Characterization of *Rhizobium* strain isolated from the roots of *Glycine max (Soybean)*

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Abstract

Glycine max (Soybean) is a species of legume native to East Asia, widely grown for its edible bean which has numerous uses. The plant is classed as an oilseed rather than a pulse by the Food and Agricultural Organization .It is known for its dietary protein source, and symbiotic nitrogen fixation by *Rhizobium* present in its root nodules. The present study describes the characterization of a *Rhizobium* strain isolated from root nodules of *Soybean*. The *Rhizobium* isolates were rod shaped, gram negative, acid and mucous producing. They were found to be pH sensitive, with optimum values of 7.0°C. The bacteria was sensitive to the antibiotics streptomycin.

It utilizes glucose, sucrose and starch as sole carbon source. The *Rhizobium* species isolated from *Soybean* roots have the potential to produce industrially important enzymes; amylase and cellulase. The *Rhizobium* can be easily immobilized onto carriers like charcoal powder which can be applied as biofertilizer.

Key words: ***Soybean***, *rhizobium* isolation, biochemical analysis, immobilization, enzyme production

Introduction

Nutrient enrichment of soils by nitrogen fixing symbiotic bacteria present in legumes has been known for centuries. Scientific demonstration of this symbiosis was started in 19th century and it established the facts that bacteria present in nodules on legume roots are responsible for fixing atmospheric nitrogen (Zsbrau, 1999). *Rhizobium* spp. are well known group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. This symbiosis reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops (Dilworth and Parker, 1969)

The soybean (U.S.) or soya bean (UK) (Glycine max) is a species of legume native to East Asia, widely grown for its edible bean which has numerous uses. The plant is classed as an oilseed rather than a pulse by the Food and Agricultural Organization (FAO).Fat-free (defatted) soybean meal is a primary, low-cost source of protein for animal feeds and most prepackaged meals; soy vegetable oil is another product of processing the soybean crop. For example, soybean products such as textured vegetable protein (TVP) are ingredients in many meat and dairy analogues. Soybeans produce significantly more protein per acre than most other uses of land. Although it is well known fact that soybean is a good source of atmospheric nitrogen fixation by *Rhizobium* present in its root nodules, effort was not made to study the indigenous *Rhizobia* present in nodules of this plant. In the present study, we have isolated a strain of *Rhizobium* from the root nodule. Further characterization was done by performing various biochemical tests.

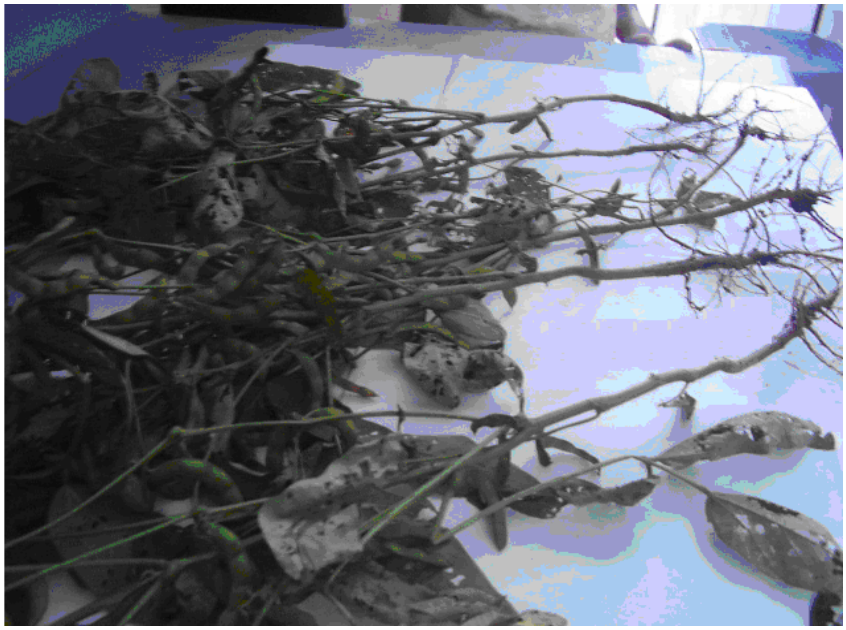

Figure 1. Roots of Soybean showing nodules developed by symbiotic bacteria, *Rhizobium*.

Materials and methods

Isolation of rhizobium from Soybean roots

The fresh and plump root nodules (Figure 1) of Soybean were collected from the plants grown in pots. The collected nodules were surface-sterilized with 75% ethanol and 0.1% mercuric chloride and washed thoroughly with distilled water. *Rhizobium* strain was obtained by streaking the crushed root nodules on YEM (yeast extract mannitol, pH 7.0) agar plates and

incubated at 29.4°C (Aneja, 2003). After 2 days of incubation, *Rhizobium* colonies were obtained. Further streaking, spreading and visual characterization of colony morphology helped in isolation of pure cultures of *rhizobium*. Pure isolates were used for further analysis and all tests were performed in triplicates.

Microbiological assays

The morphological traits evaluated comprised colony morphology, mucous production and change in pH of medium during growth and growth rate. Mucous morphology analysis was based on type, elasticity and appearance, while colony morphology parameters were diameter, form, transparency and color (Aneja, 2003). Gram staining reaction was performed to evaluate type of strain.

NaCl and pH variation assay

Rhizobium culture was grown in triplicates on YEM medium having different concentrations of NaCl ranging from 1 to 6% (w/v). Growth was determined by measuring the optical density at 600 nm after 48 h of inoculation .In order to analyze the effect of pH variations on the growth of

the organism, media were prepared with pH 4.0, 7.0 and 9.0. After inoculation, the plates were kept at 30.0°C separately to analyze the effect of pH.

Glucose peptone agar (GPA) and lactose assay

GPA assay was performed to determine the capability of the microorganism to utilize glucose as the sole carbon source for its growth.GPA medium (40 g/L glucose, 5 g/L peptone, 15 g/L agar, pH 7.0) was inoculated with *Rhizobium* culture, incubated and growth was observed. Similarly lactose assay was performed to determine the capability of the micro-organism to utilize lactose present in medium (10 g/L lactose, 5 g/L peptone, 3 g/L beef extract, 15 g/L agar, pH 7.0) as the sole carbon source for its growth.

Gelatin hydrolysis

The 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 (Aneja, 2003). The actively grown cultures were inoculated in nutrient gelatin medium (5 g/L peptone, 3 g/L beef extract, 12 g/L gelatin) and grown for 48 h. On subjecting the growing culture to low temperature treatment at 4°C for 30 min, the cultures which produce gelatinase remains liquefied while others due to presence of gelatin becomes solid.

Starch hydrolysis

The test was performed so as to determine capability of microorganism to use starch as carbon source (de Oliveira, 2007). Starch agar media (5 g/L peptone, 2 g/L potato starch, 3 g/L beef extract,15 g/L agar, pH 7.0) were inoculated with *Rhizobium*, incubated and analyzed. In the presence of starch, the production of extracellular 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 (0.1 N) were spread on 24 h old cultures grown on Petri-plates. Formation of blue color indicated non-utilization of starch and *viceversa*.

Fluorescence assay

The test was performed to determine the ability of the isolates to fluoresce (King, 1954). King's Medium (2 g/L peptone, 1.5 g/L, MgSO4, 1.5 g/L K2HPO4, 10 mL/L glycerol, 15 g/L agar, pH 7) was prepared and inoculated aseptically with *Rhizobium* cultures.Culture was incubated at 29.4°C and after 48 h observations were made under UV- light source.

Intrinsic resistance to antibiotics

The susceptibility or resistance of rhizobia to an antibiotic was assayed with the help of antibiotic disc test. The following antibiotics were used for analysis; streptomycin (25 μg/disc).

Production media optimization to scale up culture

The YEM medium for the growth of the *Rhizobium* contains various components and established to be best suited for the growth of the organism on laboratory scale. But in order to grow the *Rhizobium* on fermenter scale or other production purposes, the option of production media optimization cannot be left out. So the various components of the YEM media were varied or replaced and used at different concentrations for media optimization for *rhizobium*. The production media formulations were mainly focused on:

1) The sucrose was replaced by manitol at different concentrations while keeping the other constituents same as that in the normal YEM broth.

2) The concentration of yeast extract used was varied keeping the other constituents of the normal YEM broth same.

After inoculation with *Rhizobium* culture, the tubes were kept in orbital shaker incubator at 200 rpm at 30.0°C for 2 days. Samples were taken from each and its optical density (OD) was measured using the spectrophotometer at 600 nm.

Amylase and cellulase activity assay

The suspension cultures of *Rhizobium* were sonicated to disrupt the cells and centrifuged at 10000 rpm for 10 min to pellet down the debris. Supernatant with enzyme extract was transferred to new tube and used for assay.

Amylase activity was estimated by dinitrosalicylic acid (DNSA) method (Miller, 1959). DNSA reagent was prepared by dissolving 1g DNS, 1 g NaOH, 0.2 g phenol solid and 0.005 g sodium sulfite in 100 mL of water. 1% sucrose solution was used as enzyme substrate. In a tube, 0.4 mL of enzyme extract, 1.8 mL of the substrate and 2 mL of DNS were added and incubated at 37°C for 10 min. To stop the reaction, 1 mL of 40% solution of sodium potassium tartarate was added. Change in color was observed and OD was taken at 575 nm using a spectrophotometer. OD is proportional to the concentration of the enzyme present. The more the enzyme activity, the more the color change and thus, the higher the OD Cellulase activity was also estimated by dinitrosalicylic acid (DNSA) method. All the reagents used were the same as that used for the amylase activity assay instead of substrate, wherein 1% cellulose solution was used as substrate for the enzyme. Enzyme assay for both enzymes were performed with all media formulations as mentioned in Table 1.

Bio-fertilizer preperation

Once the pure culture of *Rhizobium* has been established and confirmed for its various activities, the next step was conversion of the rhizobia broth into a form which is easily used by farmers.*Rhizobium* cells were immobilized on carriers, which is an inert material used for mixing with broth so that inoculants can easily be handled, packed, stored, transported and used. The carrier (charcoal) was powdered and dried in sun to get 5% moisture level. Then it is screened

through 100-200 mesh sieves and neutralized by mixing with calcium carbonate powder and sterilized by autoclaving. If the carrier is neutral there is no need of mixing calcium carbonate powder. The broth containing rhizobial cells were mixed with carrier and kept in trays or tubes. The moisture content was maintained to about 35-40%. After proper mixing, it is left for 2-10 days by covering the trays with polythene at 22-24°C.During this period *Rhizobium* cells multiplied, a process called curing. Thereafter, *Rhizobium* inoculants can be used directly or packed and stored.

RESULTS AND DISCUSSION

Colonies of *Rhizobium* were obtained on YEM agar medium after incubation at 35.0°C for two days. The colonies were having sticky appearance showing the production of mucous though at lower levels. Analysis of colony morphology indicated round colonies, white colored till 3-4 days of growth and turning yellowish in color after 4 days. Typical colonies had a diameter of 5-7 mm. The pH of the medium and broth during growth of isolates was changed from 7.0 to 6.0, thus showing the production of acid which is the characteristic of *Rhizobium* to produce acid during growth (DeVries et al.1980; Baoling et al. 2007). Isolates were observed to be transient growers as colony becomes visible after 24 h of inoculation. General microscopic view of the isolates showed them to be rod cells and gram negative in nature.

pH is an important parameter for the growth of the organism. Slight variations in pH of medium might have enormous effects on the growth of organism. *Rhizobium* has been reported to grow the best at neutral pH i.e. 7.Our results indicated that cells were able to grow only at pH 7.0 and kept at 30.0°C. Less growth was observed in medium with pH 4.0 and 9.0. Rhizobial cells were able to grow on the GPA media showing the utilization of glucose as the carbon source by the *rhizobium*. It is a confirmatory test for *Rhizobium* and these are able to utilize glucose as carbon source (Kucuk et al., 2006). However, pure *Rhizobium* isolates are unable to grow on lactose. It was observed that rhizobial cells do not produce gelatinase enzymes as medium containing gelatin solidified when kept at 4°C for 30 as well as 60 min. Negative gelatinase activity is also a feature of *Rhizobium* (Hunter et al., 2007).Positive results were obtained from the starch hydrolysis assay. On subjecting inoculated plates to iodine test,clear zones around the colonies were seen and the colonies turned yellow in appearance, whereas blue color appears on no growth areas. This indicates that the isolates have the potential to hydrolyze starch present in the medium. De Oliveira et al. (2007) also observed that *Rhizobium* strains can

utilize starch obtained from different sources. *Rhizobium* cells grown on King's Medium under the UV source shows the absence of ability of the organisms to fluoresce. The *Rhizobium* isolates were susceptible to streptomycin. The diameter of zone of inhibition of bacterial growth was found to be 18 mm for streptomycin. The concept of production media optimization was to establish a media which shows the optimum conditions for the growth of the organism at cheap cost as compared to normal media. Once the production media have been optimized, this can be used to scale up biomass yields using bioreactors/fermentors. So the optimization of Mannitol concentration was done from 5 gm/ltr-20 gm/ltr and the optimum growth concentration was found to be 10 gm/ltr. The optimization of Yeast extract conc was also done & it was found that at 0.1gm per litre.conc yeast extract gave max results.(Table 1)

S.No.	Media constituent varied with Conc./litre	Absorbance at 600 nm after 6 hrs.
	Mannitol	
1.	5 gm.	0.430
2.	10 gm.	1.523
3.	15 gm.	0.855
4.	20 gm.	0.356
	Yeast extract	
1.	0.5 gm.	1.233
2.	1.0 gm	1.640
3.	1.5 gm.	0.070
4.	2.0 gm.	0.155

Carbohydrate utilization assays indicated that *Rhizobium* isolates obtained from Soybean roots were able to utilize different carbohydrate sources, thus it was assumed that they may produce important enzymes like amylase, cellulases, etc. Amylases are among the most important enzymes and are of great significance in present-day biotechnology, having approximately 25% of the enzyme market (Rao et al., 1998). With increased biomass yield, high activity of amylase and cellulase indicated that Rhizobium cells can be used at industrial scale for production of these enzymes. Although we have also developed biofertilizers using charcoal as carrier; however the field trials are yet to be conducted.

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