Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 6 (9): 715-723, 2011
ISSN 1816-4935 / DOI: 10.3923/jm.2011.715.723

Optimization and Comparative Study of the Sugar Waste for the Growth of *Rhizobium* Cells Along with Traditional Laboratory Media

[1]Ashok Kumar Singh, [1]Gauri, [2]Rajendra Prasad Bhatt and [1]Shailja Pant
[1]Department of Microbiology, Dolphin (P.G) Institute of Biomedical and Natural Sciences, Manduwala, Dehradun (Uttarakhand), India
[2]Department of Botany and Microbiology, H.N.B. Garhwal University (A Central University), SriNagar, Garhwal (Uttarakhand), India

Corresponding Author: Gauri, Department of Microbiology, Dolphin (P.G) Institute of Biomedical and Natural Science, Manduwala, Dehradun (Uttarakhand), India Tel: 9568011274

ABSTRACT

To produce good quality nitrogen inoculants, it is desired to obtain effective rhizobia with good nodulation and nitrogen fixing capacity to the host plant. It is therefore, required to find a nutrient medium for their growth and optimize the pH, temperature, incubation period and aeration. This study was conducted to evaluate the use of sugar waste (molasses) as an alternative growth medium for the cultivation of *Rhizobium* and to determine the optimum environmental parameters such as pH and temperature. Growth and population count of *Rhizobium trifolii* (MTCC 905) at different concentrations of sugar waste was monitored by recording Optical Density (OD) and Colony forming unit (cfu mL^{-1}). Growth and population count were highest at 10% concentration i.e., 0.706±0.012 and 8.98±0.12, respectively. After optimizing the concentration of sugar waste growth pattern of *R. trifolii* was observed at 10% sugar waste concentration along with different synthetic media by recording Optical Density (OD) at 600 nm after 12 to 60 h. Cells of fast growing rhizobia grow rapidly on medium containing only 10% sugar waste and growth was superior to that of the control (standard media) used for *Rhizobium*. A pH of 7.0, temperature of 28°C and 48 h incubation period were most appropriate for *Rhizobium trifolii* (MTCC 905). The present study concludes that 10% sugar waste is complete medium for the rhizobial inoculants.

Key words: Growth medium, inoculants, *Rhizobium trifolii,* sugar waste, synthetic media

INTRODUCTION

The economic and the environmental costs of agricultural production have been increased by non judicious use of chemical fertilizers and its techniques of production and application. Consequently, the environmental problems have magnified due to the pollution of air, soil and water sources. The biological fertilizers may be incorporated as additional tools which can be economical and clean alternatives for the sustainable management of ecosystems (Mishra *et al*., 2006). Thus, the initial step in the legume inoculant production is the large number of selected *Rhizobium* sp. in liquid medium (Thompson, 1991). Therefore, it is the need of the present scenario to develop an economical as well as productive formulation and nutrient medium for the growth of such rhizobial cells. Most of the researchers choose YEM (Yeast Extract Mannitol) medium for rhizobial culture which contains mannitol or glycerol as a carbon source, yeast extract as a source of nitrogen, mineral salts and growth factors (Verma *et al*., 2010; Fahmi *et al*., 2011;

Yadav *et al*., 2011) due to these costly ingredients of that medium making it not suitable for commercial production of biofertilizers (Rebah *et al*., 2002).

In view of the growing demand of rhizobial inoculants it is imperative to search cheap and readily available substances to these costly ingredients. Numerous investigators have looked for ways of producing biofertilizers using low cost media. A variety of agricultural and industrial by-products such as proteolyzed pea husks, molasses and water hyacinth (Gulati, 1979), malt sprouts (Bioardi and Ertola, 1985), deproteinized leave extracts (Chanda *et al*., 1987), cheese whey (Estrella *et al*., 2004), waste water sludge (Ben Rebah *et al*., 2007) and Jaggery solution (Jain *et al*., 2000). These products contain growth factors, carbon and/or nitrogen source and to support the growth of rhizobia equal to or better than the known growth in the available media. Molasses is also used as a substrate for the production of Biosurfactants by Panesar *et al*. (2011).

Molasses is the final effluent obtained in the preparation of sucrose by repeated evaporation, crystallization and centrifugation of juices from sugar cane and sugar beat (Tunuguntla and Sullivan, 2004). Beside carbon and nitrogen source molasses is also an excellent source of manganese, copper, iron, calcium, potassium, magnesium, vitamin B6 and selenium (Aslan *et al*., 1997) and have high Ash and crude protein content (Paviz *et al*., 2011). The objective of the present study was to optimize the molasses as a cultivation medium and growth conditions (pH and temperature) for *Rhizobium trifolii* (MTCC 905).

MATERIALS AND METHODS

This study was conducted in Microbiology Department of Dolphin (P.G) Institute of Biomedical and Natural Science, Dehradun (Uttarakhand), India in the year 2009.

Rhizobium trifolii (MTCC 905) was used as model strain and obtained from Microbial Type Culture Collection (MTCC) from the Institute of Microbial Technology (IMTECH), Chandigarh, India.

Sugar waste as a substrate: The sugar waste was collected from Uttarakhand State Sugar Corporation Ltd. Unit: Doiwala, Dehradun, Uttrakhand.

Chemical analysis of sugar waste: The pH, Dissolved Oxygen (DO), Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and reducing sugar content of sugar waste was studied (APHA, 1992).

Starter culture: The culture (50 mL) was prepared by inoculating *Rhizobium* strain into Tryptone Yeast extract (TY) broth and *Rhizobium* Minimal Medium (RMM), respectively and incubated at 28±2°C for 16-24 h.

Media optimization

Optimization of sugar waste: The starter culture (1%) was inoculated into different concentration of sugar waste i.e., 10, 20 up to 100% and growth was monitored by recording Optical Density (OD) and colony forming unit (cfu mL^{-1}) at regular time interval. The viable count of bacterial population was also observed (Cappuccino and Sherman, 2001).

Comparison of sugar waste with different lab media: Effect of different media i.e., 10% sugar waste, RMM and TY on *Rhizobium* was monitored in terms of absorbance at 600 nm after every 12 up to 60 h by inoculating the bacteria with these media.

Effect of culture parameters on growth

pH: Rhizobial culture was inoculated into 10% sugar waste media having constant temperature maintained at different pH i.e., 6.0, 6.5, 7.0, 7.5, 8.0. The growth was monitored in terms of absorbance at 600 nm after every 12 up to 60 h. The viable count of bacterial population was also observed.

Temperature: At constant pH of 7.0 and 10% sugar waste concentration, *Rhizobium* strain was inoculated into 10% sugar waste as a suitable growth media maintained at different temperature i.e., 26, 27, 28, 29, 30°C and growth was observed by recording Optical Density (OD) at 600 nm after every 12 up to 60 h. The viable count of bacterial population was also observed. The data were expressed in Mean±SE of mean values.

RESULTS

Physiochemical characteristics of sugar waste: Three representative samples of sugar waste were tested for physiochemical characteristics. The sugar waste was brown in color with a pH of 6.8±0.2 and Dissolved oxygen 40 mg L^{-1}. The Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) were found 232 and 450 mg L^{-1}, respectively. The reducing sugar content of sugar waste was 2.75±0.17 g L^{-1}.

Effect of concentration of sugar waste on growth and population count of *Rhizobium trifolii* (MTCC 905): Keeping the two parameters constant i.e., pH and Temperature, 7.0 and 28°C, respectively. Growth and population count of *Rhizobium trifolii* (MTCC 905) at different concentrations of sugar waste (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%) was monitored by recording Optical Density (OD) at 600 nm and after 48 h incubation. There was a considerable decline in OD values and cfu mL^{-1} after 10% sugar waste concentration. At 10% concentration OD values and cfu mL^{-1} are 0.706±0.012 and 8.98±0.12, respectively. However, effect of sugar waste concentration (above 10%) was clearly visible on the growth and population count of bacteria (Fig. 1 and 2). Minimum growth for *Rhizobium trifolii* was observed at 90% sugar waste concentration i.e., 0.004±0 in terms of OD and at 70% sugar waste concentration i.e., 0.21±0.05 in terms of Colony forming unit mL^{-1}.

Figure 1 represents the growth of bacteria in terms of optical density while Fig. 2 represents the population count of bacteria in terms of Colony forming unit mL^{-1}.

Growth profile of *Rhizobium trifolii* (MTCC 905) on sugar waste concentrations and synthetic media: Keeping the two parameters constant i.e., pH and Temperature, 7.0 and 28°C, respectively. Growth of *Rhizobium trifolii* was observed at 10% sugar waste concentration along

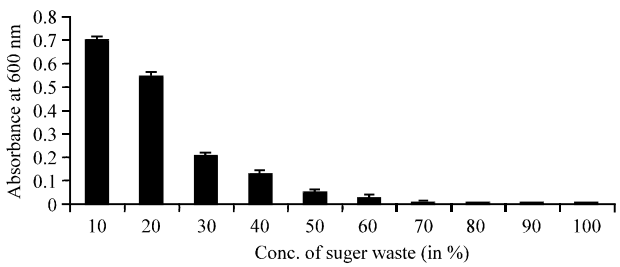

Fig. 1: Effect of concentration of sugar waste on growth of *Rhizobium trifolii* (48 h incubation)

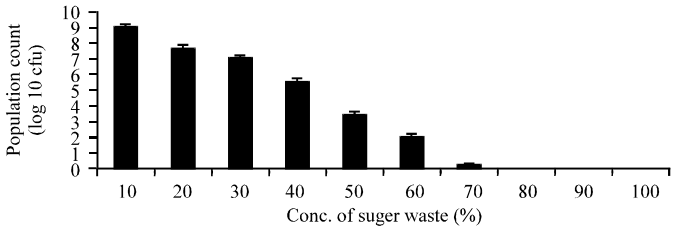

Fig. 2: Effect of concentration of sugar waste on population of *Rhizobium trifolii*

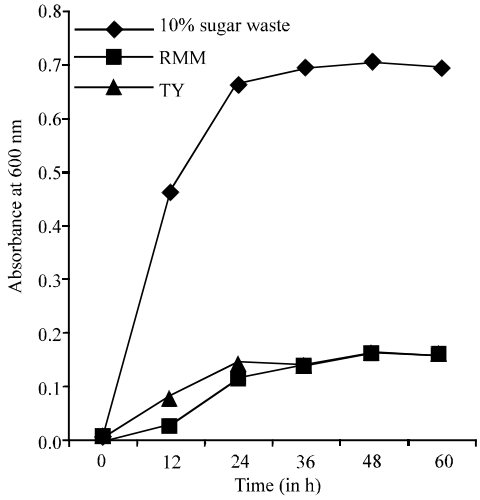

Fig. 3: Growth profile of *Rhizobium trifolii* on sugar waste and synthetic medium

with RMM and TY Medium separately within same conditions by recording Optical Density (OD) at 600 nm after every 12 h. Growth of *Rhizobium trifolii* was found maximum in 10% sugar waste concentration at 48 h i.e., 0.706±0.011 and minimum in RMM Medium i.e., 0.162±0.014 (Fig. 3). The number of bacteria reached maximum at 48 h and above which the growth was constant.

Growth and population count of *Rhizobium trifolii* (MTCC 905) at different pH: Keeping the two parameters constant i.e., concentration of sugar waste and Temperature, 10% and 28°C, respectively. The growth of *Rhizobium trifolii* at different pH (i.e., 6.0, 6.5, 7.0, 7.5 and 8.0) was recorded in terms of optical density (600 nm) after every 12 up to 60 h (Fig. 4). There was a considerable increase in OD values reaching a maximum of 0.706±0.009 with increasing pH up to 7.0 in 48 h, above which the growth was constant. However, inhibitory effect of acidic pH (below 7.0) and alkaline pH (above 7.0) was clearly visible on the growth. Minimum growth was observed at pH 6.0 and 8.0 i.e., 0.512±0.015. Population count of *Rhizobium trifolii at* different pH was monitored in terms of cfu mL^{-1} after 48 h incubation. There was a considerable increase in cfu values reaching a maximum of 8.48±0.003 with increasing pH up to 7.0 after 48 h (Fig. 5). Minimum growth was observed at pH 6.0 i.e., 8.133±0.006 in terms of cfu mL^{-1}.

Effect of different temperature on growth and population count of *Rhizobium trifolii* (MTCC 905): When the two parameters was kept constant i.e., pH and concentration of sugar waste, 7.0 and 10%, respectively, growth of *Rhizobium trifolii* at different temperature (i.e., 26,

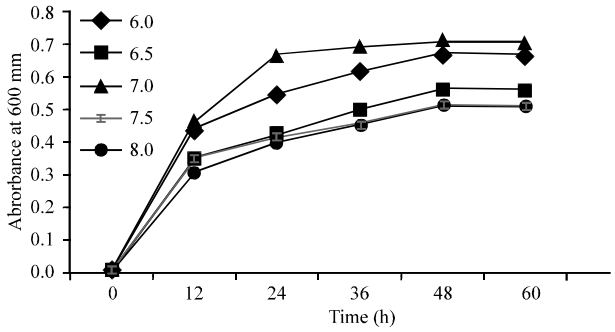

Fig. 4: pH dependent growth profile of *Rhizobium trifolii* (Temperature 28°C, 10% sugar waste)

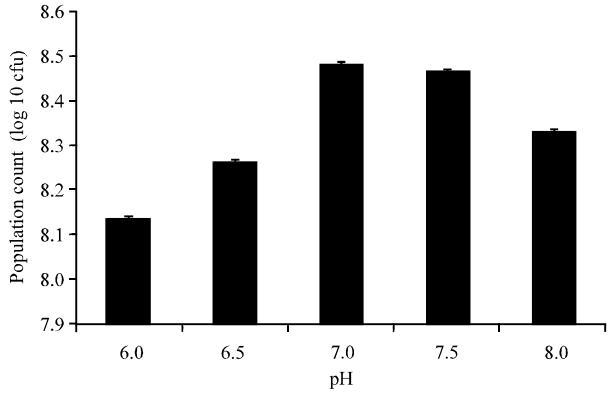

Fig. 5: Effect of pH on population of *Rhizobium trifolii* (Temperature -28°C, sugar waste-10%)

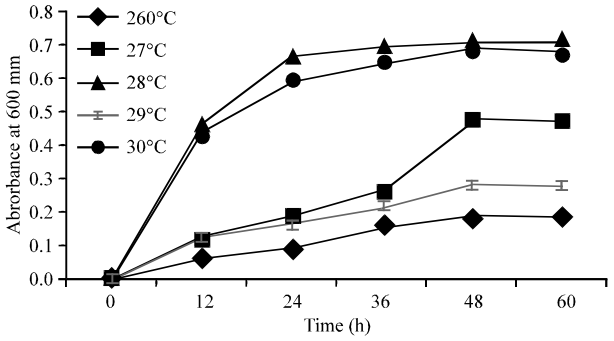

Fig. 6: Temperature dependent growth profile of *Rhizobium trifolii* (pH 7.0, 10% sugar waste)

27, 28, 29 and 30°C) was monitored by recording Optical Density (OD) at 600 nm after every 12 up to 60 h (Fig. 6). There was a significant increase in OD values reaching a maximum of 0.706±0.012 with the increase in temperature up to 28°C in 48 h. However, effect of temperature (below and above 28°C) was clearly visible on the growth. Minimum growth of *Rhizobium trifolii* was observed at 26°C i.e., 0.187±0.012. Growth of *Rhizobium trifolii* (MTCC 905) after 48 h was constant. Population count of *Rhizobium trifolii* at different temperature was monitored in terms of cfu mL^{-1} after 48 h incubation. There was a considerable increase in cfu values reaching a maximum of 8.98±0.12 with increasing temperature up to 28°C after 48 h (Fig. 7).

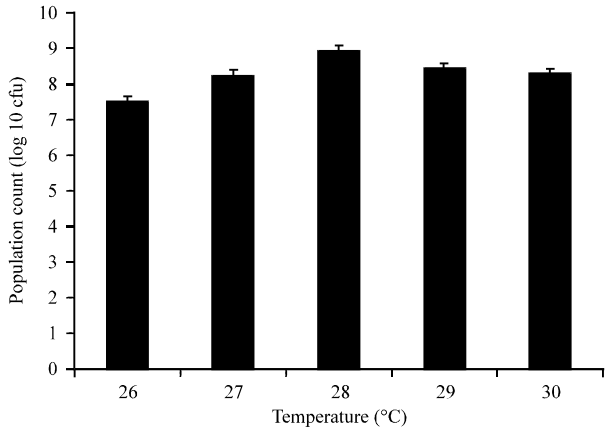

Fig. 7: Effect of temperature on population of *Rhizobium trifolii* (pH 7.0, sugar waste 10%)

DISCUSSION

The sugar waste obtained from Uttarakhand State Sugar Corporation Ltd. Unit. Doiwala, Dehradun was analyzed for various physiochemical parameters such as pH, Dissolved Oxygen (DO), Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and reducing sugar content. Simurina *et al.* (2008) also analyzed physiochemcial parameters of sugarwaste. Growth and population count of *R. trifolii* was observed at different concentrations of sugar waste (10, 20, up to 100%) by recording Optical Density (OD) at 600 nm and log 10 (cfu mL^{-1}), respectively. Our results showed that molasses could serve as a better substrate for growth of bacteria in terms of both OD and cfu Nandi and Sinha (1974) conducted the experiments for mass scale production of *Rhizobium* inoculants in different cheap carbon sources and in different concentrations of yeast extracts in the medium. Their findings suggest molasses could serve as a superior media for *Rhizobium* as molasses contain organic substances and trace elements which helps the growth of bacteria. It has been observed in our results that concentration of sugar waste is indirectly proportional to the Optical density as well as cfu. According to Baei *et al.* (2009) molasses are obtained from circulation of sugar solution in series of evaporation, it contains caramelized and invert sugars, if high sugar waste concentration (as a substrate) is implemented, it may cause cell toxicities.

At above 10% sugar waste concentration, fall in growth and population count of *Rhizobium* was observed which might be due to that at higher concentrations of sugar waste and improper aeration conditions in the medium. The aerobic condition is good for nitrogen fixing bacteria (Hassen *et al.*, 2007). Therefore, sugar waste concentration of 10% was taken optimum for the study.

After optimizing the concentration of sugar waste i.e., 10% growth of *R. trifolii* was observed at 10% sugar waste concentration along with different standard media by recording Optical Density (OD) at 600 nm after 12 to 60 h. According to present study growth of *R. trifolii* were rapidly increased after 12 h, thereafter, the growth was slowly increased, reaching maximum at 48 h and then attained stationary phase. The results of this study indicates that on medium containing only 10% sugar waste, cells of fast growing rhizobia grow rapidly and growth was superior to that of the control (standard media). These results demonstrates that 10% sugar waste can be satisfactorily used as a growth substrate of rhizobia. Estrella *et al.* (2004) found that cheese whey based growth medium efficiently protects *Rhizobium loti* cells during freezing and can maintain their growth in a manner similar to that of traditional mannitol-based medium (YEM).

Ben Rebah *et al.* (2007) reported that waste water sludge, a world wide recyclable waste showed good potential for inoculants production as a growth medium.

pH is an important parameter for the growth of organisms, slight variation in pH of medium might have enormous effects on the growth of organisms, keeping this in mind, at different pH (6.0, 6.5, 7.0, 7.5 and 8.0) growth and population count of fast growing strain of *R. trifolii* was monitored at constant temperature (28°C) and sugar waste concentration (10%). The result of the effects of different pH values on the growth and population count of *R. trifolii* in culture shows that these strains grow well at pH between 6.0-8.0 with maximum at pH 7.0 on observing OD and c.f.u. This is in accordance with Mensah *et al.* (2006). They recorded maximum absorbance for broth experiment as well as exhibited heavy growth as measured by population count at pH 7.0 for *Rhizobium* species. Ali *et al.* (2009) also observed that there was considerable increase in OD values with increasing pH up to 7.0.2. This is according to the earlier findings (Hussain *et al.*, 2002; Yadegari *et al.*, 2008).

Temperature also plays an important role in the growth of an organism, so after optimization of pH (7.0) and sugar waste concentration (10%), growth of *R. trifolii* was observed at different temperatures (26, 27, 28, 29, 30°C). In our results it was observed that fast growing strains grow well at temperature between 26-30°C, when growth was measured in terms of OD and c.f.u. after 48 h incubation period. It has been reported in literature that temperature for growth of root nodulating bacteria ranged from 25-0°C (Pongsilp *et al.*, 2010). In our study maximum growth of *R. trifolii* was observed at 28°C therefore, it is assumed to be the most suitable temperature for *Rhizobium*. This is in agreement with the earlier findings (Ogutco *et al.*, 2008; Agarwal and Ahmad, 2010; Parthiban *et al.*, 2011).

CONCLUSION

The present study recommends that the use of 10% sugar waste (molasses) as a suitable growth media because it is readily available, inert, cheap, eco friendly and superior to the standard media used for *Rhizobium*. It use as an alternate of synthetic media in commercial preparations of biofertilizers will considerably reduce the production cost.

REFERENCES

APHA (American Public Health Association), 1992. Standard Methods for the Examination of Water and Wastewater. 18th Edn., APHA., WEF and AWWA., Washington DC., USA.

Agarwal, S. and Z. Ahmad, 2010. Contribution of the *Rhizobium* inoculation on plant growth and productivity of two cultivars of berseem (*Trifolium alexandrinum* L.) in saline soil. Asian J. Plant Sci., 9: 344-350.

Ali, S.F., L.S. Rawat, M.K. Meghvansi and S.K. Mahna, 2009. Selection of stress-tolerant rhizobial isolates of wild legumes growing in dry regions of Rajasthan, India. ARPN J. Agric. Biol. Sci., 4: 13-18.

Aslan, Y., E. Erduran, H. Mocan, Y. Gedik, A. Okten, H. Soylu and O. Deger, 1997. Absorption of iron from grape molasses and ferrous sulfate: A comparative study in normal subjects and subjects with iron deficiency anemia. Turk. J. Pediatr., 39: 465-471.

Baei, M.S., G.D. Najafpour, H. Younesi, F. Tabandesh and H. Eisazadeh, 2009. Poly (3-hydroxybutyrate) synthesis by *Cupriavidus necator* DSMZ 545 utilizing various carbon sources. World Applied Sci. J., 7: 157-161.

Ben Rebah, F., D. Prevost, A. Yezza and R.D. Tyagi, 2007. Agro-industrial waste materials and waste water sludge for rhizobial inoculant production: A review. Bioresour. Technol., 98: 3535-3546.

Bioardi, J.L. and R.J. Ertola, 1985. Rhizobium biomass production in batch and continuous culture with a malt-sprouts medium. World J. Microbiol. Biotechnol., 1: 163-172.

Cappuccino, S. and N. Sherman, 2001. Serial Dilution Agar Plating Procedure to Quantitate Viable Cells. In: Microbiology: A Laboratory Manual, Cappuccino, J.G. and N. Sherman (Eds.). 6th Edn., Benjamin-Cummings Publishing Co., San Francisco, CA., pp: 119-124.

Chanda, S., S. Matai and S. Chakrabatri, 1987. Deproteinized leaf juice as a medium for growth of *Rhizobium*. Indian J. Exp. Biol., 25: 573-575.

Estrella, M.J., F.L. Pieckenstain, M. Marina, L.E. Diaz and O.A. Ruiz, 2004. Cheese whey: An alternative growth and protective medium for *Rhizobium loti* cells. J. Indian Microbiol. Biotechnol., 31: 122-126.

Fahmi, A.I., H.H. Nagaty, R.A. Eissa and M.M. Hassan, 2011. Effects of Salt Stress on Some Nitrogen Fixation Parameters in Faba Bean Pak. J. Biol. Sci., 14: 385-391.

Gulati, S.L., 1979. New nonsynthetic medium for *Rhizobium* culture production from wastes. Biotechnol. Bioeng., 21: 1507-1515.

Hassen, A.A., J. Xu and J. Yang, 2007. Growth conditions of associative nitrogen-fixing bacteria *Enterobacter cloace* in rice plants. Agric. J., 2: 672-675.

Hussain, N., F. Mujeeb, M. Tahir, G.D. Khan, N.M. Hassan and A. Bari, 2002. Effectiveness of *Rhizobium* under salinity stress. Asian J. Plant Sci., 1: 12-14.

Jain, S.K., D.V. Pathak and H.R. Sharma, 2000. Alternate carbon substrate for mass production of *Rhizobium* inoculants. Haryana Agric. Univ. J. Res., 30: 1-6.

Mensah, J.K., F. Esumeh, M. Iyamu and C. Omoifo, 2006. Effects of different salt concentrations and pH on growth of *Rhizobium* sp. and a cowpea–*Rhizobium* association. Am.-Eurasian J. Agric. Environ. Sci., 3: 198-202.

Mishra, R.P., R.K. Singh, H.K. Jaiswal, V. Kumar and S. Maurya, 2006. *Rhizobium*-Mediated induction of phenolic and plant growth promotion in rice (*Orizza Sativa* L). Curr. Microbiol., 52: 383-389.

Nandi, P.N. and N. Sinha, 1974. Mass scale production of *Rhizobium* culture. Proc. Indian Natl. Acad., 40: 479-481.

Ogutco, H., O.F. Algur, E. Elkoca and F. Kantar, 2008. The determination of symbiotic effectiveness of *Rhizobium* strains isolated from Wild Chickpeas collected from high altitudes in Erzurum. Turk. J. Agric. For., 32: 241-248.

Panesar, R., P.S. Panesar and M.B. Bera, 2011. Development of Low Cost Medium for the Production of Biosurfactants Asian J. Biotechnol., 3: 388-396.

Parthiban, K., S. Manikandan and S. Ganesapandian, 2011. Production of cellulose I microfibrils from *Rhizobium* sp. and its wound healing activity on mice. Asian J. Applied Sci., 4: 247-254.

Paviz, M.M., T. Ghoorchi and F. Ghanbari, 2011. Effects of molasses and bacterial inoculant on chemical composition and aerobic stability of sorghum silage. Asian J. Anim. Vet. Adv., 6: 385-390.

Pongsilp, N., C. Leelahawonge, A. Nuntagij, N. Teaumroong and N. Boonkerd, 2010. Characterization of *Pueraria mirifica* nodulating rhizobia present in Thai soil. Afr. J. Microbiol. Res., 4: 1307-1313.

Rebah, F.B., D.T. Rajeshwar, P. Danielle and Y.S. Rao, 2002. Wastewater sludge as a new medium for rhizobial growth. Water Qual. Res. J. Canada, 372: 353-370.

Simurina, O.D., B.V. Filipev, L.B. Levi and V.D. Pribis, 2008. Application of sugar beet molasses in the production of tea biscuits. Food Process. Qual. Saf., 4: 201-206.

Thompson, J.A., 1991. Legume Inoculant Production and Quality Control. In: Report on the Expert Consultation on Legume Inoculant Production and Quality Control, Thompson, J.A. (Ed.). FAO., Rome, pp: 15-32.

Tunuguntla, A. and M.J. Sullivan, 2004. Black strap molasses for the treatment of inflammatory bowel disease-associated anemia. South Med. J., 97: 794-794.

Verma, J.P., J. Yadav and K.N. Tiwari, 2010. Application of *Rhizobium* sp. BHURC01 and plant growth promoting rhizobactria on nodulation, plant biomass and yields of Chickpea (*Cicer arietinum* L.). Int. J. Agric. Res., 5: 148-156.

Yadav, J., J.P. Verma, V.K. Rajak and K.N. Tiwari, 2011. Selection of effective indigenous *Rhizobium* strain for seed inoculation of chickpea (*Cicer arietinum* L.) production. Bacteriol. J., 1: 24-30.

Yadegari, M., H.A. Rahmani, G. Noormohammadi and A. Ayneband, 2008. Evaluation of bean (*Phaseolus vulgaris*) seeds inoculation with *Rhizobium phaseoli* and plant growth promoting rhizobacteria on yield and yield components. Pak. J. Biol. Sci., 11: 1935-1939.