Original Article

Biochemical And Physiological Characterization Of Actinomycetes Isolated From Rhizospheric Regions In The Soils Of Arachis Hypogea L. And Gossypium Herbaceum L. Near The Gir Wildlife Sanctuary

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Abstract

30 isolates of actinomycetes were collected from the soil samples of Arachis hypogea L. and Gossypium herbaceum L. near the fields located surrounding the Gir Wildlife Sanctuary area of Junagadh District,Gujarat State,India. All the isolates were subjected to serial dilution process. The present study aimed to identify and characterize these isolates using biochemical and physiological tests. From the morphological tests and gram staining procedures conducted prior to the biochemical and physiological tests, it was already observed that Actinomycetes are Gram positive , filamentous shaped bacteria. It was also observed that these bacteria contain hyphae, mycelium as well as sporangia which is able to release spores. It was able to produce yellow pigments, posses an earthy aroma and showed optimum growth under aerobic conditions at temperature about 28 ºC, pH 7 and 5% w/v NaCl Concentration. Therefore, it was considered as a mesophilic, basophilic and moderate Salt tolerant by nature. The isolates showed variations in carbon utilization but able to utilize almost all carbon sources. These bacteria were also able to give positive results in methyl red test, nitrate reduction tests, urea hydrolysis, citrate utilization tests etc. Altogether, the results indicated that these isolates i.e. Actinomycetes are able to utilize almost all nutrient sources available in the rhizosphere region of Arachis hypogea L. and Gossypium herbaceum L.

Keywords: Actinomycetes, Arachis hypogea L., Gossypium herbaceum L , Ray Fungus.

INTRODUCTION:

Actinomycetes is a group of bacterium that are Gram positive. They are often filamentous in appearance and exhibit sporulation having DNA rich in G+C from 55-75%. (Ho *et al*). Actinomycetes name is derived from Greek words aktis means “a ray” and mykes means “fungi”. This nomenclature was given to this group of bacteria observing their external appearance. The main genus of this group of bacterium is “Streptomycetes”. The group which is not belonging to *streptomycetes* are considered as “rare Actinomycetes*”*.Comparing approx. 100 genus of the Actinomycetes, which live in marine habitat, are inadequately understood and only few reports are obtainable of Actinomycetes that live in mangroves. (Kumar *et al*). Actinomycetes are the most prolific source for production of antibiotics. Nearly eight thousand compounds has been identified up to the end of twentieth century of which the contribution of Actinomycetes is the most. In present times, more interest has been targeted on the utilization of immobilized microbial cells for generating useful bioactive compounds (Manjula *et al*). The isolation and characterization of this group of bacteria were performed by using different biochemical methods. (Dhanshekaran *et al*). Various biochemical tests were performed to characterize this group of bacteria viz. starch hydrolysis, citrate utilization test, indole test, methyl red test, vogus-proskauer (Acetone Production) test, Catalase test(Holt et al).

MATERIALS AND METHODS

Sample Collection: Soil samples were collected from the fields of *Arachis hypogea* L. & *Gossypium herbaceum* L. in the sterilized bags and transported to the research laboratory and stored in a refrigerator below 5º C until use.

Isolation Procedure: The media utilized for the isolation and cultivation of actinomycetes was Actinomycetes Isolation Agar (AIA). Soil samples were autoclaved at optimum temperature and pressure conditions.

After autoclaving, soil samples were serially diluted,filtered and sterilized upto 10^{-2} dilutions. From each dilution, 0.1 mL was used and spread evenly with sterile glass rod over the surface of AIA and kept for incubation at 30º C. Streak Plate method was utilized to purify the colonies of rhizospheric actinomycetes. (Williams *et al*). Transfer of pure culture on slants was done and for future use, culture was preserved at 4ºC.

Biochemical Characterization:

Various biochemical tests were performed for the characterization of actinomycete isolates. These tests include Indole test, Methyl red test, Vogus proskaur(VP) test, Citrate utilization test, Urea hydrolysis, Nitrate reduction, H_2S reduction, Catalase test.
Standardized protocols of above mentioned biochemical tests are as follows:

1. Sugar Fermenatation Test:
Using 1 gram percent of Fructose, Mannitol, Arabinose, Inositol, Lactose Media, inoculation of test culture was done in sugar broth followed by incubation at 28º C for 5-7 days. Methyl red was used as indication dye for color change. For gas production Derham's vial (inverted position) was utilized.

2. IMViC (Indole Methyl red Voges Proskeuor Citrate) test:

i) Indole production test : Using 1 percent tryptone broth, inoculation of test culture was done followed by incubation at 28 º C for 5-7 days. Post incubation, 4 drops of xylene were added shaking the culture vigorously followed by addition of 1ml Ehlrlich's reagent.

ii) Methyl red test: Using GPB(Glucose Phosphate Broth), test culture was inoculated and incubated at 28º C for 5-7 days. Addition of 5 drops of methyl red was done after incubation expecting the development of red color in the test culture.

iii) V-P (VOGES-PROSKAUER) test: Using GPB (Glucose Phosphate broth), loopful of test culture was inoculated followed by incubation at 28 ° C for 5-7 days. Post incubation, 0.6 ml α-napthol & 0.2 ml Potassium Hydroxide were added per ml of broth recording the result after 15-60 mins expecting the development of Cherry red color in the test culture.

iv) Citrate utilization test : After Heavily streaking the test culture with Simmon citrate agar slant, incubation at 28° C for 5-7 days expecting growth of colonies with/without deep blue color in test culture.

3. Urea hydrolysis test : Using Urea broth, test culture was inoculated followed by incubation at 28° C for 5-7 days expecting the purple red color throughout the medium.

4. Catalase test (Slide method): Placing the slide in Petri-dish, loopful of test culture without medium was placed on slide followed by adding a drop of 3% hydrogen peroxide expecting the test culture not to produce any effervescence.

5. Gelatin hydrolysis test : Using Nutrient gelatin broth, test culture was inoculated followed by incubation at 28° C for 5-7 days. Post incubation , tubes were refrigerated for 30 mins to check for liquefaction.

6. H_2S Production test or Lead Acetate Paper strip test. : Using 2% peptone broth, Test culture was inoculated followed by incubation 28º C for 5-7 days. White filter paper strips soaked in lead acetate solution were placed at the neck of tube such that ¼ to ½ of strip projects below the cotton plug expecting it to turn black due to lead sulfide formation which indicated H_2S production.

7. Nitrate reduction test : Using Peptone nitrate broth (PNB), test culture was inoculated and then incubated at 28° C for 5-7 days followed by the addition of 0.5 ml α-Napthaline & Sulphanilic Acid reagent to each test culture expecting the development of red color within 30 seconds.

Physiological Characterization:

1. **NaCl resistance test:** Using 5% , 8% and 10% NaCl containing Actinomycetes Isolation Agar medium test culture was spreaded followed by Incubation at 28°C for 5-7 days expecting the Growth of test culture in medium.

2. **pH resistance test**: Spreading of Test culture was performed on AIA media of pH 5, 7 and 9 followed by incubation at 28° C for 5-7 days expecting the growth of test culture in medium.
3. **Temperature resistance test :** Spreading of Test culture was performed on AIA media at temperature followed by incubation at 28° C, 4 °C & 55 °C for 5-7 days expecting the growth of test culture in medium.
4. **Starch hydrolysis test :** Spreading of Test culture was performed on starch agar media followed by incubation at 28° C for 5-7 days expecting the growth of test culture in medium. (Transparent zone surrounding colony after adding lugol's iodine)
5. **Cellulose hydrolysis test :** Dividing the petriplates in 2 equal parts, 2 different isolates were streaked in each sector of plate as single line on cellulose agar medium followed by incubation at 28° C for 5-7 days expecting the growth of test culture in medium. (Transparent zone surrounding colony)

RESULTS & DISCUSSION:

Sr no.	Isolate name	Fructose	Lactose	Mannitol	Arabinose	Inositol
1	C-2	-	+	+	+	+
2	C-5	+	-	+	-	-
3	C-6	+	+	+	+	+
4	C-8	+	+	+	-	+
5	C-10	+	+	+	+	+
6	C-11a	+	+	+	+	-
7	C-12	+	+	-	-	+
8	C-13	+	+	-	+	-
9	C-14	+	+	+	-	+
10	C-15	+	+	-	-	+
11	C-17	+	+	+	+	-
12	C-20	+	+	+	-	+
13	C-21	+	-	-	-	-
14	C-24	+	+	+	-	-
15	C-25	+	+	+	+	+
16	C-27	+	-	+	+	-
17	C-29	+	+	+	+	+
18	GC-2	+	+	+	+	+
19	GC-3	+	+	+	+	+
20	G-1	+	+	+	-	+
21	G-2	+	+	+	-	+
22	G-3	+	+	+	+	+
23	G-4	+	+	+	-	+
24	G-5	+	+	+	+	+
25	G-6	+	+	+	+	+
26	G-7	+	+	-	+	+
27	G-8	+	+	-	+	+
28	G-9	+	+	+	+	+
29	G-10	+	+	+	+	+
30	G-13	+	+	+	+	+

Table 1 a: Inference of Different Sugar Utilization Tests by all 30 isolates.

After performing various biochemical tests/assays, 30 isolates showed varied results in different tests. In sugar fermentation test, decoloration of the sample was observed in most of the samples indicating acid productiona and thus giving positive results for sugar fermentation test. In the case of indole production test, after adding Ehlrich's reagent, pink colored ring was observed immediately at the lower surface of xylene in few samples only which means that most of the samples gave the negative result. Most of the isolates/test
samples gave methyl red test positive developing a red colour in the GPB medium. More than half of the total number of isolates gave negative results in the case of Vogus-Proskauer Test. All 30 isolates gave citrate test positive forming a deep blue coloration concluding that all isolates are able to utilize citrate forming alkaline products. Out of 30 isolates,10 isolates gave both methyl red and Vogus-Proskauer test positive. It was also observed by performing catalase test that no effervescence was produced in any test sample. In contrast to the

above two tests, all isolates showed growth in Gelatin Test and in case of urea and nitrate test, 6 isolates and 8 isolates gave positive results respectively. The detailed inferences of all thirty isolates is mentioned in Table 1 a & b.

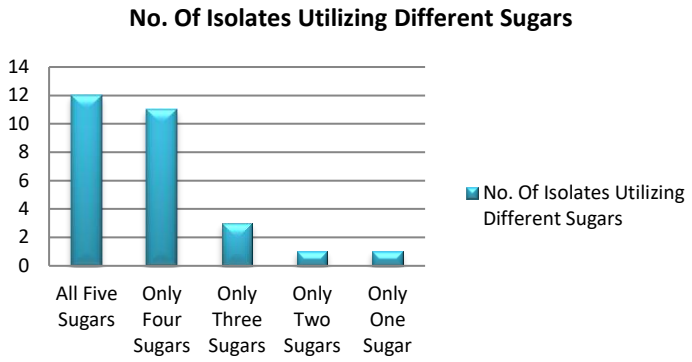

Figure 1 a: The graph values showing total number of isolates/test samples that were able to utilize different sugars.

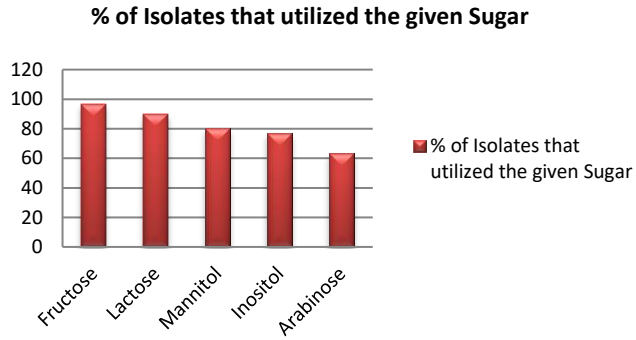

Figure 1 b: The graphical representation of the percentage of different sugars that are being utilized by isolates after performing sugar fermentation biochemical test.

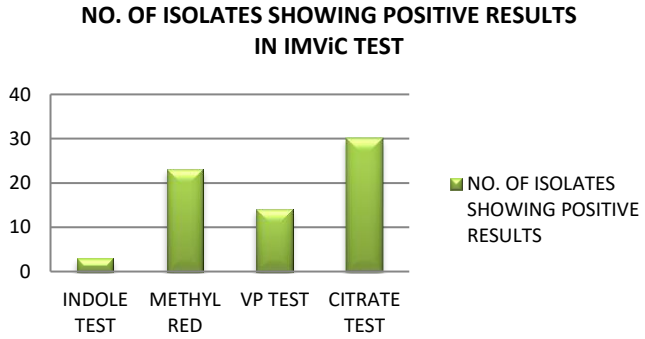

Figure 1 c: Graphical Representation of number of isolates that gave positive results in IMViC tests.

Sr no.	Isolate name	Indole	Methyl red	VP	Citrate
1	C-2	-	+	-	+
2	C-5	-	+	+	+
3	C-6	-	+	+	+
4	C-8	+	+	+	+
5	C-10	-	-	+	+
6	C-11A	-	-	-	+
7	C-12	-	+	-	+

8	**C-13**	+	-	-	+
9	**C-14**	-	-	-	+
10	**C-15**	+	+	-	+
11	**C-17**	-	-	+	+
12	**C-20**	-	-	+	+
13	**C-21**	-	-	+	+
14	**C-24**	-	+	-	+
15	**C-25**	-	+	-	+
16	**C-27**	-	+	-	+
17	**C-29**	-	+	-	+
18	**GC-2**	-	+	+	+
19	**GC-3**	-	+	+	+
20	**G-1**	-	+	-	+
21	**G-2**	-	+	-	+
22	**G-3**	-	+	-	+
23	**G-4**	-	+	-	+
24	**G-5**	-	+	+	+
25	**G-6**	-	+	+	+
26	**G-7**	-	+	+	+
27	**G-8**	-	+	-	+
28	**G-9**	-	+	-	+
29	**G-10**	-	+	+	+
30	**G-13**	-	+	+	+

Table 1 b: Inferences of all 30 isolates showing positive and negative results after performing IMViC tests.

In case of physiological test results, only three isolates showed growth on all three salt concentrations,sixteen isolates showed growth on one salt concentration. Five islolates didn't showed growth on any of the salt concentration. Fifteen isolates showed growth on 5% salt concentration. Thirteen isolates showed growth on 8% salt
concentration. Seven isolates showed growth on 10% salt concentration. Fifteen isolates showed growth on all three pH values i.e.pH 5, pH 7 and pH 9. Thirteen isolates showed growth on two different pH. Two isolates showed growth on only one pH value. Fifteen isolates showed growth on pH- 5. All isolates showed growth on pH-7. Twenty eight isolates showed growth on pH-9. The detailed inferences of all thirty isolates is mentioned in Table 2.

Table 2: Inferences of all 30 isolates showing positive and negative results after performing various Physiological Tests (Salt Concentration Test, pH test and Temperature Test)

Sr no	Isolate name	Salt tolerance test			pH resistance test			Temperature tolerance test			Starch hydrolysis (Based on Zone of clearance)	Cellulose hydrolysis (Based on Zone of clearance)
		5% NaCl	8% NaCl	10% NaCl	5	7	9	4°	28°	55°		
1	**C 2**	+	+	+	+	+	+	-	+	-	-	-
2	**C 5**	+	-	-	+	+	+	-	+	-	-	+
3	**C 6**	+	-	-	-	+	-	-	+	-	+	+
4	**C 8**	-	-	+	-	+	+	+	+	-	+	+
5	**C 10**	-	-	-	-	+	+	-	+	-	+	+
6	**C 11A**	-	+	-	-	+	+	+	+	-	+	+
7	**C 12**	+	+	-	+	+	+	+	+	+	+	+
8	**C 13**	-	-	+	-	+	+	+	+	-	+	+
9	**C 14**	+	-	+	+	+	+	+	+	-	+	+

10	**C 15**	+	+	-	-	+	+	-	+	-	+	+
11	**C 17**	-	+	-	-	+	+	+	+	-	+	+
12	**C 20**	-	-	+	+	+	+	-	+	+	+	+
13	**C 21**	-	+	-	+	+	+	-	+	-	+	+
14	**C 24**	+	-	-	-	+	+	-	+	-	-	+
15	**C 25**	-	-	-	+	+	+	+	+	+	+	+
16	**C 27**	+	+	-	-	+	+	-	+	-	+	-
17	**C 29**	-	+	-	-	+	+	-	+	-	+	+
18	**GC 2**	+	-	-	+	+	+	+	+	-	+	+
19	**GC 3**	+	-	-	+	+	+	+	+	-	+	+
20	**G 1**	-	-	-	-	+	+	+	+	-	+	+
21	**G 2**	-	-	-	+	+	+	+	+	-	+	+
22	**G 3**	+	+	+	+	+	+	-	+	-	+	+
23	**G 4**	+	-	-	+	+	+	-	+	-	+	+
24	**G 5**	+	-	-	+	+	+	-	+	-	+	+
25	**G 6**	-	+	-	-	+	+	-	+	-	-	+
26	**G 7**	+	-	-	+	+	+	-	+	-	+	+
27	**G 8**	-	-	-	-	+	-	-	+	-	+	-
28	**G 9**	-	+	-	-	+	+	-	+	-	+	+
29	**G 10**	-	+	-	-	+	+	-	+	-	+	+
30	**G 13**	+	+	+	+	+	+	+	+	-	+	+

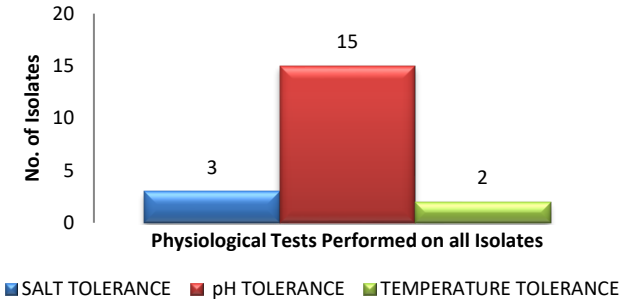

Figure 2(left): Graphical Representation of total number of isolates giving positive tests in all three different pH(5,7 & 9) salt concentrations(5%,8% & 10%) and temperature values (4ºC,28ºC and 55ºC).

CONCLUSION:

The isolated strains showed up the utilization of most of sugars. Fructose was best utilized followed by lactose, Mannitol, Inositol & Arabinose. All Isolates Produced citrate permease, carried out mixed acid & butanediol fermentation. Tryptophanase production was observed in few isolates. All isolates produced gelatinase, most isolates produced amylase & cellulase, few produced Urease & Nitrate reductase. None of the isolates produced catalase & amino acid desulfurase.Isolates can resist up to 8% salt. Some are halophiles growing at 10% . Best pH for growth is 7-9. So isolates are basophilic. Best temperature for growth is 28°C. But many are psycrophilic growing at 4°C. Few are thermophilic growing at 55°C.The microscopical, biochemical, physiological, morphological and cultural studies reveal that the isolated and investigated strain is an actinomycetes. Results of the present study also concludes that Actinomycetes are extremely vague in their carbon requirements and almost all the carbon compounds were utilized by one or more actinomycete isolates. The ability of actinomycete isolates to decompose various substrates vary significantly.More studies will be done on the isolated actinomycetes to utilize potential actinomycetes for anti-microbial & Plant Growth Promoting(PGP) studies.

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