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Isolation, Identification, Biochemical and Antibiotic Sensitivity Characterization of *Rhizobium* Strains from *Vigna mungo* (L) Hepper, *Cicer arietinum* L and *Vigna radiata* (L) R Wilczek in Muzaffarnagar, Uttar Pradesh, India

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A B S T R A C T

In present investigation uard (*Vigna mungo* (L) Hepper), chickpea (*Cicer arietinum* L) and moong (*Vigna radiata* (L) R Wilczek) were studied for isolation, identification, biochemical and antibiotic activities characterization of *Rhizobium* strains. A total of 37 samples were screened out of which 27 samples of *Cicer arietinum*, 5 samples of *Vigna mungo* and 5 samples of *Vigna radiata* were collected from different fields of surrounding area of Muzaffarnagar, Uttar Pradesh. On the basis of white mucoid growth on YEMA medium only 3 samples from *Vigna mungo*, 18 (*Cicer arietinum*) and none of sample *Vigna radiata* from were found positive for the presence of *Rhizobium*. In Gram staining reaction all the 21 (18 from chickpea and 3 from uard) isolates were found Gram-negative and rod shaped. Biochemical characterization of *Rhizobium* strains showed that all the 21 isolates have negative reaction with Citrate utilization test, Starch utilization test was positive with all isolates except for U1, C3, C13 and C24, Glucose peptone agar test was positive with all isolates except for C 18, negative results were found with Gelatin test for all isolates and Catalase test was found positive for all isolates. All the isolates were found sensitive to Ciprofloxacin (Cf) except C12 which was partially resistant, Ofloxacin (Of) except C2 which was partially resistant. Norfloxacin (Nx) was susceptible to 11 isolates, resistant to 7 isolates and partial resistant to 3 isolates. Levofloxacin (Le) and Gatifloxacin (Gf) were susceptible to all isolate but Levofloxacin (Le) was partial resistant to C3, C8 and C14. All the isolates, on the other hand, showed resistance to antibiotics Aztreonam (Ao) and Co-trimoxazole (Co) were showed resistant while Nitrofurantoin (Nf) showed partial resistance to isolates with few exception.

Keywords

Isolation, Biochemical characterization, Antibiotic activity, *Rhizobium.*

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Introduction

Nitrogen is an essential nutrient for plant growth and development. Nitrogen is an important element for the synthesis of amino acids which are used by the plant to form protein. Plants primarily take nitrogen in the ionic form of either ammonium or nitrate. Intensive farming practices that accomplish high yields need chemical fertilizers, which

are costly and also create environmental problems (Rigby and Caceres, 2001). The extensive use of chemical fertilizers in agriculture is currently under debate due to environmental concern and fear for consumer's health. Consequently, there has recently been a growing level of interest in environment friendly sustainable agricultural practices and organic farming systems (Lee and Song, 2007). Leguminous plants are also able to utilize nitrogen derived from the symbiotic relationship they form with root nodule bacteria. This phenomenon is extremely important and the value of this "free" fertilizer N_2 can be placed in global perspective if one considers that an estimated 50 million tons of nitrogen is manufactured industrially each year against an estimated 90 million tons fixed by plant processes (Cleyet *et al.,* 1990). The genus *Rhizobium* was given by Frank in 1890, based on its characters to form nodules on roots of legume plants (Graham and Parker, 1964). Root-nodule bacteria are separated into two genera *Rhizobium* which are fast growing bacteria and *Bradyrhizobium* which are slow growers (Jordan, 1984). Rhizobia are soil bacteria that fix N_2 (diazotroph) after becoming established inside root nodules of legumes (Fabaceae). There are several different genera of Rhizobia, all of them belong to the Rhizobiales, a probably-monophyletic group of protobacteria and they are soil bacteria characterized by their unique ability to infect root hairs of legumes and induce effective N_2-fixing nodules to form on the roots. Unlike many other soil microorganisms, Rhizobia produce no spores and they are rod shaped, aerobic and motile (Chaintreuil *et al.*, 2000). Inoculation plants and spoil with *Rhizobium* can improved soil fertility and reduce the production cost of next crop through reduced input in the form of nitrogen fertilizers and which also minimize hazard effects of fertilizers on human, soil and environmental health (Mia and Shamsudin, 2010).

There is one more beneficial aspect of exploitation of *Rhizobium* in agricultural practice that the excessive use of chemicals to control plant diseases lead to groundwater contamination, development of resistant races of pathogen, human health hazardous and environmental detrimental (Akhar *et al.*, 2009) and this shift the attention towards the plant growth-promoting Rhizobacteria (PGPR) which in recent years proved to be an alternative to chemicals by controlling the plant diseases effectively especially the soil and seed-borne pathogens (Beneduz *et al.*, 2012, Gupta *et al.*, 2015).

Materials and Methods

The experiments were carried out at the Department of Pathology and Microbiology, College of Biotechnology, SVP University of Agriculture and Technology, Modipuram, Meerut (U.P.).

Collection of samples

Plant samples were collected from the different locations of Muzaffarnagar such as Charthawal, Thanabhawan, Khatauli, Bhagra, Janshat, Budhana, and Purkaji. A total of 5 samples of urad, 27 samples of chickpea and 5 samples of moong were collected and taken from the field to the laboratory for the isolation of Rhizobium strains. The detail of the plant samples collection location was given in Table 1.

Isolation

The uproot plant samples are brought into the laboratory, tagged properly and named as U1–U5 for urad, C1-C27 for chickpea, and M1-M5 for moong. These samples were washed carefully so that nodules are not getting separated from the plant roots. Nodules from the respective leguminous crop plants are picked with sterile forceps. These nodules are

then surface sterilized (with the help of 1% sodium hypochloride) followed by washing with alcohol and distilled water. Then the nodules are placed onto a clean glass slide and smoothly crushed with another slide, so to obtain a juicy liquid containing the bacteria. This liquid substance is used as the inoculum for the isolation of *Rhizobium*.

The isolation is done on selective medium for *Rhizobium* i.e. YEMA (Yeast Extract Mannitol Agar) media through streak plate method. The streaked plates were incubated at 35^0C for 24 hours and are observed for the appearance of growth. White mucoid colonies appear on streaked Petri plates than it proved the presence of *Rhizobium*. These white and mucoid colonies are picked up separately and inoculated on nutrient agar slants in two replicates and incubated at 35^0C for 24 h. After incubation, if proper growth is there, slants were stored at 4^0C in refrigerator for further characterization.

Gram staining

Bacterial smear of different strains was prepared separately and fixed in flame. Smears were fixed by passing over a Bunsen burner flame and then stained with ammonium oxalate crystal violet for one minute. Then, it was washed with tap water and immersed in Gram's iodine for one minute. Again washed with tap water and blot dried smear was flooded with 95% ethyl alcohol (decolorize) for 30 sec.

It was again washed with water and blot dried carefully. Then it was counter stained with safranin, again washed with tap water and finally dried and examined under oil immersion objective on the microscope. Gram-negative bacteria retain the pink/red colour while Gram-positive bacteria retain the crystal-violet.

Biochemical characterization of Rhizobium

Biochemical characterization of different isolates was done for the identification of *Rhizobium* on the basis of different biochemical tests viz., Citrate utilization test, Triple sugar iron test, Glucose peptone agar utilization test, Gelatin liquefaction test, Starch utilization test and Catalase test.

Citrate utilization test

In this medium citrate is the only carbon source available to the bacteria; however, the Rhizobium cannot grow on the citrate and therefore, no change in colour occurs. To inoculate the slant, a loopfull of culture of Rhizobium was used; the slant was inoculated following stab and streak method and finally observed after incubation period of 24 h at 37^0C.

Triple sugar iron agar test

It was performed to determine the capability of isolates to use various carbohydrate sources (e.g. sucrose, glucose, lactose) as media for growth (Kligler, 1918; Hajnaa, 1945).

Starch utilization test

The test was performed to determine the capability of microorganism to use starch as carbon source (De Oliveria *et al.*, 2007). Starch agar media were inoculated with Rhizobium, incubated and analyzed. In the presence of starch, the production of extra cellular enzymes occurs indicating the potential of the organism to use starch as carbon source. Iodine test was used to determine capability of microorganisms to use starch. Drops of iodine solution were spread on 24 h old cultures grown in Petri-plates. Formation of blue color indicated non-utilization of starch and vice versa.

Glucose peptone agar (GPA) test

GPA assay was performed to determine the capability of the microorganism to utilize glucose as the sole carbon source for its growth (Singh *et al.,* 2008). GPA medium was inoculated with *Rhizobium* culture, incubated and growth was observed.

Gelatin test

This test was performed to determine capability of microorganisms to produce gelatinase enzyme and use gelatin as media source. Degradation of gelatin indicates the presence of gelatinase enzyme. The actively grown cultures were inoculated in nutrient gelatin medium and grown for 48 h. On subjecting the growing culture to low temperature treatment at 4^0C for 30 min, the cultures which produce gelatinase remain liquefied while others due to presence of gelatin become solid (Aneja, 2003).

Catalase test

This test was performed to study the presence of catalase enzyme in bacterial colonies. *Rhizobium* colonies (24 h old) were taken on glass slides and one drop of H2O2 (30%) was added. Appearance of gas bubble indicated the presence of catalase enzyme (MacFaddin, 1980).

Antibiotic sensitivity test

Antimicrobial discs used were of Ciprofloxacin (Cf), Ofloxacin (Of), Norfloxacin (Nx), Levofloxacin (Le), Aztreonam (Ao), Gatifloxacin (Gf), Nitrofurantoin (Nf), Co-trimoxazole (Co). All the isolates were inoculated in YEM broth separately and incubated at 37^0C for 12 h. The culture broth of each isolate was spread as thin layer separately on the nutrient agar medium (NAM) using a sterile cotton swab. Antimicrobial discs were placed on the inoculated plates of different isolates at a distance of approximately 2.5 cm and then incubated overnight at 37^0C. The sensitivity or the resistance of *Rhizobium* isolates to antibiotics was determined by observing the absence or presence of growth around the discs. Those isolates which show growth around a particular antibiotic are resistant to that corresponding antibiotic, whereas, the isolates whose growth is inhibited by a particular antibiotic seem to be sensitive to that particular antibiotic (Persuna, 2014)

Results and Discussion

A total of 37 root nodule samples collected from different leguminous crops (5 samples of urad, 27 samples of chickpea and 5 samples of moong) of Muzaffarnagar region were used for the isolation and identification of *Rhizobium* strains in the laboratory (Figure 1,2,3 and 4). The data in Table 2 revealed that among 37 samples tested, 21 (18 of chickpea, 3 of uard and none from moong) samples were found positive for the presence of *Rhizobium* on the basis of white mucoid growth on YEMA medium when incubated for 24 h at 35^0C (Figure 5 and 6). All the 21 samples that showed the presence of *Rhizobium* when subjected to Gram staining and all the isolates (18 from chickpea and 3 from uard) were found Gram-negative as the cells appeared pink after Gram staining. After Gram staining, all the isolates are preserved on nutrient agar medium slants for further characterization.

Biochemical characterization

Characterization of different isolates was done on the basis of different biochemical tests viz. Citrate utilization test, Triple sugar iron test, Glucose peptone agar utilization test, Gelatisn liquefaction test, Starch utilization test and Catalase test). The results of different biochemical tests are represented in Table 3 and Figures 7 and 8.

Table.1 Collection details of plant samples

S.No.	Location of sample	No. of samples	Crops
1.	Charthawal	10	Chickpea (C)
2.	Thanabhawan	10	Chickpea (C)
3.	Khatauli	5	Chickpea (C)
4.	Bhagra	2	Chickpea (C)
5.	Janshat	3	Urad (U)
6.	Budhana	2	Urad (U)
7.	Purkaji	5	Moong (M)

Table.2 Different samples showing the presence or absence of white mucoid colonies on YEMA after 24 h incubation

S.No.	Crop	Sample No.	Location	Creamy/white mucoid colonies
1.	Chickpea	C_1	Charthawal	-
		C_2	"	+
		C_3	"	+
		C_4	"	+
		C_5	"	-
		C_6	"	-
		C_7	"	-
		C_8	"	+
		C_9	"	+
		C_{10}	"	+
		C_{11}	Thanabhawan	-
		C_{12}	"	+
		C_{13}	"	+
		C_{14}	"	+
		C_{15}	"	+
		C_{16}	"	-
		C_{17}	"	-
		C_{18}	"	+
		C_{19}	"	+
		C_{20}	"	+
		C_{21}	Khatauli	+
		C_{22}	"	-
		C_{23}	"	+
		C_{24}	"	+
		C_{25}	"	+
		C_{26}	Bhagra	+
		C_{27}	"	-
2.	Urad	U_1	Janshat	+
		U_2	"	-
		U_3	"	+
		U_4	Budhana	+
		U_5	"	-
3.	Moong	M_1	Purkaji	-
		M_2	"	-
		M_3	"	-
		M_4	"	-
		M_5	"	-

*+: Positive result, -: no result (Negative results)

Table.3 Biochemical characterization of Rhizobium isolates

S. No.	Samples No.	Citrate utilization test	Starch utilization test	Glucose peptone agar test	Gelatin test	Catalase test
1.	U_1	-	-	+	-	+
2.	U_3	-	+	+	-	+
3.	U_4	-	+	+	-	+
4.	C_2	-	+	+	-	+
5.	C_3	-	-	+	-	+
6.	C_4	-	+	+	-	+
7.	C_8	-	+	+	-	+
8.	C_9	-	+	+	-	+
9.	C_{10}	-	+	+	-	+
10.	C_{12}	-	+	+	-	+
11.	C_{13}	-	-	+	-	+
12.	C_{14}	-	+	+	-	+
13.	C_{15}	-	+	+	-	+
14.	C_{18}	-	+	-	-	+
15.	C_{19}	-	+	+	-	+
16.	C_{20}	-	+	+	-	+
17.	C_{21}	-	+	+	-	+
18.	C_{23}	-	+	+	-	+
19.	C_{24}	-	-	+	-	+
20.	C_{25}	-	+	+	-	+
21.	C_{26}	-	+	+	-	+

*+: Positive result, -: no result (Negative results)

Table.4 Antibiotic sensitivity of *Rhizobium* isolates from leguminous root nodule samples

Sample/isolate	Reaction							
	Cf	Of	Nx	Le	Ao	Gf	Nf	Co
U_1	S	S	R	S	R	R	R”	R
U_3	S	S	R	S	R	S	S	S
U_4	S	S	S	S	R	S	S	R
C_2	S	R”	R”	R”	R	S	R”	R
C_3	S	S	S	S	R	S	R”	R
C_4	S	S	R	S	R	S	R	R
C_8	S	S	S	R”	R	S	R”	S
C_9	S	S	R”	S	R	S	S	R
C_{10}	S	S	S	S	R”	S	S	R
C_{12}	R”	S	S	S	R	S	S	R”
C_{13}	S	S	R	S	R	S	R”	R”
C_{14}	S	S	S	R”	R	S	R”	R
C_{15}	S	S	R	S	R	S	R	R
C_{18}	S	S	R”	S	R	S	S	R
C_{19}	S	S	S	S	R	S	R”	R
C_{20}	S	S	R	S	R”	S	S	S
C_{21}	S	S	S	S	R	S	S	S
C_{23}	S	S	R	S	R	S	R”	R
C_{24}	S	S	S	S	R	S	R”	R”
C_{25}	S	S	S	S	R	S	S	R
C_{26}	S	S	S	S	R	S	R”	R

*S:- Sensitive, R:- Resistance, R”:- Partial resistance

Fig.1 Field of Urad (*Vigna mungo*)

Fig.2 Collection of Urad sample from the field

Fig.3 Root nodules of Urad plant (*Vigna mungo*)

Fig.4 Root nodules of chickpea plant (*Cicer arietinum)*

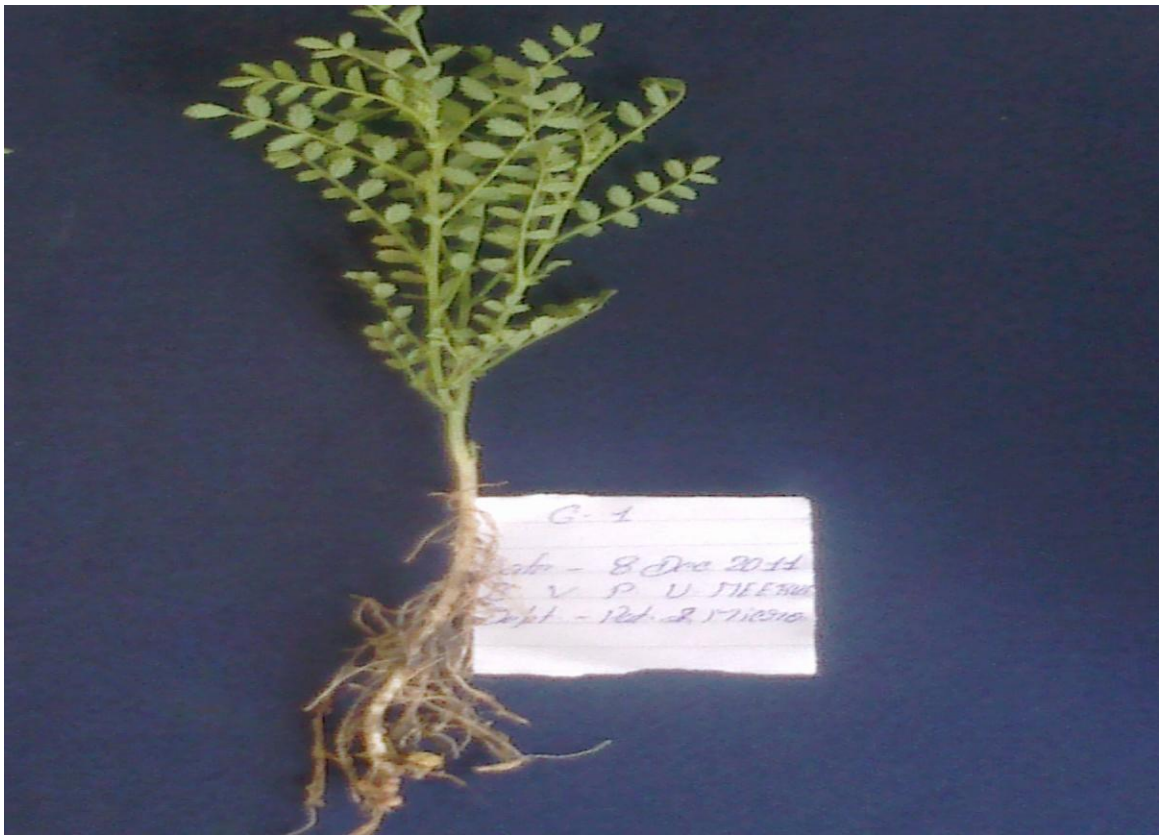


Fig.5 Isolation of a *Rhizobium* from Urad root nodules on yeast extract mannitol agar

Fig.6 Isolation of a *Rhizobium* from Chickpea root nodules on yeast extract mannitol agar

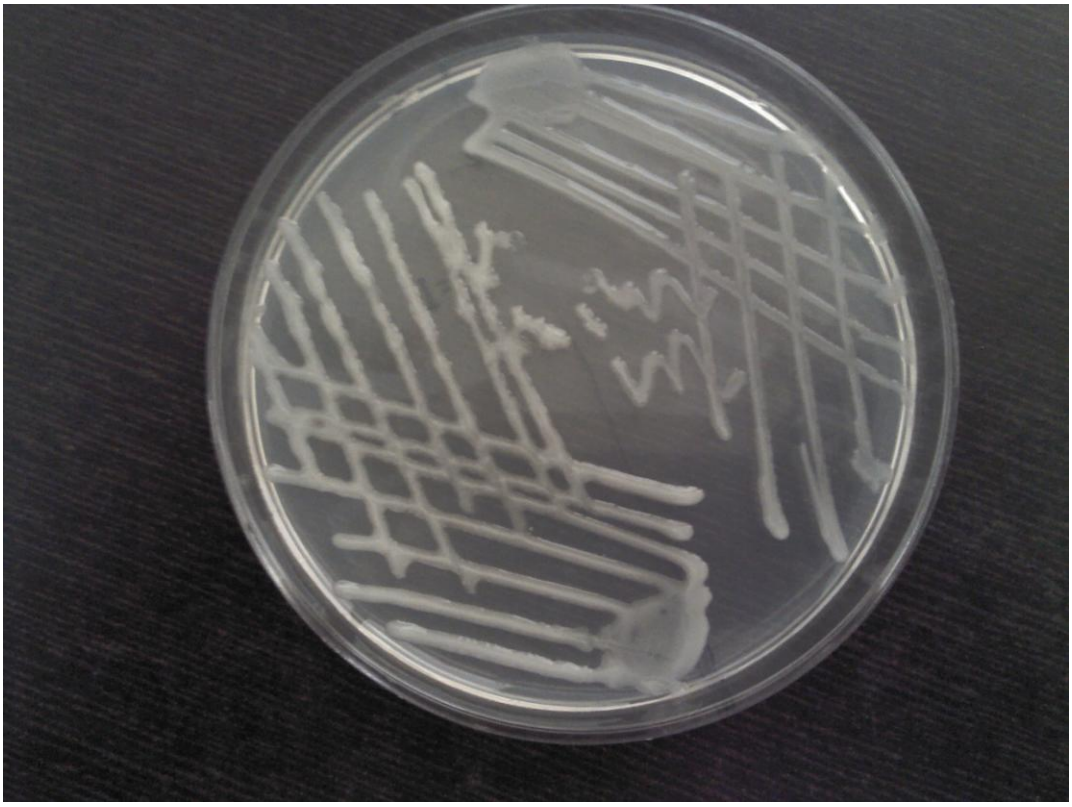


Fig.7 Biochemical reaction of *Rhizobium* on triple sugar iron agar slants

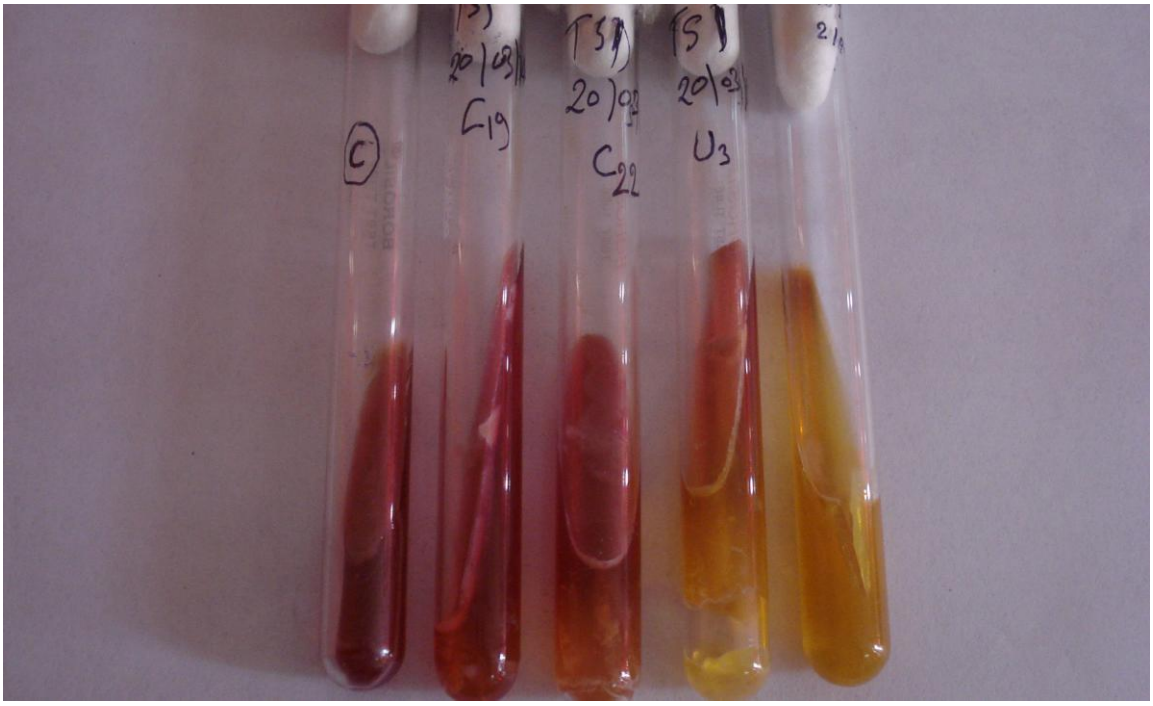
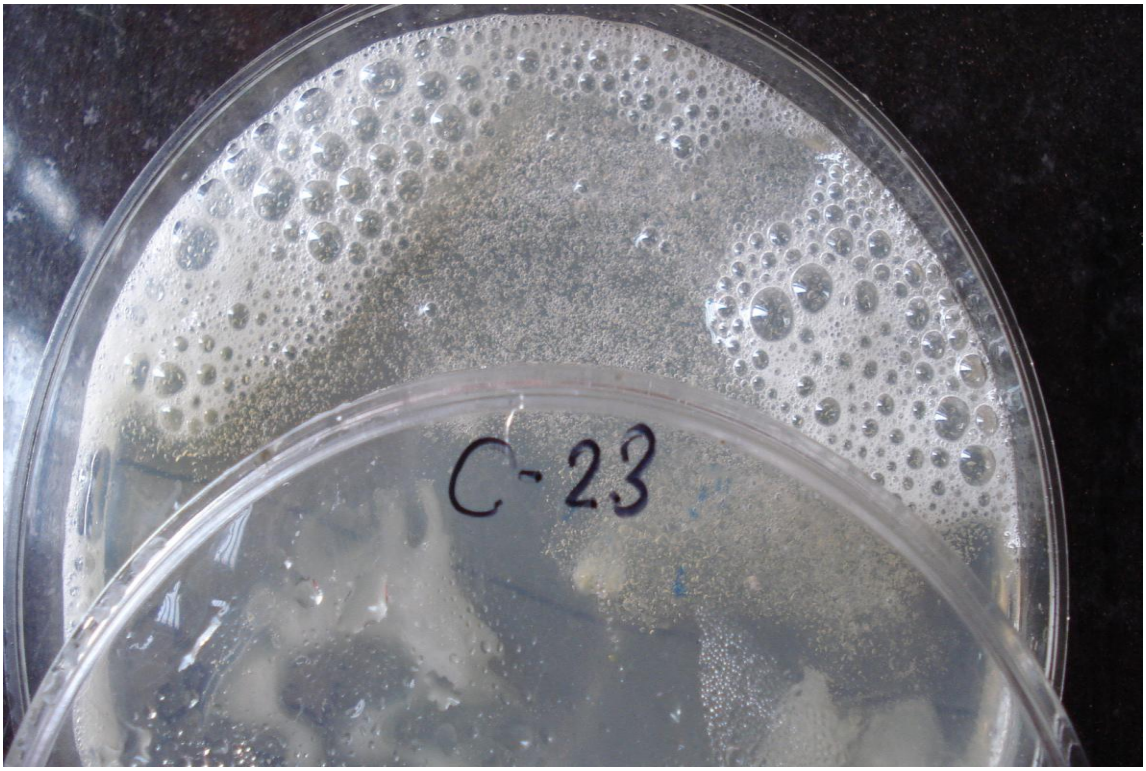


Fig.8 Bubble formation on inoculated yeast extract mannitol agar plate show positive catalase test



Triple sugar iron test

All the positive isolates were subjected to the (TSI) test by stab and streak method and were observed for fermentation of the sugar, such as glucose, sucrose and lactose. It was observed that nearly all the isolates except for U1, C3, C13 and C24, fermented the glucose sugar while they lack the ability to ferment both sucrose and lactose. While Glucose peptone agar test was positive with all isolates except for C 18, negative results were found with Gelatin test for all isolates and Catalase test was also found positive for all isolates.

Antibiotic sensitivity test

Antibiogram tests of all the 21 isolates of *Rhizobium* (comprising 3 uard samples and 18 chickpea samples) were performed. The results of antibiotic sensitivity are presented in Table 4. All the isolates were found sensitive to Ciprofloxacin (Cf) except C12 which was partially resistant, Ofloxacin (Of) except C2 which was partially resistant. Norfloxacin (Nx) was susceptible to 11 isolates, resistant to 7 isolates and partial resistant to 3 isolates. Levofloxacin (Le) and Gatifloxacin (Gf) were susceptible to all isolate but Levofloxacin (Le) was partial resistant to C3, C8 and C14. All the isolates, on the other hand, showed resistance to antibiotics Aztreonam (Ao) and Co-trimoxazole (Co) were showed resistant while Nitrofurantoin (Nf) showed partial resistance to isolates with few exception.

Rhizobium is a Gram negative bacterium which has the capability to fix atmospheric nitrogen. It is particularly important in legumes. In our study, *Rhizobium* was found in 21 samples out of 37 samples taken from leguminous crops. Samples are surface sterilized with $HgCl_2$ and the plating was done on YEMA medium. As a result, total 21 samples showing mucoid white colonies on YEMA plates including 3 from urad and 18 from chickpea were subjected to Gram staining and then characterized through biochemical tests. The isolates were identified as of *Rhizobium* on the basis of different biochemical tests

Our results match with those of De Oliveira *et al.*, (2007), who observed that *Rhizobium* strains have capability to use starch. Our *Rhizobium* strains found positive for utilization of glucose as carbon source; similar results were reported by Kucuk *et al.*, (2006). Almost all isolates are incapable of utilizing citrate as carbon source. The *Rhizobium* strains tested here are found negative for the production of gelatinase enzyme, similar to the findings of Hunter *et al.*, (2007). Negative gelatinase activity is a feature of *Rhizobium.* All the isolates were found positive for catalase test indicating the presence of catalase enzyme (Irum Naz, 2009). Most of the isolates were resistant to Aztreonam (Ao) and co-trimoxazole (Co), for other drugs they were mostly sensitive. It might be because of their (isolates) environmental origin, and therefore, sensitive to majority of the drugs.

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