African Journal of Biotechnology Vol. 7 (20), pp. 3671-3676, 20 October 2008
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB08.752
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Characterization of *Rhizobium* strain isolated from the roots of *Trigonella foenumgraecum* (fenugreek)

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Accepted 29 September, 2008

***Trigonella foenumgraecum* (fenugreek) is known for its dietary protein source, medicinal properties 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 fenugreek. The *Rhizobium* isolates were rod shaped, gram negative, acid and mucous producing. They were found to be temperature and pH sensitive, with optimum values of 29.4 and 7.0°C, respectively. The bacteria was sensitive to the antibiotics; chloramphenicol, kanamycin and streptomycin. It utilizes glucose, sucrose and starch as sole carbon source. The *Rhizobium* species isolated from fenugreek roots have the potential to produce industrially important enzymes; amylase and cellulase. Immobilizing the organism in agar and agarose does not affect its activity; indeed increased biomass yield and enzyme production was observed. The *Rhizobium* can be easily immobilized onto carriers like charcoal powder which can be applied as biofertilizer.**

Key words: Fenugreek, *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).

Fenugreek (*Trigonella foenumgraecum* L.), like other legumes, is a good source of dietary protein for consumption by man and animals. From ancient times, Greeks (and the Romans) used it as medicine, spice and cattle fodder and so it was and still known as Greek hay. Seeds of fenugreek are used as a yellow dye, in cosmetics and for medicinal purposes. Fenugreek is a good soil renovator and widely used as a green manure (Saeed and Elsheikh, 1995). *Rhizobium* inoculation of fenugreek has been reported to increase the biomass of plant and seed production (Poi et al., 1991). Fenugreek was reported to fix 48% of its total N_2 during the growing season (Desperrier et al., 1985). Although it is well known fact that fenugreek 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 and we also determined if the rhizobial cells can be efficiently immobilized on matrices to produce industrially important enzymes.

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

MATERIALS AND METHODS:

Isolation of rhizobium from fenugreek roots

The fresh and plump root nodules (Figure 1) of fenugreek 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, pH and temperature 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 29.4 and 37°C separately to analyze the effect of temperature along with pH.

Methylene blue and gentian violet treatment

In this assay, methylene blue dye was added to a concentration of 0.1% to the growth medium and inoculated with *Rhizobium*. Incubation was given at 29.4°C for 2-7 days and observations were made. Similar experiment was done with gentian violet at the concentration of 0.1% (Gao et al., 1994).

Glucose peptone agar (GPA) and lactose assay

GPA assay was performed to determine the capability of the micro-organism 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 micro-organims 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 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 (0.1 N) were spread on 24 h old cultures grown on Petri-plates. Formation of blue color indicated non-utilization of starch and *vice versa*.

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 $MgSO_4$, 1.5 g/L K_2HPO_4, 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.

Triple sugar iron agar test

The test was performed to determine the capability of isolates to use various carbohydrate sources e.g. sucrose, glucose, lactose, etc as media for growth. Triple Sugar Iron Agar media consisted of beef extract 3 g/L, yeast extract 3 g/L, peptone 15 g/L, NaCl 5 g/L, lactose 10 g/L, sucrose 10 g/L, dextrose 1 g/L, ferrous sulfate 0.2 g/L, sodium thiosulfate 0.3 g/L, phenol red 0.24 g/L, agar 15 g/L, pH 7.0 (Kligler, 1918; Hajnaa, 1945). After inoculation and incubation,

Table 1. Composition of various media formulation used for production media optimization and also for amylase and cellulase activity assays.

Media name	YEM medium Glucose	Other components	
G1	6 g/L	Yeast extract	4.0 g/L
G2	8 g/L	K_2HPO_4	0.5 g/L
G3	10 g/L	$MgSO_4$	0.2 g/L
G4	12 g/L	NaCl	0.1 g/L
G5	14 g/L		
	Sucrose		
S1	6 g/L	Yeast extract	4.0 g/L
S2	8 g/L	K_2HPO_4	0.5 g/L
S3	10 g/L	$MgSO_4$	0.2 g/L
S4	12 g/L	NaCl	0.1 g/L
S5	14 g/L		
	Yeast extract		
Y1	0.5 g/L	Sucrose	10 g/L
Y2	2 g/L	K_2HPO_4	0.5 g/L
Y3	6 g/L	$MgSO_4$	0.2 g/L
		NaCl	0.1g/L
F	$FeSO_4$ (0.2 g/L) instead of $MgSO_4$	sucrose	10 g/L
		Yeast extract	1 g/L
		K_2HPO_4	0.5 g/L
		NaCl	0.1 g/L
N	Na_2HPO_4 (0.5 g/L) instead of K_2HPO_4	sucrose	10 g/L
		Yeast extract	1 g/L
		$MgSO_4$	0.2 g/L
		NaCl	0.1 g/L

color on the butt and the slant was observed. On this basis capability of organisms to use carbohydrates, three possible observations could occur.

Alkaline (red) slant and acid (yellow) butt

Only glucose fermentation has taken place. In the slant, acid produced is oxidized and alkali is formed. In the butt, acid reaction is maintained because of less oxygen and the slow growth of organisms.

Acid (yellow) slant and acid (yellow) butt

Lactose and sucrose fermentation has occurred. Because of presence of these substances in higher concentrations in the media, acid reaction is maintained in both slant and butt.

Alkaline (red) slant and alkaline (red) butt

No carbohydrate fermentation occurred. Peptones are catabolised to form alkaline pH.

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; erythromycin (10 µg/disc), ampicillin (25 µg/disc), chloramphenicol (30 µg/disc), Kanamycin (30 µg/disc) and 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 are summarized in Table 1 and were mainly focused on:

1) The sucrose was used in different concentrations as compared to normal YEM medium.
2) The sucrose was replaced by glucose at different concentrations while keeping the other constituents same as that in the normal YEM broth.
3) The concentration of yeast extract used was varied keeping the other constituents of the normal YEM broth same.
4) Instead of $MgSO_4$, $FeSO_4$ was used.
5) Potassium salt used was replaced by the sodium salt i.e. instead of K_2HPO_4, Na_2HPO_4 was used.

After inoculation with *Rhizobium* culture, the tubes were kept in orbital shaker incubator at 185 rpm at 29.4°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 3000 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 1 g 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.

Immobilization of *Rhizobium* cells

The purpose was to study the effect of immobilization on *Rhizobium* cells in matrices. *Rhizobium* cells were immobilized onto two matrices: agar-agar and agarose, as described by Adinarayana et al. (2005). Inoculum was prepared by adding five milliliters of sterile distilled water to a 48-h old slant of *Rhizobium*. The cells were scrapped from the slant into sterile distilled water and the resulted cell suspension was transferred asceptically into 250 mL Erlenmeyer flasks containing 45 mL of sterile inoculum medium. The flask was kept in an incubator shaker at 185 rpm at 29.4°C. The content of the flasks was centrifuged at 3000 rpm for 10 min and the supernatant was decanted. The cell pellet was washed thoroughly with sterile potassium chloride (20.0 g/L) solution and sterile distilled water subsequently. Finally the cell mass was suspended in sterile sodium chloride solution (9.0 g/L). This cell suspension was used as inoculum for immobilization.

A definite quantity of agar-agar was dissolved in 18 mL of 0.9% sodium chloride solution to get final concentration of 2% and sterilized by autoclaving. The cell suspension (2 mL) was added to the molten agar-agar maintained at 40°C, mixed well and poured into sterile Petri plates. The solidified agar blocks were cut into small equal size cubes. Cube of each agar and agarose were used as inoculum for fresh media. Inoculated flasks were kept in an incubator shaker at 185 rpm at 29.4°C for 2-3 days and assayed for biomass yield and enzyme production (amylase and cellulase). Similarly, immobilization was done using agarose.

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 29.4°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.

It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumes. Hashem et al. (1998) have proposed that salt stress may decrease the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis, and hence nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting very early symbiotic events, such as chemotaxis and root hair colonization, thus directly interfering with root nodule function. To date, some rhizobial isolates have been shown to grow under high salt conditions (4-5%) (Kucuk et al., 2006). Our results indicated that cells were able to grow on 1% NaCl containing medium but were unable to grow on higher concentrations, thus showing that the isolate was sensitive to the salt.

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 29.4°C. No growth was observed in medium with pH 4.0 and 9.0. Also cells were unable to grow when incubated at 37°C even in pH 7.0. Thus medium with pH 7.0 and temperature 29.4°C are the optimum parameters for growth of the organism. Our results were in agreement with previous studies (Gao et al., 1994; Kucuk et al., 2006; Baoling et al., 2007).

Methylene blue and gentian violet are used as an agent against the growth of the microorganisms. Rhizobial

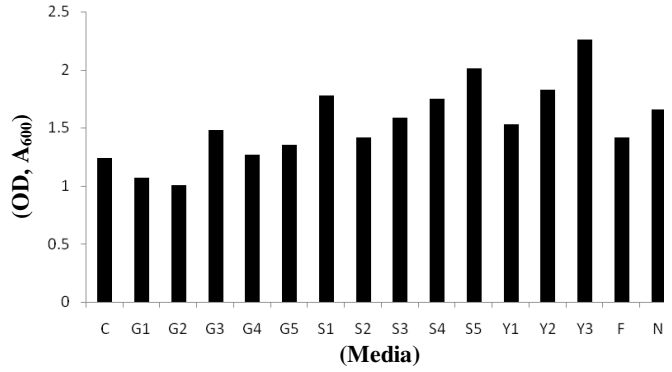

Figure 2. Biomass yield (OD_{600}) of *Rhizobium* in various media formulations.

cells were unable to grow either on medium containing 0.1% methylene blue and 0.1% gentian violet. Earlier studies also indicated that rhizobial cells were unable to grow in the presence of the two dyes (Wei et al., 2003).

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. Yellow slants and red butt were obtained showing the utilization of glucose and sucrose in the triple sugar iron agar medium (Hajnaa, 1945). No such studies have been conducted on *Rhizobium* strains.

The *Rhizobium* isolates were resistant to erythromycin and ampicillin at the amount of antibiotics under observation. However, it was susceptible to chloramphenicol, kanamycin and streptomycin. The diameter of zone of inhibition of bacterial growth was found to be 3.5, 2.5 and 2 cm for chloramphenicol, kanamycin and streptomycin, respectively. There are 3 known determinants of bacterial permeability to an antibiotic: hydrophobicity, electrical charge, and amount of the antibiotic (Hungaria et al., 2000) and the *Rhizobium* that showed a high level of resistance did not take up the antibiotics. However detailed study is needed to evaluate this fact.

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. *Rhizobium* strains were able to utilize glucose and sucrose more efficiently than normal YEM medium (Kucuk et al., 2006). Once the production media have been optimized, this can be used to scale up biomass yields using bioreactors/fermentors. There was overall increase in biomass yield for media formulations except G1 and G2 (Table 1) as compared to normal YEM medium (Figure 2). The increase in the biomass yield was very high for G3, S5 and Y3 (Table 1). Also the change in salts showed increased biomass yield as compared to normal YEM broth. These results indicated that these can be used as the production media for the growth of rhizobium in the fermentation and other processes where higher yield of the biomass is desired.

Rhizobium being a micro-organism produces various enzymes like nitrogenase (Baoling et al., 2007). Carbohydrate utilization assays indicated that *Rhizobium* isolates obtained from fenugreek 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). Cellulase is another such industrially important enzyme (Morales et al., 2005). Indeed, rhizobium strains can produce amylase (De Oliveira et al., 2007) and cellulase (Morales et al., 2005). The amylase production was maximum when Na_2HPO_4 was used instead of K_2HPO_4. Also good yield of the enzyme was also seen in the case of G1, S2, Y1 and N (Figure 3A). Cellulase activity was negligible in YEM medium (C) (Figure 3B). The highest production was observed in N medium as compared to other media formulations. Morales et al. (2005) also reported that production of cellulase was less in YEM broth as compared to other media.

Earlier AbdelGadir and Alexander (1997) reported that rhizobial cells can be immobilized on calcium alginate beads; their work was mainly focused on isolation of heat tolerant strains of rhizobia not on production of useful enzymes. The use of immobilized cells as industrial catalysts can be advantageous compared to batch fermentation process. Whole cell immobilization has been a better choice over enzyme immobilization (Adinarayana et al., 2005). Biomass yield was found to be high in immobilized cells as compared to normal YEM broth culture. A_{600} was found to be 1.852 in immobilized cells as compared to 1.242 for YEM broth cultures.

For amylase activity, A_{575} was found to be 1.110 in case of immobilized cells as compared to 1.097 for normal YEM culture. For cellulase activity, A_{575} was found to be 0.73 in case of immobilized cells as compared to 0.001 for normal YEM culture. Similarly, an increase in biomass yield, amylase and cellulase activities was observed when rhizobial cells were immobilized on agarose. These

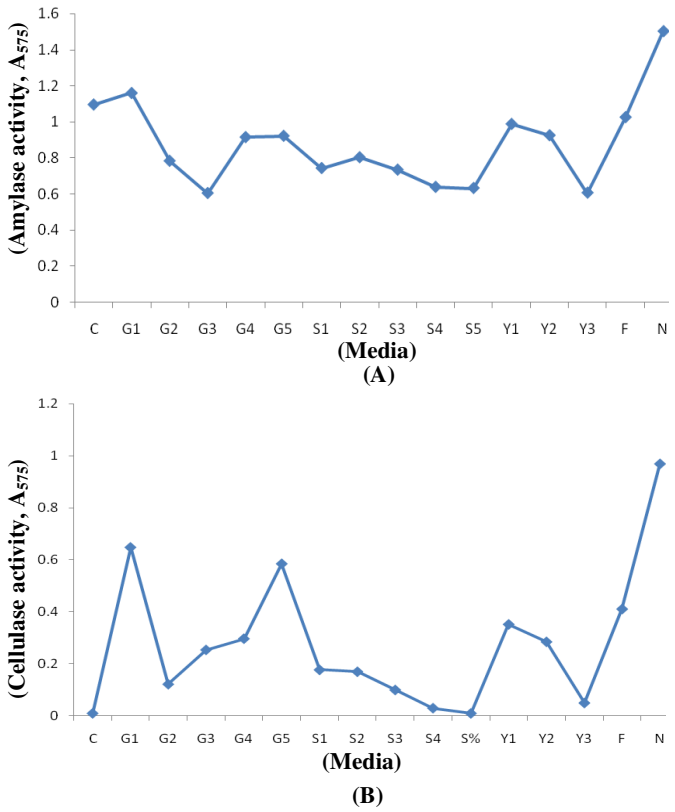

Figure 3. Activities of amylase (A) and cellulase (B) in different media formulations.

results with increased biomass yield, high activity of amylase and cellulase indicated that immobilized *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.

ACKNOWLEDGEMENT

Authors are thankful to the Principal, Shaheed Udham Singh College of Engineering and Technology, Mohali Punjab, India for providing facilities to carry out a part of research work.

REFERENCES

AbdelGadir AH, Alexander M (1997). Procedures to enhance heat resistance of *Rhizobium*. Plant Soil 188: 93-100.

Adinarayana K, Jyothi, B, Ellaiah P (2005). Production of Alkaline Protease With Immobilized Cells of *Bacillus subtilis* PE-11 in Various Matrices by Entrapment Technique. AAPS Pharm. Sci. Tech. 6, E391-E397. (http://www.aapspharmscitech.org).

Aneja KR (2003). Experiments in Microbiology Plant Pathology and Biotechnology. 4th edition, New Age International Publishers, New Delhi, India.

BaoLing H, ChengQun L, Bo W, LiQin F (2007). A rhizobia strain isolated from root nodule of gymnosperm *Podocarpus macrophyllus*. Sci. Chin. Ser. C-Life Sci. 50: 1-6.

de Oliveira AN, de Oliveira LA, Andrade JS, Chagas JAF (2007). *Rhizobia* amylase production using various starchy substances as carbon substrates. Braz. J. Microbiol. 38: 208-216.

Desperrier N, Baccou JC, Sauvaire Y (1985). Nitrogen fixation and nitrate assimilation in field grown fenugreek (*Trigonella foenumgraecum*). Plant Soil 92: 189-199.

DeVries GE, Veld I, Kijne P (1980). Production of organic acids in *pisum sativum* root nodules as results of oxygen stress. Plant Sci. Lett. 20: 115-123.

Dilworth MJ, Parker CA (1969). Devolopment of the nitrogen fixing system in legumes. J. Theor. Biol. 25: 208-218.

Gao JL, Sun JG, Li Y, Wang ET, Chen WX (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province, Chin. Int. J. Syst. Bacteriol. 44: 151-158.

Hajnaa A (1945). Triple-Sugar Iron Medium for the identification of the intestinal group of bacteria. J. Bacteriol. 49: 516-517.

Hashem FM, Swelim DM, Kuykendall LD, Mohamed AI, Abdel-Wahab SM, Hegazi NI (1998). Identification and characterization of salt and thermo-tolerant Leucaena nodulating *Rhizobium* strains. Biol. Fert. Soil. 27: 335-341.

Hungaria M, Andrade DS, Chueira LM (2000). Isolation and characterization of new efficient and competitive bean (*Phaseolus vulgaris* L.) rhizobia in Brazil. Soil Biol. Biochem. 32: 1515-1528.

Hunter WJ, Kuykendall LD, Manter DK (2007). *Rhizobium selenireducens* sp. nov.: A Selenite-Reducing *α*-Proteobacteria Isolated From a Bioreactor. Curr. Microbiol. 55: 455-460.

King EO, Ward MK, Raney DE (1954). Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44: 301-307.

Kligler IJ (1918). Modifications of culture media used in the isolation and differentiation of typhoid, dysentery, and allied bacilli. J. Exp. Med. 28: 319-322.

Kucuk C, Kivanc M, Kinaci E (2006). Characterization of *Rhizobium* Sp. Isolated from Bean. Turk. J. Biol. 30: 127-132.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31: 426.

Morales VM, Martinez-Molina E, Hubbell DH (2005). Cellulase production by Rhizobium. Plant Soil. 80: 407-415.

Poi SC, Basu TK, Behari K, Srivastav A (1991). Symbiotic e€ectiveness of di€erent strains of *Rhizobium* meliloti in selecting inoculants for improvement of productivity of *Trigonella foenum-graecum*. Environ. Ecol. 9: 286-287.

Rao MB, Tanksale AM, Gathe MS, Deshpande VV (1998). Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 62: 597-635.

Saeed HA, Elsheikh EAE (1995). Response of fenugreek (*Trigonella foenumgraecum*) to inoculation as influenced by salinity, soil texture, chicken manure and nitrogen. *Univ. Khartoum* J. Agric. Sci. 3: 77-90.

Wei GH, Tan ZY, Zhu ME, Wang ET, Han SZ, Chen WX (2003) Characterization of rhizobia isolated from legume species within the genera *Astragalus* and *Lespedeza* grown in the Loess Plateau of China and description of *Rhizobium* loessense sp. nov. Int. J. Syst. Evol. Microbiol. 53: 1575-1583.

Zsbrau HH (1999). *Rhizobium*-Legume symbiosis and nitrogen fixation under sever conditions and in arid climate. Microbiol. Mol. Biol. Rev. 63: 968-989.