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Isolation and Biochemical Identification of N_2 Fixing Bacteria (*Azospirillium* Sp.) From Saurashtra Region

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Abstract

Azospirillium species are widely used as Biofertilizer to increase crop yield and are free-living nitrogen-fixing bacteria, one of the best-studied plant growth-promoting Rhizobacteria. It is commonly found along with the roots of many different plant species. The present study included the isolation of *Azospirillium* species from roots and soils in Rhizospheric regions. 50 isolates were isolated from fields in different districts of Saurashtra region in Gujarat against NFB Medium. Significant isolates were isolated from the root surface and root internal tissues. These 50 were morphologically identified and biochemically characterized isolates included the techniques documented in Bergey's Textbook of Identified Bacteria 9th edition (TSI- Triple Sugar Iron, GPB- Glucose Phosphate Broth, MR- Methyl Red VP- Voges Proskauer, TRP-Tryptophan, PNB- Peptone Nitrate Broth, Carbohydrate) out of which fifteen isolates were biochemically positive and were also capable of producing IAA (Indole Acetic Acid), and were also capable of producing IAA.

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Introduction

Today's global scenario presents many constraints to agricultural production, leading to a decline in soil and soil fertility as well as a decline in agricultural output in general. Things are even worse for resource-poor farmers, who cannot afford to use high doses of organic fertilizers for crop production.[1] By 2020, 28.8 million tons of nutrients are needed to produce million tons (approximately 321 million) of grain. However, available nutrients were 21.6 million tons - a wide gap of 7.2 million tons between nutrient supply and nutrient removal.[2] In addition to reducing urea-N and preventing soil depletion, biological nitrogen fixation (BNF) technology also considerably lowers environmental pollution.[3] Since they are beneficial to the environment and economical for farmers, organic fertilisers are seen to be a key component in productivity, soil sustainability, and environmental

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protection.[4] Farmers may gain financially from using this organic fertiliser, which also assures continued food production. The worldwide green revolution will begin with the application of chemical-free fertilisers or microbial inoculation.[5]

Bio-fertilizers are mainly live microbial products including bacteria, algae and fungi, singly or in combination with the effect of improving nutrients available in the soil for plants. Essential nutrients change from unusable to usable form with the assistant of microbial activities; activities also include phosphate solubilization, nitrogen fixation, soil biodegradation and plant growth hormone secretion. Bio-fertilizers are ecological, high-yielding, easier for farmers to use and more efficient.[2]

***Azospirillium* Species**

The genera *Azotobacter* and *Azospirillium* increase the yield of cereals and legumes under field conditions.[4] *Azospirillium* is solitary of the earliest identified/discovered and best-characterized plants that promote rhizobacteria covering tropical and temperate regions commonly applied to grass, rice, wheat, sugarcane and many other crops.[6] It grows profusely at pH-7 and can form spores in acidic soils at pH 4.8. *Azosprilllium* is a motile, non-fermenting, vibrioid, gram-negative nitrogen-fixing rhizome. It is an encapsulated microcapsule, Sheath are composed of a network of inverse polysaccharides ensuring stress tolerance and increased shelf life. *Azospirillium* is an α subgenus of proteobacteria with the original name Spirillum lipoferum- isolated by *Beijerinck* from the Netherlands Laterly, by Schroder in 1932 from the soil of Germany and Austria.[7] The organism showed increased nitrogen content in the N2-free medium. *Azospirillium* can grow aerobically and anaerobically in different geographical climates. It uses organic acids on sugars as carbon sources, ammonium or nitrates as nitrogen sources, and it can fix nitrogen under microscopic and free living conditions.[8] A number of processes, including as the production and release of amino acids, IAA (indole acetic acid), Cytokines, Gibberellins, and other polyamines, as well as root development and the subsequent increase in water and nutrient intake, all work to promote *Azospirillium*. A huge genus, *Azospirillium*, has around 113 plant species from 35 different plant families.[9,10] Analyzed the results of multiple field trials with different non-legume crops, representing more than 20 years worldwide in a variety of weather and soil conditions, examining that 30% yield increase can reach 70% response time with *Azospirillum* inoculation.[11] reported that *Azospirillum* sp. inoculated on upland crops showed yield responses in winter cereals (14.0%), summer cereals (9.5%), and also in legumes (6.6%).[12] reported *Azospirillum* spp. isolated from Himalaya valley and other places from Lake Baiyang. The species isolated in 2019 were - *A.palustre*,[13] *A. griseum*,[14] *A. Ramasamy,*[15] *A.Agricola*,[16] *A.soli*,[17] *Nitrospirillum & Niveispirillum*,[18] *A.Himalayense*,[19] *A.fermentation*,[20] *A.Humicireducens*,[21] *A.formosense*,[22] *A.thiophilum*,[23] *A.palatum*,[24] *A.picis*,[25] *A .rugosum*,[24] *A.canadense & A.zeae*,[26] *A.melinis,*[27] *A.oryzae*,[28] *A.doeberinerae*,[29] *A.largimobile*,[30] *A.irakense*,[31] *A. halopraeferens*,[32] *A.amazonense*,[33] *A.lipoferum & A.brasilense*.[34]

Root Attachment to Surface and Site of Root Colonization

Sites suitable for colony colonisation have been shown to be where *Azospirillum* colonisation occurs on the surface of roots. In the most studied plant species, the bacteria form aggregation colonies that are supported by enormous fibrous material around the tips of elongated hairs and roots. Two distinct phases of *Azospirillum*'s attachment to wheat have been examined by.[35] Bacterial proteins rule the quick and weak adsorption phase. The second phase is more prolonged, active, and irreversible, it could be based on the extracellular surface polysaccharides of bacteria.

The country becomes more self-sufficient in food production thanks to the use of chemical fertilisers, but this practise damages the environment, has negative effects on human health, contaminates groundwater, and promotes soil acidification owing to a decline in organic matter. Owing to inadequate plant absorption of these chemical fertilisers, they enter water bodies through rains, create eutrophication there, and have an impact on aquatic life. Finding management practises that can impact plant development, a general improvement in root growth, significant bio-control activities, and numerous mechanisms of the plant-microorganism interplay will be a future issue.

Materials and Methods

In the present investigation, the *Azospirillium* isolates mentioned in Table 1 were derived from the rhizomes of different non-legumes collected from different districts of Saurashtra region.

Sample Collection

The Isolates were collected at a depth of 5-6 cm from the Rhizome Zone of the Plants (Maize, Cotton, Jowar, Sugarcane, Bajra, Wheat, Groundnut etc.) stored at 4 °C. Species were isolated from roots and soil by serial dilution.[36]

Root Isolation

Roots, washed thoroughly with water, cut into small pieces and shaken in a shaker for 30 min in sterile distilled water; 0.1 ml of water was inoculated into NFB semi-solid medium and incubated for 5-7 days at 30-35 °C to form a white film.[37,38]

Soil Isolation

1 g soil was diluted in order to 10-6 with sterile distilled water. 1 ml of 10-4 to 10-6 diluent was incubated in tubes containing semi-solid NFB medium (Nitrogen-Free Bromothymol) and incubated for 5-7 days at 30-35°C to form films. These films have demonstrated positive results for the development of *Azospirillium*. Colonies were spread on Petri dishes containing solid NFB medium (Bromothymol without Nitrogen) and incubated at 30-35 °C for 72 h to isolate single colonies.[39]

Media Formulation

NFB medium contains 5 g malic acid, 4.0 g sodium chloride, 0.5 g potassium phosphate, 0.05 g ferric sulfate, 0.01 g manganese sulfate, 0.10 g magnesium sulfate, 0.02 g sodium chloride, 0.01 g calcium chloride, 0.002 g sodium molybdate dihydrate, 1000 ml water distillation, 2.0 ml bromothymol blue (BTB) - (0.5% alcohol solution) Final pH 6.6 to 6.8, 1.75 g of semi-solid medium and 15 g of solid medium.[40,41]

Morphology and Classification

The juvenile inoculum from the semi-solid medium was applied on a clean slide, thermostated, then flooded with iodine for 1 minute, decolored with ethanol 95% in a matter of seconds, gently rinsed with tap water, and cleaned with Safranin stains. For viewing with an oil immersion lens, slides were air-dried.[42] Gram-negative, rod-, spiral-, spiral-, and filamentous bacteria were seen under a microscope. The physical characteristics of the *Azospirillium* isolates are listed in Table 2.

Biochemical Characteristics

According to Bergey's Guide to Determining Bacteria, 9th Edition, isolates were characterized for different biochemical assays. The tests were carbohydrate fermentation, catalase test, mobility test, citrate utilization, triple sugar iron, indole production, MR-VP test, peptone nitrate reduction, ammonia production, urea hydrolysis, gelatin hydrolysis and lipid hydrolysis,[3,34]

Production of Phytohormones

24 hour old explants were allowed to develop in tryptophan culture at 30 °C for 24 hours in order to measure the quantity of Indole Acetic Acid (IAA) produced in each isolate using Salkowski's reagent. After incubation, the broth was centrifuged, and Salkowski reagent 2 ml were combined with 1 ml of the supernatant. IAA generation is shown by the colour shift and absorption at 540 nanometers. Before to using the set in potted culture studies, each isolate was maintained in three sets.[44]

Results and Discussion

In this present study, 50 isolates were isolated from different Rhizome Regions of the roots and soil samples. Samples were taken from 10 districts of Saurashtra region - Rajkot 19, Junagadh 9, Amerali 5, Botad 5, Morbi 2, Dwarka 2, Kutch 3, After Ahemdabad 2, Surat 2 and Vadodara 1 are presented in Table 1

Characteristics of the Colony

All 50 isolates were morphologically classified by Gram staining based on their characteristics such as shape, size, margin texture, elevation, and opacity. Of these isolates, 44 strains of bacteria were gram-negative, all of which were circular in shape with an entire margin and smooth texture, followed by a predominantly small, but some are medium sized and slightly raised or flat elevation. 14 colonies were opaque white, 32 colonies were transparent and 4 colonies were translucent. 6 Strain of bacteria were Gram Positive.

Table no. 1: shows isolates from Saurashtra region

Sr. No.	Sample	Sample Code	City/Village	District	Sr. No.	Sample	Sample	City/ Code	District Village
1	Maize	S1M1V	Vichhiya	Rajkot	26	Jowar	S49JoF	Fareni	Junagadh
2	Maize	S2M2V	Vichhiya		27	Sugarcane	S50SuF	Fareni	
3	Maize	S3M3V	Vichhiya		28	Maize	S26MJG	Gir Somnath	
4	Maize	S4MRJ	Rajkot		29	Cotton	S40CKH	Khijadiya	Amreli
5	Maize	S5MAT	Atkot		30	Jowar	S41JKH	Khijadiya	
6	Bajra	S6B1V	Vichhiya		31	Groundnut	S42GKh	Khijadiya	
7	Bajra	S7B2V	Vichhiya		32	Mung	S38MuME	Medi	
8	Bajra	S8B3V	Vichhiya		33	Maize	S39MME	Medi	
9	Bajra	S9BRJ	Rajkot		34	Bajra	S15BPD	Padiyad	Botad
10	Jowar	S10J1V	Vichhiya		35	Jower	S17JBPD	Padiyad	
11	Jowar	S11J2V	Vichhiya		36	Groundnut	S20GSR	Sarangpur	
12	Jowar	S12JV	Vichhiya		37	Ekad	S21ESR	Sarangpur	
13	Jowa	S13JRJ	Rajkot		38	Wheat	S22WSV	Sarva	
14	Tuver	S27TuRJ	Rajkot		39	Groundnut	S23GMO	Morbi	Morbi
15	Tuver	S37TuG	Gondal		40	Cotton	S24CMO	Morbi	
16	Cotton	S30CJM1	Jamkandona		41	Wheat	S45WMP	Mirzapar	Kutch
17	Cotton	S31CJM2	jamkandona		42	Maize	S46MBJ	Bhuj	
18	Wheat	S43WJU	Juthard		43	Tomato	S47ToBJ	Bhuj	
19	Grass	S44GJU	Juthard		44	Tuver	S28TuDH	Dholka	Ahemdabad
20	Bajra	S25BJG	Junagadh	Junagadh	45	Tomato	S29ToDH	Dholka	
21	Sugarcane	S33SuF	Fareni		46	Sugarcane	S32SuBH	Bharthana	Surat
22	Jower	S16JUP	Upleta		47	Chana	S33ChV	Bharthana	
23	Cotton	S18CUP	Upleta		48	Mung	S34MuV	Sokhda	vadodara
24	Groundnut	S19GUP	Upleta		49	Wheat	S14WBNV	Bhanvad	Dwarka
25	Hardar	S48HF	Fareni		50	Cotton	S35CoBNV	Bhanvad	

*Sample- Plant sample, Sample Code S- Sample, No.- Sample number, Plant Initials, Plant Numbers and City/Village Initials, City/Village-Sample collected, District

Table no. 2: Colony Characteristic of different isolates

Sample	+/-	Shape	Size	Margin	Texture	Elevation	Opacity
S1	-ve	Round	Small	Entire	Smooth	Slight Raised	Opaque
S2	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S3	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S5	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S6	-ve	Round	Small	Entire	Smooth	Slight Raised	Opaque
S7	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S9	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S10	-ve	Round	Medium	Entire	Smooth	Raised	Transperant
S11	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S12	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S13	-ve	Round	Small	Entire	Smooth	Flat	Opaque
S14	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S15	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S16	-ve	Round	Small	Entire	Smooth	Flat	Transperant

S18	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S19	-ve	Round	Small	Entire	Smooth	Raised	Opaque
S20	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S21	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S22	-ve	Round	Small	Entire	Smooth	Slight Raised	Opaque
S23	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S24	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S26	-ve	Round	Medium	Entire	Smooth	Raised	Transperant
S27	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S28	-ve	Round	Small	Entire	Smooth	Slight Raised	Opaque
S29	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S30	-ve	Round	Small	Entire	Smooth	Raised	Opaque
S31	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S32	-ve	Round	Small	Entire	Smooth	Raised	Transperant
S33	-ve	Round	Small	Entire	Smooth	Raised	Transperant
S34	-ve	Round	Small	Entire	Smooth	Flat	Opaque
S35	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S36	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S37	-ve	Round	Small	Entire	Smooth	Slight Raised	Opaque
S39	-ve	Round	Small	Entire	Smooth	Raised	Transperant
S40	-ve	Round	Medium	Entire	Smooth	Raised	Translucent
S41	-ve	Round	Medium	Entire	Smooth	Raised	Transperant
S42	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S43	-ve	Round	Small	Entire	Smooth	Slight Raised	Opaque
S44	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S46	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S47	-ve	Round	Small	Entire	Smooth	Slight Raised	Transperant
S48	-ve	Round	Small	Entire	Smooth	Slight Raised	Opaque
S49	-ve	Round	Small	Entire	Smooth	Flat	Transperant
S50	-ve	Round	Small	Entire	Smooth	Flat	Transperant

* S-Sample number, +ve - Gram Positive Bacteria and –ve – Gram Negative Bacteria

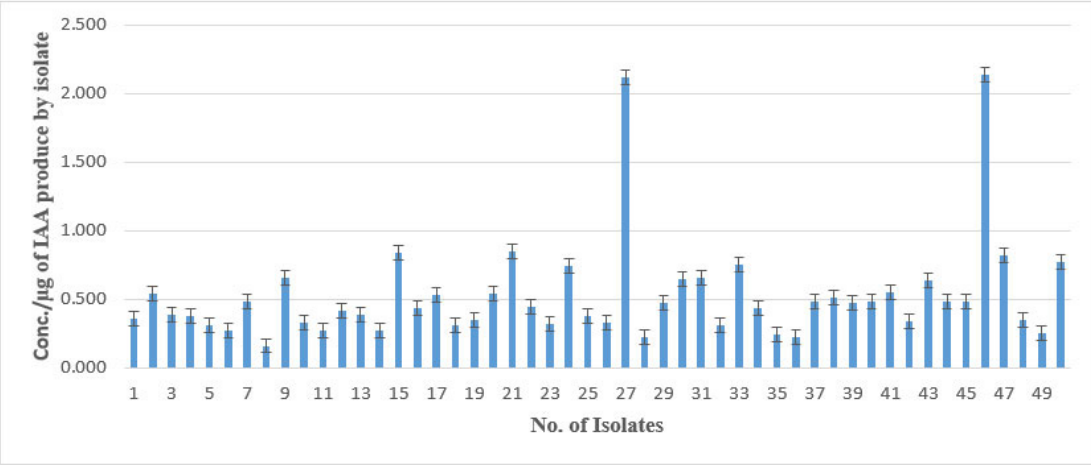

Figure 1: Shows IAA (Indole Acetic Acid) Produced by isolates

Table No. 3: Shows Biochemical Characterization of different Isolates

Sample	Motility	Citrate	TSI	GPB		Ammonia	TRP	Urea	PNB	Catalase	Gelatin	Carbohydrate			
				MR	VP							Glucose	Lactose	Sucrose	Maltose
S2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S3	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
S7	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
S11	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-
S12	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+
S13	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
S15	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-
S16	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+
S17	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+
S21	+	-	-	+	-	+	-	+	+	+	-	+	-	-	-
S27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S29	-	+	-	+	-	+	+	+	+	+	-	-	+	-	-
S30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S41	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
S46	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*TSI- Triple Sugar Iron, GPB- Glucose Phosphate Broth, MR- Methyl Red VP- Voges Proskauer, TRP-Tryptophan, PNB- Peptone Nitrate Broth

Biochemical Characteristics

Models: - S-2, S-3, S-7, S-11, S-12, S-13, S-15, S-16, S-17, S-21, S-27, S- 29, S-30, S-41, S-46 are portable Fermenting sugars such as glucose, fructose, lactose, mannitol, it hydrolyzes gelatin, and urea, they can also produce H_2S, indol and ammonia. The results are shown in Table 3. These isolates were then screened for potted culture experiments.

Production of Phytohormones

Salkowski reagent was used to assess the IAA generation of 50 isolates at 540 nm. Figure 4 displays the three isolates that produced the greatest IAA, S-27 190.19 g/ml, S-46 195.20 g/ml, and S-21 90.04 g/ml. The histogram's X-axis and l-Y axis both display the number of isolates, shows the amount of IAA created by Isolate.

Discussion

One of the most well researched plant growth-promoting bacteria, *Azospirillum*, is found specifically in the rhizomes and intercellular regions of cereal roots and other plant roots.[43] The favourable benefits of this bacterium on plant development have been demonstrated through a number of pathways indicated by plant-*Azospirillum* interactions. The concept of the multiple mechanism hypothesis was developed through the study of numerous phytohormones, plant regulators, nitrogen fixation, phosphate solubilizers, a wide range of molecules and enzymes, enhanced membrane activity, root system proliferation, increased water and mineral absorption, reduced pathogens, environmental stressors, and competition against pathogens.[12] The most significant agricultural crop in the world is wheat, which is farmed in a variety of habitats, from marshlands to desert areas. Since the 1970s, scientists have been examining the results of *Azospirillum* inoculating grass.[45] *Azospirillum*, one of 113 species in 35 plant families, is a soil bacterium that encourages plant development.[8] The number of primary root nodules dramatically increased when A. *brasilense*, an efficient generator of IAA, was applied to alfalfa seeds; this increase was associated with inoculum size.[11] One isolation (Sp1) and the second isolate (Sp2) revealed 99% similarity to *Azospirillum brasilense* and *Azospirillum zeae*, respectively, based on 16S rRNA sequence

analysis. In a pot experiment, the impact of these two isolates on bread wheat was investigated.[46] The greatest obstacle to effective inoculation is the toxicity of pesticides employed in seed treatments. In order to avoid direct bacterial exposure to pesticides, we investigated different approaches to inoculating maize and wheat seeds in this work.[47]

Conclusion

The current study includes 50 isolates from the Saurashtra Area, of which 2 isolates, S-27 and S-46, were shown to produce the greatest IAA (Indole Acetic Acid) production when tested against Salkowski's reagent. These 2 isolates will be examined for 16s rRNA sequencing as well as evaluated against pot cultures. In order to create bio-fertilizers and field characteristics, it can be employed. The Saurahtra area would be a better place to isolate species of *Azospirillium* sp., which might help improve numerous growth indices, mineral uptake, and water absorption.

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Conflict of Interest

The authors do not have any conflict of interest.

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