Journal of Academia and Industrial Research (JAIR)
Volume 2, Issue 8 January 2014

ISSN: 2278-5213

SHORT COMMUNICATION

Isolation and Characterization of *Rhizobium leguminosarum* from Root nodule of *Pisum sativum* L.

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Abstract

Root nodules were collected from young and healthy seedling of *Pisum sativum* L. from farmer's field at different locations of Dehradun district, Uttarakhand state, India. Eighty one *Rhizobium* strains were isolated from the root nodule of *Pisum sativum* and characterized by standard biochemical tests. Average generation time was between 3.0 to 3.6 h which indicated that isolated rhizobia were fast grower. All strains were gram-negative and did not absorb red colour when cultured in YEMA containing congo red. Only 11.11% *Rhizobium* strains showed growth in the presence of 8% KNO_3 in the broth. Strains showed urea hydrolysis (9.87%), gelatinase activity (12.34%) and precipitation in calcium glycerophosphate (14.81%). All the strains utilized D-glucose, mannitol, D-fructose, L-arabinose as fermentation sugar. Only 16% *Rhizobium* strains tolerated 2% NaCl. Results confirmed that isolated strains were *Rhizobium leguminosarum*.

Keywords: Root nodules, *Pisum sativum*, average generation time, fermentation sugar, *Rhizobium leguminosarum*.

Introduction

Bacteria of family Rhizobiaceae are symbiotic and effectively convert atmospheric nitrogen which is utilized by the host. Rhizobiaceae family contains six genera namely *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium* and *Bradyrhizobium* (Okazaki *et al.*, 2004). Biofertilizer promotes plant growth and productivity has internationally been accepted as an alternative source of chemical fertilizer. Rhizobacteria effectively colonize plant root and increases plant growth by production of various plant growth hormones, P-solubilizing activity, N_2 fixation and biological control activity (Deshwal *et al.*, 2011).

Abd-Alla *et al.* (2013) mentioned that alkaline soils have fertility problems due to poor physical properties which adversely affect the growth and the yield of crops and inoculation with *Rhizobium* and AMF biofertilizer is more effective for promoting growth of faba bean grown in alkaline soils than the individual treatment, reflecting the existence of synergistic relationships among the inoculants. There are about 750 genera of legumes (Young and Haukka, 1996). Although most rhizobia are host specific, but it is also true that several different bacterial species are also isolated from a single legume species and it is only from limited hosts which have been examined as far as microsymbionts are concerned (Arora *et al.*, 2001). These rhizobia are characterized into two groups on the basis of growth rate. First group is fast grower rhizobia and second is slow grower rhizobia (Lohis and Hansen, 1921).

The slow growing bacteria have mean generation time greater than 6 h and fast growing bacteria have less than 6 h in selective broth medium (Elkan, 1992). Deshwal *et al.* (2003) reported the association of slow grower and fast growing rhizobia with *Arachis hypogaea* L. Both *Rhizobium* and *Agrobacterium* have placed in the family Rhizobiaceae in the order Eubacterials. Fred *et al.* (1932) observed that the *Agrobacterium* spp. too show colonies on YEMA medium indistinguishable from fast growing species of *Rhizobium*. Allen and Allen (1950) observed that YEMA medium containing congo red (1:400) is absorbed by *Agrobacterium*. On the other hand, *Bradyrhizobium* rapidly utilized hexose (galactose, gluconate, glucose and mannose) were able to lower the pH when supplied with any one of arabinose, galactose, glucose, mannose or xylose (pH 5-8) (Padmanabhan *et al.*, 1990). Rhizobia is characterized on the basis of biochemical tests. Gachande and Khansole (2011) isolated *Rhizobium joponicum* syn. and *Bradyrhizobium japonicum* from root nodules of Soy bean (*Glycine max* L.) on YEMA medium and its morphological, cultural and biochemical characteristics were studied. Against these backdrops, the aim of present study is to isolate rhizobia from nodules of pea plant and characterize them to ascertain their taxonomic position.

Materials and methods

Collection of plant: Root nodules were collected from young and healthy seedling of Pea plant (*Pisum sativum* L.) from farmer's field at different locations of Dehradun district, Uttarakhand state, India (Fig. 1).

Fig. 1. *Pisum sativum* L.

Isolation of root nodulating bacteria: Pea plants were uprooted carefully so as to get intact are obtained. These were brought in laboratory without any delay. Healthy peanut nodules were detached from the root and further isolation of root nodulating rhizobia was carried out (Vincent, 1970). The detached root nodules were washed in tap water to remove the adhering soil particles from nodule surface. Nodules were dipped in 0.1% mercuric chloride ($HgCl_2$) solution for 30 sec and later washed successively ten times with sterilized distilled water to remove the traces of toxic $HgCl_2$. Surface sterilized nodules were transferred in test tube containing 5 mL sterilized distilled water. These nodules were crushed with the help of sterilized glass rod to obtain a milky suspension of bacteriods. These were streaked on YEMA containing congo red. The plates were sealed by parafilm to avoid contamination and incubated at 28±1°C for 24-48 h. *Bradyrhizobium* or *Rhizobium* colonies were remained white, translucent, elevated and mucilaginous, after 24-72 h, where as contaminations turned red. The colony were picked up and transferred to YEMA slant for further characterization.

Generation time: The time required for a given cell or cell population to become double is referred as doubling time or generation time. Generation time can be determined by inoculating rhizobial isolate in 50 mL sterilized YEM broth in 150 mL conical flask and incubated at 28±1°C at 150 rpm for 24 h. The growth in term of turbidity was measured by taking optical density (OD) at 610 nm of the culture (sample) at every 4 h interval. The graph was plotted between OD vs. time and generation time was calculated using following formula:

$$\text{Generation time} = \frac{(T_2 - T_1)}{3.3\,(\log_{10} OD_2 - \log_{10} OD_1)}$$

Where (T_2-T_1) is the difference of two time intervals at any two point in log phase in growth curve; $(\log_{10} OD_2 - \log_{10} OD_1)$ is the difference between the $\log_{10}$ value of OD_2 at time T_2 and OD_1 at time T_1.

Biochemical tests: Biochemical tests such as growth on glucose peptone agar (Kleczkowska *et al.*, 1968), ability to produce 3-ketolactase (Gaur *et al.*, 1973), growth in presence of 8% KNO_3 (Idrissi *et al.*, 1996), growth on hofer's alkaline medium (Hofer, 1935), hydrolysis of urea (Lindstrom and Lehomarki, 1988), growth on 1, 2% NaCl (Sadowsky *et al.*, 1983), gelatinase activity (Idrissi *et al.*, 1996), H_2S (Zobell and Feltham, 1934), acid production in YEM broth (Jordan, 1984), catalase activity (Graham and Parker, 1964), acid reaction in litmus milk (Jordan, 1984), precipitation in calcium glycerophosphate (Hofer, 1941), starch hydrolysis, utilization of different carbon source was done.

Results and discussion

Root nodulating bacteria were isolated from pea plant. Average generation time was between 3.0 to 3.6 h which indicated that isolated rhizobia were fast grower. All strains were gram negative and did not absorb red colour when cultured in YEMA containing congo red (Fig. 2). Further, all strains were failed to grow in glucose peptone agar (GPA) medium. Hence, cultures were transferred on plates containing lactose in place of mannitol in YEMA medium (Fig. 3). After 24 h, plates were flooded by pouring Benedict's reagent, further incubated for 1 h. No yellow colour ring was formed around the colony. Rhizobia were not able to produce 3-Ketolactose. Only 11.11% *Rhizobium* strains showed growth in the presence of 8% KNO_3 in the broth. Strains showed urea hydrolysis (9.87%), gelatinase activity (12.34%) and precipitation in calcium glycerophosphate (14.81%). All the strains utilized D-glucose, mannitol, D-fructose, L-arabinose as fermentation sugar but only 86.41%, 80.24%, 86.41% *Rhizobium* strains utilized D-mannose, rhamnose and sucrose respectively (Table 1).

Fig. 2. Growth on CRYEMA medium.

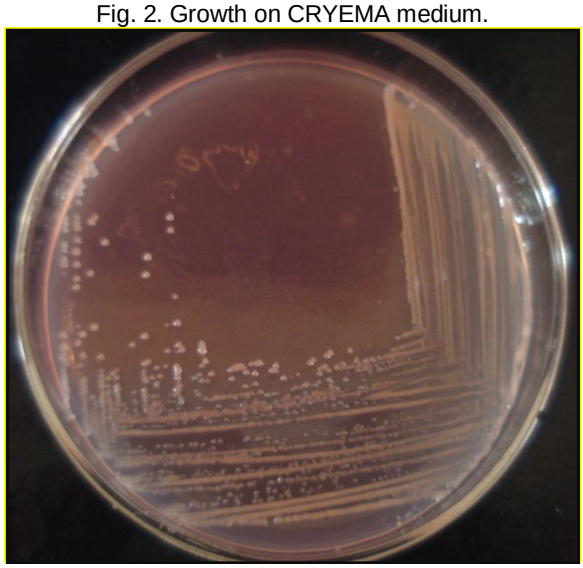
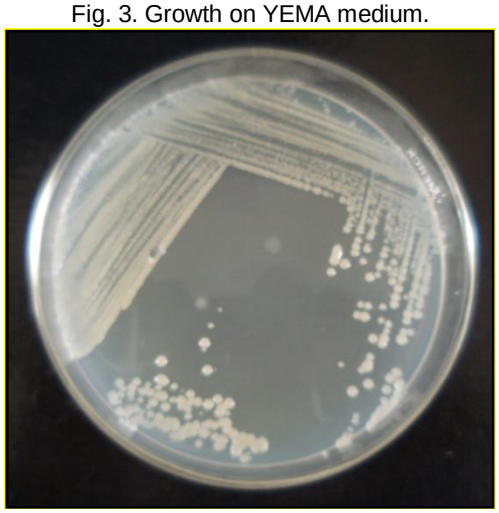


Fig. 3. Growth on YEMA medium.



Morphology and growth on CRYEMA medium increased the possibility of rhizobia. Wolde-Meskel *et al.* (2004) emphasized on the classical phenotypic characterization of *Rhizobia* which is helpful for primarily classification of rhizobia. Our isolated *Rhizobium* strains did not absorbed red colour from CRYEMA medium and all the biochemical tests confirmed that isolated strains were *Rhizobium leguminosarum*. Similarly Shetta *et al.* (2011) mentioned that *Rhizobium* strains failed to absorb congo red stain in the CRYEMA medium and generation time for YEMB cultures at 28°C ranged between 2.07 and 3.85 h.

Similarly, Shahzad *et al.* (2012) isolated *Rhizobium* from root nodules of Alfalfa (*Medico sativa*) plant and characterized on the basis of various biochemical tests. Previously, Sadowsky *et al.* (1983) mentioned that fast-growing soybean rhizobia were positive for catalase, urease, oxidase, nitrate reductase, tolerated 2% NaCl, capable to grow at pH 9.5 and fermented L-arabinose, D-fructose, D-galactose, D-glucose, D-mannitol, D-mannose, L-rhamnose and D-xylose. Similarly, Singh *et al.* (2008) also characterized *Rhizobium* strains on the basis of biochemical tests. *Rhizobium* is symbiotic bacteria which form nodule in leguminous plant. But *Agrobacterium* infects the root and forms false nodule (pseudo nodule) therefore, biochemical tests are essential to differentiate *Rhizobium* and *Agrobacterium.* All the biochemical test and cited literature suggested that isolated strains were *Rhizobium leguminosarum*.

Conclusion

All 81 *Rhizobium* strains did not absorb red colour when cultured in YEMA containing congo red medium. Pseudo-nodule forming bacteria *Agrobacterium* utilized congo red but *Rhizobium* strains didn't utilize congo red. This test is essential to differentiate *Rhizobium* and *Agrobacterium*. Other biochemical tests confirmed that isolated strains were *Rhizobium leguminosarum*. All *Rhizobium* strains tolerated 1% NaCl but only 16% *Rhizobium* strains tolerated 2% NaCl.

Table 1. Biochemical characterization of *Rhizobium leguminosarum* isolated from Pea plant.

Biochemical test	Test results	Percentage (%)
Gram reaction	-	100
Motility test (0.4%)	+	100
Motility test hanging drop method	+	100
Absorb congo red + YEMA	-	100
Growth in GPA	-	100
Ability to produce 3-Ketolactose	-	100
Growth in Hofer's alkaline medium	+	28.39
Growth in 8% KNO_3	-	11.11
Hydrolysis of Urea	-	9.87
Gelatinase activity	-	12.34
Growth in 1% NaCl	+	100
Growth in 2% NaCl	+	16
H_2S production	-	100
Catalase	+	100
Acid reaction in litmus milk	-	100
Ppt. in calcium glycerophosphate	-	14.81
Starch hydrolysis	-	00
Fermentation test		
D-glucose	+	100
Mannitol	+	100
D-fructose	+	100
L- arabinose	+	100
D-mannose	+	86.41
Rhamnose	+	80.24
Sucrose	+	86.41
Generation time (h)	3.2	

+ = Positive reaction, - = Negative reaction.

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