacteria and fungi have been seen to fix nitrogen available in the form of nitrogen gas (N_2), into a form which can be used by plants. However less information is available on nitrogen fixing bacteria and their impact on growth and development of crop plants.

In this context attempts were made to isolate different N_2 fixing bacteria from paddy field soil sample. For the isolation Ashbey's medium was used. On this medium typical N_2 fixing bacteria were obtained. Total four isolates were obtained and they were characterized appropriately.

The problem of deficiency of N_2 fixation in soil can be solved by using fertilizers containing of N_2 fixing bacteria. These microorganisms fix the nitrogen which is released into the soil, where it can be easily absorbed by the plant roots. This allows the plant to take up nitrogen more efficiently, leading to improved growth, development, and overall productivity.

CHAPTER 7

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CHAPTER 6

APPENDIX

APPENDIX

A. List of Media Used:-

1. Nitrogen free mannitol agar (Atlas, 1983):

Mannitol	10 gm
K_2HPO_4	1.0 gm
NaCl	0.2 gm
$MgSO_4.7H_2O$	0.5 gm
$CaCO_3$	5.0 gm
Ferric chloride	0.005 gm
$MnSO_4$	0.005 gm
Agar-Agar	20 gm

(pH adjusted to 7.2)

Preparation of medium: -

All components in proportion to 1000 ml were added to distilled water in flask and final volume was brought to1000 ml. pH was adjusted and medium was autoclave sterilized at 121°C for 15 min.

2. Starch Agar (Atlas, 1983):-

Starch	1gm
K_2HPO_4	0.2gm
$MgSO_4$	0.2gm
K_2SO_4	0.1gm
NaCl	4gm

$CaCO_3$	0.5gm
Agar-Agar	3gm
Distilled water	100 ml
Final pH	7.2

Preparation of medium: -

All components in proportion to 100 ml were added to distilled water in flask and final volume was brought to100 ml. PH was adjusted and medium was autoclave sterilized at 121^0c for 15 min.

3.Gelatin agar:

Gelatin	4 gm
Glucose	0.05gm
KH2PO4	0.5gm
K2HPO4	1.5gm
Nutrient agar	1000ml (melted)

Preparation of medium: -

All components in proportion to 100 ml were added to distilled water in flask and final volume was brought to100 ml. PH was adjusted and medium was autoclave sterilized at 121^0c for 15 min.

5.Nutrient agar

Peptone	10gm
NaCL	5.0gm
Meat extract	3.0gm
Agar – agar	25.0gm

Distilled water	1000ml
pH (7.2)	

Preparation of medium: -

All components in proportion to 100 ml were added to distilled water in flask and final volume was brought to100 ml. PH was adjusted and medium was autoclave sterilized at 121^0c for 15 min.

B. Media for Biochemical test –

1. Hugh and Leif sons Medium (Atlas, 1983):

Peptone	0.2 gm
NaCl	0.5 gm
Di potassium Phosphate	0.3 gm
Bromothymol Blue 1%	0.03 ml
Glucose 10%	10 ml
Distilled water	90 ml
Agar-Agar	0.8 gm
Final PH	7.2

Preparation of medium:-

All components in proportion to 100 ml were added to distilled water in flask and final volume was brought to100 ml. PH was adjusted and medium was autoclave sterilized at 121^0c for 15 min.

3. Koser's Citrate Broth (Atlas, 1983)

Sodium Ammonium Phosphate	1.5 gm

Potassium Dihydrogen Phosphate	1gm
Magnesium Sulphate	0.2 gm
Sodium Citrate	3 gm
Distilled Water	1000 ml
Final pH	7.2

Preparation of medium:-

All components in proportion to 100 ml were added to distilled water in flask and final volume was brought to 100 ml. pH was adjusted and medium was autoclave sterilized at 121 ^{0}C for 15 min.

C. Reagents for Biochemical test –

1. Oxidase Reagent (Atlas, 1983):

Tetra methyl paraphenylene diamine hydrochloride	1 gm
Ascorbic acid	0.1 gm
Distilled water	100 ml

Preparation of Reagent: -

Dissolve 1g of Tetra methyl phenylene diamine hydrochloride and 0.1 gm of ascorbic acid in 100 ml distilled water.

2. Catalase Reagent (Atlas, 1983):

Hydrogen peroxide	10%

Preparation of Reagent: -

Dissolve 10 ml of hydrogen peroxide in 90 ml distilled water.

3. Frazier's reagent (Atlas, 1983)

$HgCl_2$	12 gm
Concentrated HCl	20 ml
Distilled water	80 ml

D. Stains preparation:

1. Crystal violet stain (Atlas, 1983):

Solution A:

Crystal violet	2 gm
Ethyl alcohol (95%)	20 ml

Solution B:

Ammonium oxalate	0.8 gm
Distilled water	80 ml

Solution A and B mixed thoroughly to prepare crystal violet stain.

2. Basic fuchsine stains (Atlas, 1983):

Basic Fuchsin	0.5 gm.
Distilled water	1000 ml

3. Gram's Iodine solution (Atlas, 1983):

Potassium Iodide (Kl)	2gm
Iodine crystals	1 gm
Distilled water	300 ml