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Isolation and Identification of Nitrogen fixing Bacteria from Little rann of Kachchh.

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ABSTRACT

The little rann of Kachchh having unique ecosystem of dry and wet land. The present work describes cultivation dependent diversity of bacteria that involved in Nitrogen transformation. 10 sample were collected from different places of little rann of Kachchh. Isolate 04 bacterial strain from the collected sample using different nitrogen free medium.

Bacillus lichaniformis and *Azotobzcter chrococcum* were isolated from collected soil sample using Nitrogen free medium. They are generally Present various environments like alkaline soil, marine sediments, marsh water etc. The Present study was done to isolate strain of *Bacillus lichaniformis* and *Azotobzcter chrococcum* from collected saline soil sample from different location. Relevant biochemical test and 16srRNA were done to identify both species.

INTRODUCTION

Little rann of Kachchh is situated between $22^{O}55$" -$24^{O}35$" N and $70^{O}30$"-7145" E near the white desert of Kachchh, Gujarat. Little rann of Kachchh represented both dry and wet land depending the season of year, wide variation of soil composition and salt deposition may see during year. Major region of the desert consists of 60% clay unlike another desert (Gupta and Ansari 2014)

Little ran of Kachchh is reservoir of Microorganisms ranging from Normal flora to extreme organism which can survive in such harsh environments. Lots of Work done in term of diversity studies in hyper saline system. Arid and Semi-arid areas affected by salt, due to salinisation of arable land, it decrees the fertility. According to a

report of the Indian Agriculture Research Institute, the central government has declared 67 lakhs hectares of alkaline land in country, causing a loss of 56.6 lakh tonnes of agriculture produce (https://allgujaratnews.in/en/gujarat). Many Countries having major challenges of Agriculture lander under saline condition.

The ability to perform various types of transformations, such as the oxidation of nitrogen compounds to nitrate or nitrite, the reduction of oxidised nitrogen compounds to ammonium, or the "fixation" of N2 from the atmosphere into ammonium, is a Unique talent of many types of microorganisms. Over the years, various types of diazotrophic bacteria have been isolated from different soil and plant. *(Dobereiner, 1992)* The nitrogen element is carried up by the plant as ammonium (NH4 +) and nitrate molecules (NO3-). This element is biologically obtained by specific microorganisms called nitrogen-fixing bacteria, or more formally known as diazotroph bacteria. (Cleveland, C *et al.,*1999) In general, nitrogen can only be utilised by most organisms when it is coupled with oxygen, hydrogen, or carbon. The ability to convert atmospheric nitrogen from its abundant gaseous form to a useful form is only possessed by microbes. *(Dobereiner, 1992)* environmental factors like temperature, pH, oxygen, and mineral nutrients are affect the diazotrophic bacteria (BURESH et al. 1980). Presence of salt in soil also affect the growth of Microorganism. Nitrogen fixing bacteria provide an alternative source to chemical nitrogen fertilization in agricultural systems with positive environmental effects and lower costs for agricultural production. (Turner *et al*. 2013).

Diverse species of Bacilli having ability to fix atmospheric Nitrogen. Bacillus species like *B.cerus,B. Circulance, B. firmus, B.pumilus, B.licheniformis, B. megaterium, Bvietnamensis* and *B. aerophilus* are known to fix the atmospheric Nitrogen.(Saxena,A.K. et al.2020)

B.licheniformis and *P.Stuztzeri* involved in the denitrification process of a frequently used aerobic denitrifier. NAR reduction activity and nitrate transport process, also significantly promote by B. licheniformis. B. licheniformis promoted the membrane permeability of aerobic denitrifying through secreting lipopolysaccharide lichenysin. (Jiang,M et al.2022)

The ecological balance is affected by a variety of other elements as well, such as plant type and age, climate, soil type, agricultural practises, and the makeup of the microbial population (Bowen, G D and Rovlra, AD 1976). It could be able to decrease the use of chemical fertilisers which damaging to the environment and halt further deterioration of soil biological productivity, which are essential to the sustainability of agriculture. (Pacovasky et al. 1985; Dobereiner and Pedrosa, 1987). The Azotobacter is known to fixes the nitrogen non-symbiotically, degrades cellulose, phosphates and most importantly it degrades lignin also in trace amounts. Consequently, it is referred to as a multitasking organism. (Rao, N. S. 1999)

Materials and Methods

Study site

Soil sample were collected from different saline region of little run of Kachchh. The soil is Semiarid to dry in nature, which was characterized by lack of moisture and neutral to alkaline pH, which influences the activities of soil microorganisms.

Sample collection

Soil samples were collected from six different sites at different depth 0-10 cm and 20-30 cm during monsoon and post monsoon seasons of current Year. The sample collected into sterile polythene bags were transported to laboratory and stored at 4^0C for further analysis.

Soil analysis

Soil physical and chemical characteristics like soil texture, moisture, WHC, FC were measured while Chemical characters like pH-by-pH meter, Organic Carbon & Total Nitrogen (by Micro-Kjeldahl method)

Isolation medium

NFb medium, LGI and Burk's Nitrogen free medium was used to isolate the diazotrophs from saline soil was done by following methods. 1 gm of soil sample was serially diluted using sterile distilled water up to 10^{-7} dilution. 1 ml of aliquot was inoculated in tubes containing LGI, NFb and Burk's medium. All the tubes were incubated at 35^0C for 48-72 hrs and observed the growth by formation of pellicles. Pellicles were streaked on Petri plates containing N-free medium and incubated all plates at 35^0C for 48-72 hrs. The colonies develop on medium were transferred to slant of same medium and stored at 4^0C for 16rRNA sequencing and further analysis.

16S rRNA sequencing

16S rRNA gene sequence analysis DNA was isolated from the colonies. Quality of DNA was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 16s rRNA gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 357F & 1391R primers using BDT v3.1 Cycle Sequencing Kit on ABI 3500xl Genetic Analyzer. The 16s rRNA sequence was used to carry out BLAST with the database of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple sequence alignment software programs.

Result and Discussion

Physiochemical Properties of sample soil

Soil pH

The average pH values of different depth of soil, were recorded in Table 1**.** The highest pH 7.67 recorded in kodhdhi site of 10-20 cm. The lowest pH was recorded 7.21 in kodhdhi-1 soil. The overall soil pH was found to be neutral to alkaline that is quite favourable for cultivation practices. (See figure 1)

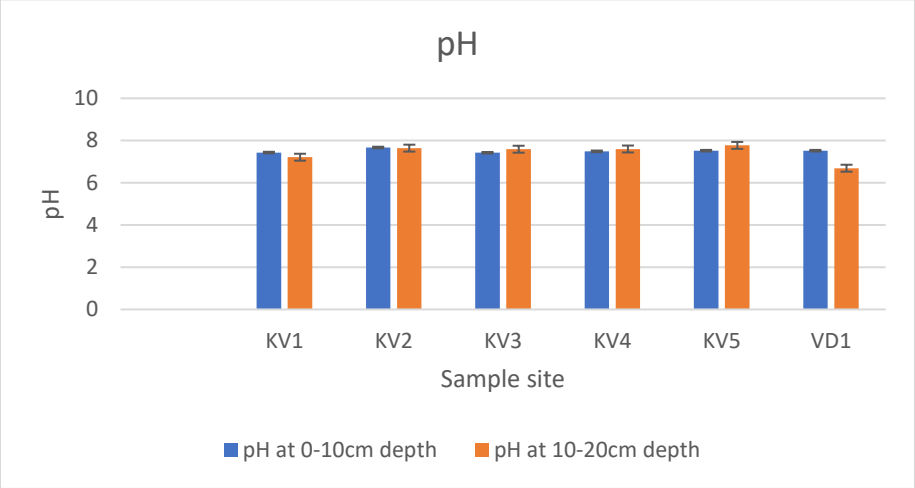

Figure 1 pH of soil sample

Total Nitrogen and Carbon concentration

Total nitrogen & Carbon concentration content of this soil is recorded in Table 1**.** The average value of total nitrogen content varies between 0.36% and 0.95%. Comparatively these values of total micro kjeldahl nitrogen are not too low as expected in a rid to semi-arid soil. The data indicates that the metabolic activities are higher because the nitrogen content in arid to semi- arid soil is expected to be 0.001 to 0.01%. The highest total nitrogen recorded at Kodhdhi 1 site. While, the lowest was recorded at Kodhdhi 2 site.(see fig 2)

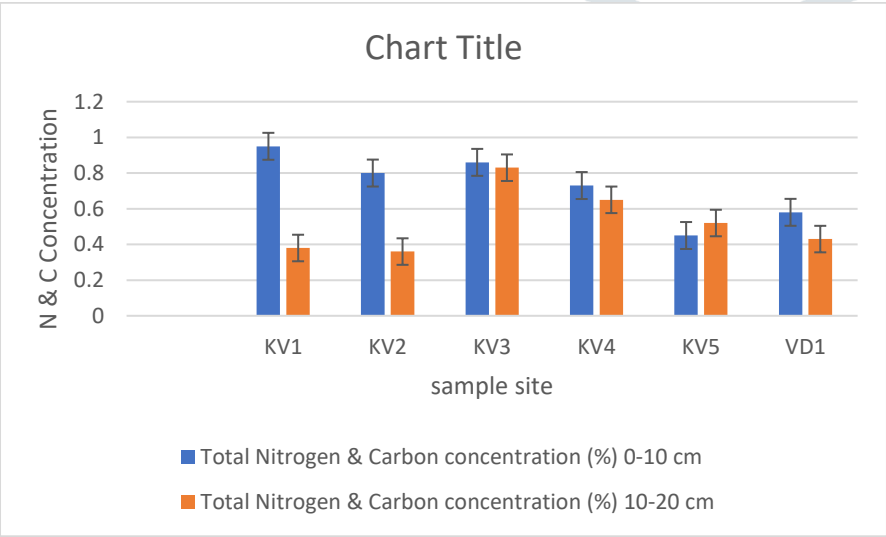

Fig 2 Total Nitrogen and Carbon concentration (%)

Biochemical test

Biochemical tests like, Catalase test, Citrate utilization test, Indole test Starch hydrolysis test, carbohydrate Fermentation, Motility test Methyl red and Voges-prousker test are performed. According to Bergey's manual and with the results obtained from biochemical test. The isolates A1, and A2 were positive to Indole, Voges-prousker, Catalase, Urease, Oxidase and Nitrate reductase test. B2 and Bh1 were positive to Methyl red, Voges-prousker, Oxidase, and Catalase test. All the isolates were motile in nature. (Table 1).

Sr. No	Biochemical tests	A1	A2	B2	Bh1
1	Gram's Reaction	-	-	+	+
2	Motility	+	+	+	+
3	Indole test	+	+	-	-
4	Methyl red test	-	-	+	+
5	Voges-prousker test	+	+	+	+
6	Citrate test	-	-	-	-
7	Oxidase test	+	+	+	+
8	Catalase test	+	+	+	+
9	Urease test	+	+	-	-
10	Nitrate reduction test	+	+	-	-

Table 1: biochemical characteristics of isolated species

Culturable diazotrophic bacterial population

The Isolation of diazotrophic bacteria from collected Soil were done by different N-free semisolid media (NFb, LGI and Burk's). A total of 10 diazotrophic isolates were obtained from the different soil sample. Four Azotobacter species were isolated from saline soil and their cultural features was observed and identified by various biochemical characteristics and 16SrRNA sequencing analysis. Microscopic examination of the A1 and A2 strains showed that they are gram negative, short rod and polymorphic in nature. B2 and Bh1 strain shows that they are gram positive in nature. The 16SrRNA sequence analysis of the A1 and A2 strain showed maximum similarity of 96% with *Azotobacter chroococcum* and B2and Bh1 showed maximum similarity with *Bacillus licheniformis* of the reference type strain. All the four isolates tested on nitrogen free agar medium for their nitrogen fixing activities. The 16s rDNA amplification and sequencing reveals that the species is Azotobacter chroococcum and *Bacillus licheniformis*. Nitrogen fixation occurred due to the production of organic acids or secretion of nitrogenase enzyme by the Azotobacter species in the medium and this is the main principal mechanism of nitrogen fixations. These isolates corelate with previous studies. This oxygen supply helps to effectively fix atmospheric nitrogen into a broth medium that lacks a nitrogen source but has a small amount

of carbon source. The secretion of the nitrogenase enzyme, which accelerates the process of nitrogen fixation on the medium, is aided by a high oxygen input into the culture media. [9]. The fluctuation in the amount of nitrogen produced by each Azotobacter strain may be influenced by environmental factors like as oxygen supply and the nutritional composition of the medium.

CONCLUSION

On the basis of present work it can be concluded that strains of *Azotobacter chroococcum* and *Bacillus licheniformis* were screened among the total isolates from different soil site and was confirmed by biochemical and 16s rRNA study.

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