Indian Journal of Biochemistry & Biophysics
Vol. 56, October 2019, pp. 378-383

Molasses growth medium for production of *Rhizobium* sp. based biofertilizer

Garcha S*, Kansal R & Gosal SK

Department of Microbiology, Punjab Agricultural University, Ludhiana- 141 004, Punjab, India

Received 31 December 2018; *revised 18 March 2019*

Rhizobium forms symbiotic relationship with leguminous crops and is recommended for use in various legumes. *Rhizobium* sp. fix atmospheric nitrogen and make it available to legumes through formation of root nodules. *Rhizobium* biofertilizer production is carried out mostly by using semi-synthetic microbiological medium which forms major expense of this activity. Successful commercial production of biofertilizer can be enhanced by use of natural substrates, as molasses, cheese whey, corn steep liquor, for bacterial biomass production. The present work centers around the use of sugarcane molasses as a source of fermentable sugars. It was supplemented with various organic/inorganic nitrogen sources, chemical compounds to increase biomass yield and to increase the shelf life of the product thus prepared. Compliance to Fertilizer Control Order specifications was demonstrated in wet lab analysis.

Keywords: Biofertilizers, Bioinoculant, Molasses, Yeast extract mannitol agar (YEMA)

Biofertilizer is defined as a substance which contains living microorganisms that when applied to the seed or soil, colonizes the rhizosphere or the inner part of the plant and promotes growth by increasing the supply or accessibility of primary nutrients to the host plant[1]. They contain bacteria and fungi as bioinoculants to improve chemical and biological characteristics of soil and agricultural production[2]. Being inexpensive and eco-friendly, the use of biofertilizers promotes the sustainability of agriculture and protects the environment from pollutants[3,4]. *Rhizobium* is an agriculturally important bacterium, helps in nitrogen fixation in the root nodules[4,5]. Inoculation with *Rhizobium* also improves soil fertility[6]. Biomass generation is a crucial activity of biofertilizer production. Microbial biomass is generally produced by using semi-synthetic media which increases the cost of production. Success of commercial production can be enhanced by using natural substrates as growth medium to reduce the cost of biomass production. Molasses is a viscous, dark and sugar rich product of sugar extraction from the sugarcane[7,8]. It contains sucrose (32%), glucose (10.5%), fructose (8%), nitrogen (0.98%) and has a pH of approximately 6.0[9]. Molasses is a good source of carbon, energy and fermentative sugars[10,11]. In the present investigation, molasses selected as a natural substrate for mass multiplication of *Rhizobium* sp. biofertilizer due to its easy availability and high sugar content.

*Correspondence:
Phone: 0161-2401960, 91-9417602272 (Mob)
Fax: 0161-2400945
E-mail: sgarcha@pau.edu

Materials and Methods

Chemicals and glassware

Analytical grade chemicals (Molychem) and standard laboratory glassware were used in the present investigation.

Microbial culture used

Rhizobium sp. was procured from the Department of Microbiology, Punjab Agricultural University, Ludhiana. It is recommended for use as biofertilizer in Mungbean crop.

Proximate analysis of composition of molasses

The molasses used in the present study was procured from local market and also Budhewal Industries, Ludhiana (an undertaking of SUGARFED, Government of Punjab). Since the composition of molasses varies depending upon method of sugar extraction, efficacy of the process, *etc.* so the proximate analysis of molasses was done periodically throughout the time period of the study. Proximate analysis *i.e.* moisture content, pH, TSS, sugar and nitrogen estimation were done to elucidate the composition of molasses. Sugar content was estimated by standard Dubois method with no modifications[12]. Nitrogen content of molasses was estimated according to Kjeldahl method[13]. Total Soluble Solids (% TSS) in molasses was determined by using handheld Refractometer.

Optimization of molasses concentration to support growth of *Rhizobium* sp.

Molasses was chosen to serve as a principle carbon source and base in the preparation of natural medium. Molasses of different concentration *i.e.* 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 20%, 30%, 40%, 50% (*w/v*) was prepared. pH was adjusted to 6.2-6.5 with the help of 0.1N NaOH. After sterilization, it was inoculated with 7% (*v/v*) of metabolically active cultures of *Rhizobium* sp. which contained a total of 10^8 to 10^9 cells. Molasses containing inoculum of *Rhizobium* sp. was incubated for 72-96 h at 30°C. Post incubation, appropriate dilution of the molasses was plated on YEMA medium to obtain total viable count of *Rhizobium* sp. growing in it. It was compared to the count obtained in the chemically defined medium used for growth of *Rhizobium* sp. *i.e.* YEMA (Yeast Extract Mannitol Agar). Standard microbiological method, pour plating, was used for enumerating viable cell count of *Rhizobium* sp. throughout the study.

Supplementation of molasses based medium

Molasses alone does not support appreciable growth of biofertilizers as evidenced by the results of experiment outlined in 2.4. Molasses does not contain sufficient amount of nitrogen to support bacterial growth as demonstrated by the results of proximate analysis of molasses. Supplementation with nitrogen sources was thus necessitated. These nitrogen sources were selected keeping economy of production in mind. Molasses was supplemented with both organic and inorganic nitrogen sources *i.e.* yeast extract, beef extract, peptone, gelatin, ammonium-sulphate and urea and these formulations were designated as M1, M2, M3, M4, M5 and M6 medium, respectively. *Rhizobium* sp. is propagated in Yeast Extract Mannitol medium which contains 1 g/L yeast extract. Yeast extract of the commercial packing used in the study contained 10% available nitrogen (Molychem). This was used as point of reference. The amount of nitrogen supplements used in the present investigation was commensurate with the total nitrogen present in chemically defined YEMA medium. YEMA medium also contains dipotassium hydrogen phosphate which provides buffering action, magnesium sulphate which is essential for the growth of *Rhizobium* and sodium chloride which maintains the osmotic equilibrium of the organism. Since no component of molasses extends these benefits so these chemicals were added to the molasses-based medium in an amount equal to that present in YEMA. Additionally, molasses was also supplemented with potassium iodide (@ 0.2 g/L) to prevent mold contamination. It was then sterilized and inoculated with 7 % (*v/v*) metabolically active culture of *Rhizobium* sp., as explained earlier. It was later incubated at both ambient and low temperature. Four specifications are important for compliance to Fertilizer Control Order (2016) which governs quality of biofertilizers marketed in India. Total viable count and pH are the most important specifications. They were recorded fortnightly. Two other specifications include, absence of contamination and nodule forming ability. Absence of contamination was ensured by use of aseptic techniques. Nodule forming ability of this strain has been demonstrated in previously conducted field trials which merited its recommendation by Punjab Agricultural University, Ludhiana for use as bio-inoculant in Mungbean.

Results

Molasses was procured in batches from Budhewal Industries, Ludhiana and also local markets. Proximate analysis was performed for every batch procured. The results are presented in (Table 1). There was variation in sugar content among three batches of molasses (28%, 53.6% and 49.2%) which is probably due to the method of extraction of sugar, efficacy of the process, variety of sugarcane used, *etc*. Nitrogen content, percent moisture, Brix and pH did not differ greatly, recording between 0.54-0.61%, 14-16%, 86-88 and 4.4-4.6, respectively. In previous studies conducted by various authors, molasses has been reported to contain 43% sugars, 27% moisture content and 79.5 Brix. Protein content of cane molasses can vary between 3 to 10.6% depending upon origin, processing and variety of sugarcane[14]. Molasses has been reported as acidic in nature and can have 70% organic substances which include 35-55% sugar and 15-25% non-sugar substances[15]. According to the research carried out for poly-lysine production by using molasses, it has been reported that molasses contains 32% sucrose, 10.6% glucose, 8% fructose, 0.98% of nitrogen and pH of 6.0[9].

Initial step in formulation of molasses based natural medium for biomass generation of *Rhizobium* sp. was to determine the concentration of molasses

Table 1 — Proximate analysis of molasses

S. No.	Components	Batch 1	Batch 2	Batch 3
1.	% Moisture	15.3	14.2	16.1
2.	% Sugar	28.0	53.6	49.2
3.	% Nitrogen	0.54	0.57	0.61
4.	pH	4.42 ± 0.2	4.57 ± 0.2	4.53 ± 0.2

which would form the base of the natural medium. Consequently, *Rhizobium* sp. was inoculated and incubated in different concentrations of molasses (2.5 to 50% *w/v*). Results of total viable count of *Rhizobium* sp. cells obtained post inoculation and incubation in different concentrations of molasses *i.e.* 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20, 30, 40 and 50% (*w/v*) are presented in (Table 2). Total viable count of *Rhizobium* sp. recorded was 5.30 and 4.30 log cfu in 40% and 50% (*w/v*) molasses, respectively, compared to 7.48 log cfu in YEMA, an under performance of approximately 29% in 40% (*w/v*) molasses. Accordingly, 40 % (*w/v*) of molasses was selected to form base of the molasses based medium. 50% (*w/v*) molasses yielded lesser cell count compared to 40% (*w/v*) molasses. Moreover, ease of operation and miscibility of molasses in water was also taken into consideration. Un-supplemented molasses evidently lacks growth factors and nitrogen.

Subsequently, 40% (*w/v*) molasses was supplemented as explained in 2.5 and used for propagation of *Rhizobium* sp. The results of total viable count of *Rhizobium* sp. in M1, M2, M3, M4, M5, M6 and YEMA medium are presented in ambient temperature and low temperature (Fig. 1). High viable cell count must be delivered to the plant to maximize benefit of application of bacterial biofertilizer. All six

Table 2 — Growth of bioinoculants in different concentrations of molasses and YEMA

S. No.	Concentration of molasses (%)	*Rhizobium* spp. (log cfu/mL)
1	2.5	-
2	5.0	-
3	7.5	-
4	10.0	-
5	12.5	-
6	15.0	-
7	20.0	-
8	30.0	2.84
9	40.0	5.30
10	50.0	4.30
11	YEMA broth	7.48

(-) = Not Detected

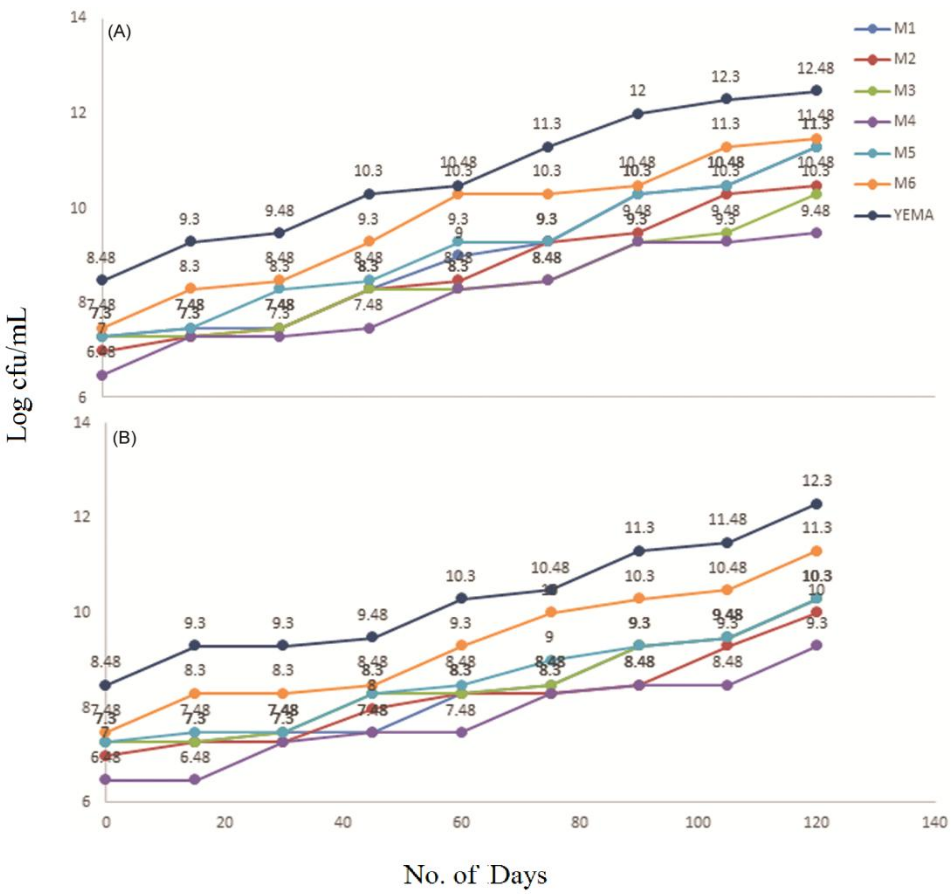

Fig. 1 — Total viable count (log cfu) of *Rhizobium* in molasses based medium incubated at (A) Ambient temperature; and (B) Low temperature

media (M1, M2, M3, M4, M5 and M6) supported appreciable growth of *Rhizobium* sp. After over four months of ambient temperature incubation, total viable cell count of 11.48 log cfu/mL of *Rhizobium* sp. was recorded in M6 medium which was 91.86% of the biomass obtained in YEMA medium in the same time period (12.48 log cfu/mL). Viable cell counts obtained in M1, M2, M3 and M5 media were comparable, 10.3 log cfu/mL to 11.3 log cfu/mL. Least cell count was obtained in M4 medium *i.e.* 9.48 log cfu/mL which was 75% of that obtained in YEMA medium (12.48 log cfu/mL). Similar results were obtained with low temperature incubation. M6 medium yielded total viable count of 11.3 log cfu/mL, 91.98% of that obtained in YEMA medium, 12.3 log cfu/mL. Among other media, least cell count of 9.3 log cfu/mL was attained in M4 medium. Organic nitrogen supplements, namely, yeast extract, beef extract, peptone and gelatin performed appreciably though not at par with M6. Among inorganic supplements, least advantage was extended by ammonium sulphate. Thereafter, statistical techniques were utilized to extrapolate the total viable cell count for a time period for twelve months. It revealed that the viable cell count would be nearly mathematically same as the cell count obtained in chemically defined YEMA medium. In this context, it is important to mention that aseptic techniques are vital for obtaining cell biomass of the desired organism and also to prevent contamination. pH of this natural molasses based medium did not exhibit significant change throughout the time period of incubation. pH recordings varied between 6.5-6.8.

Discussion

Increase in awareness about biofertilizers warrants use of a cost-effective substrate for the bacterial biomass generation. This activity can be enhanced by the use of natural medium instead of chemically defined microbiological medium for biomass production technology. It comprises three important steps: (1) Strain development, (2) Upscaling of biomass and (3) Inoculant preparation. It is generally endorsed that product, free from contaminants and having a microbial load of approximately 1×10^8 log cfu/mL cells (liquid biofertilizer) and 5×10^7cfu/g (solid carrier based biofertilizer) be used to give optimum results of plant growth promotion in recommended crop. Focus is required to obtain soil and crop specific strains and to make them easily available to production units for the up scaling of biomass in industries. Appropriate procedures with cost effective calculation of biofertilizer production and marketing, especially at small scale level in rural areas has not been developed to attract industrialists to adopt biofertilizer technology as agribusiness[33].

A variety of agricultural and industrial by-products such as pea husks, molasses, water hyacinth, malt sprouts, paddy straw, cheese whey, waste water sludge, pulp of the coffee, saw dust, carrot and many more agricultural residues have been used for biomass production[16-20]. These byproducts act as growth factors, carbon source and nitrogen source for bioinoculants and are used for the production of different bacteria, yeast and fungal cultures. Utility of molasses as natural medium is very well documented in literature. It has been used for microorganisms like lactic acid bacteria, yeast, *Bacillus* spp. *Pseudomonas fluorescens* P35 and *Bacillus subtilis* B3[21-26]. Besides, being good carbon source, molasses also contains minerals, organic compounds, vitamins that are important in the fermentation process. Molasses was used as a growth medium for high cell mass and lactic acid production by *Lactobacillus salivarius* L29[27]. Sugarcane molasses were used successfully for spore production of fungi *Beauveria bassiana* (Balsama)[28]. Two fungi *Isaria fumosorosea* and *Isaria farinose* were also grown successfully on agro-industrial molasses and rice broth (liquid fermentation) for conidia production[29]. Rice supplemented with molasses (10 g/L) and yeast extract (3 g/L) generated highest conidia for mycoherbicide production using fungal pathogens *viz. Cochliobolus lunatus* and *Alternaria alternate* was also reported[30]. Molasses medium has also been used for mass multiplication of entomopathgenic fungi *Beauveria brongniartii* (Saccardo) Petch, *Beauveria bassiana* (Balsamo) *Vuilemin* and *Metarhizium anisopliae*[31].

Rhizobium sp. has also been reported to be propagated in molasses by various workers. 10% to 100% sugar waste *i.e.* molasses based cultivation medium was optimized and compared with lab medium for the production and growth of *Rhizobium trifolii* MTCC 905 which was found to be maximum at 10% level of molasses[32]. Use of molasses and baker's yeast as source of carbon and nitrogen, respectively, has been described as suitable natural medium for biomass generation of *Rhizobium leguminosarum* bv phaseoli. Its nodule forming ability was found to be significantly higher than that of cells generated in chemically defined medium[33]. *Rhizobium* *meliloti* MTCC 100 yielded appreciable biomass when inoculated and incubated in 10% molasses[34]. Anaerobic digest of molasses and corn straw successfully supported the growth of *Rhizobium radiobacter* F2 as evidenced by increased production of bio-flocculant by the organism[35]. The present investigation was undertaken to formulate cost-effective molasses based natural medium composition for biomass generation of *Rhizobium* sp. 40% (*w/v*) molasses containing sugar content between 50-55% supported optimal growth as deduced from the results. Growth of *Rhizobium* spp. was found to be significantly higher in 40% (*w/v*) molasses, supplemented with urea (M6 medium) and other chemicals of YEMA medium, as compared to other media *i.e.* M1, M2, M3, M4 and M5. Use of molasses in excess of 10% (*w/v*) as reported in other studies was pursued intentionally for increased shelf life of the finished product. Bacterial strain used in this study has demonstrated its ability to grow well at both ambient and low temperature. This trait largely addresses problem of storage and transport of the product. Cost-effectiveness of use of molasses for biofertilizer production was examined. Cost of molasses was approximately ₹ 150/q in 2017-18 (personal communication with SUGARFED, Government of Punjab). At 400 g/L is its cost worked out to be approximately ₹ 0.6/L. Urea was added at the @ 0.05 g/L, the cost of which was calculated to be less than one rupee. In YEMA medium, average price of 10 g of Mannitol added to one litre of YEMA is ₹ 27 and yeast extract added @ 1 g/L is expected to add ₹ 4/L to cost of YEMA. Use of two key ingredients, molasses and urea reduced the cost of production of liquid molasses based biofertilizer significantly. It is pertinent to mention that other ingredients as dipotassium hydrogen phosphate, magnesium sulphate and sodium chloride were added to molasses based medium in an amount equal to that present in YEMA. The finished product is under field trials, for Mungbean crop, with collaboration from Department of Plant Breeding and Genetics (Pulses Section) at Punjab Agricultural University, Ludhiana.

References

1 Vessey JK, Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil*, 255 (2003) 571.

2 Yosefi K, Galavi M, Ramrodi M & Mousavi SR, Effect of bio-phosphate and chemical phosphorus fertilizer accompanied with micronutrient foliar application on growth, yield and yield components of maize (Single Cros 704). *Aust J Crop Sci*, 5 (2011) 175.

3 Chen JH, The combined use of chemical and organic fertilizers and/or biofertilizer for crop growth and soil fertility, In: *Proceedings of International workshop on sustained management of the soil-rhizosphere system for efficient crop production and fertilizer use*, (2011) 1.

4 Kumudha P, Studies on the effect of biofertilizers on the germination of *Acacianilotica* Linn. Seeds. *Adv Plant Sci*, 18 (2005) 679.

5 Matiru VN & Dakora FD, Potential use of rhizobial bacteria as promoters of plant growth for increased yield in landraces of African cereal crops. *Afr J Biotech*, 3 (2004) 1.

6 Mahdi SS, Hassan GI, Samoon SA, Rather HA, Dar SA & Zehra B, Bio-fertilizers in organic agriculture. *J Phytol*, 2 (2010) 42.

7 Ilyas N, Bano A & Iqbal S, Variation in *Rhizobium* and *Azospirillum* strains isolated from maize growing in arid and semiarid areas. *Int J Agri Biol*, 10 (2008) 612.

8 Bhattacharyya P & Tandon HLS, Product characteristics, crop specificity, advantages and limitations. In *Biofertilizer Technology- Research, Production and Application*, (Fertilizer Development and Consultation Organization, New Delhi) 2012, 28.

9 Weller MD, Biological control of soil borne plant pathogens in the rhizosphere with bacteria. *Annu Rev Phytopathol*, 26 (1988) 379.

10 Shukla SC & Mishra A, ε-Poly-lysine production from sugar cane molasses by a new isolates of *Bacillus* spp. and optimization of the fermentation condition. *Ann Microbiol*, 63 (2012) 1513.

11 Sarlin PJ & Philip R, A molasses-based fermentation medium for marine yeast biomass production. *Int J Res Mar Sci*, 2 (2013) 39.

12 Dumbrepatil A, Adsul M, Chaudhari S, Khire J & Gokhale D, Utilization of molasses sugar for lactic acid production by *Lactobacillus delbrueckii* sub spp. *delbrueckii* mutant Uc-3 in batch fermentation. *Appl Environ Microbiol*, 74 (2008) 333.

13 Dubois M, Gillies KA, Hamilton JK, Rebers PA & Smith F, Colorimetric method for determination of sugars and related substances. *Anal Chem*, 28 (1956) 350.

14 McKenzie HA & Wallace HS, The Kjeldahl determination of nitrogen-a critical study of digestion conditions-temperature, catalyst and oxidizing agents. *Aust J Chem*, 7 (1954) 55.

15 Younis MAM, Hezayen FF, Moustafa A, Eldein N & Shabeb MSA, Optimization of cultivation medium and growth conditions for *Bacillus subtilis* KO strain isolated from sugarcane molasses. *Am Eur J Agric Environ Sci*, 7 (2010) 31.

16 Perera TA, TL Tirimanne S, Seneviratne G & Kulasooriya SA, *Azorhizobium caulinodans* ORS 571–*Aspergillus* spp. biofilm in the presence of flavonoid naringenin: An extremely effective association for rice root colonization with a definite future as a nitrogen bio-fertilizer. *Indian J Biochem Biophys*, 54 (2017) 214.

17 Bioardi JL & Ertola RJ, *Rhizobium* biomass production in batch and continuous culture with a malt-sprouts medium. *MIRCEN J Appl Microbiol Biotechnol*, 1 (1985) 163.

18 Estrella MJ, Pieckenstain FL, Marina M, Diaz LE & Ruiz OA, Cheese whey-An alternative growth and protective medium for *Rhizobium loti* cells. *J Ind Microbiol Biotechnol*, 31 (2004) 122.

19 Rebah FB, Prévost D, Yezza A & Tyagi RD, Agro-industrial waste materials and wastewater sludge for rhizobial

inoculant production: A review. *Bioresour Technol*, 98 (2007) 3535.

20 Khandelwal M, Mehta J, Naruka R, Makhijani K, Sharma G, Kumar R & Chandra S, Isolation, characterization and biomass production of *Trichoderma viride* using various agro products- A biocontrol agent. *Adv Appl Sci Res*, 3 (2012) 3950.

21 Gajdos P, Nicaud JM & Rossignol T, Single cell oil production on molasses by *Yarrowia lipolytica* strains overexpressing DGA2 in multicopy. *Appl Microbiol Biotechnol*, 99 (2015) 8065.

22 Gomaa EZ, Production of polyhydroxyalkanoates (PHAs) by *Bacillus subtilis* and *Escherichia coli* grown on cane molasses fortified with ethanol, *Braz Arch Biol Technol*, 57 (2014) 145.

23 Nair AM, Annamalai K, Kannan SK & Kuppusamy S, Utilization of sugarcane molasses for the production of polyhydroxyalkanoates using *Bacillus subtilis*. *Malaya J Biosci*, 1 (2014) 24.

24 Younis MAM, Hezayen FF,Nour-Eldein MA & Shabeb MSA, Production of protease in low-cost medium by *Bacillus subtilis* KO strain. *J Biotech Biochem*, 4 (2009) 132.

25 Keshavarzi M, Salimi H & Mirzanamadi F, Biochemical and physical requirements of *Bacillus thuringiensis* sub spp. *kurstaki* for high biomass yield production. *J Agric Sci Technol*, 7 (2005) 41.

26 Peighami-Ashnaei S, Sharifi-Tehrani A, Ahmadzadeh M & Behboudi K, Interaction of different media on production and biocontrol efficacy of *Pseudomonas fluorescens* P-35 and *Bacillus subtilis* B-2 against grey mould of apple. *J Plant Pathol*, 91 (2009) 65.

27 Latifian M, Rad B, Amani M & Rahkhodaei E, Mass production of entomopathogenic fungi *Beauveria bassiana* (Balsamo) by using agricultural products based on liquid-solid diphasic method for date palm pest control. *Int J Agric Crop Sci*, 5 (2013) 2337.

28 Lee KB, Kang SK & Choi YJ, A low-cost *Lactobacillus salivariu* L29 growth medium containing molasses and corn steep liquor allows the attainment of high levels of cell mass and lactic acid production. *Int J Biotech*, 12 (2012) 2013.

29 Mascarin GM, Alves SB & Lopes RB, Culture media selection for mass production of *Isaria fumosorosea* and *Isaria farinose*. *Braz Arch Biol Technol*, 2 (2010) 753.

30 Jyothi G, Reddy KRN, Reddy KRK & Podile AR, Exploration of suitable solid media for mass multiplication of *Cochlio boluslunatus* and *Alternaria alternate* used as mycoherbicide for weed management (Barnyard grass) in rice. *J Exp Biol Agric Sci*, 1 (2013) 280.

31 Tamizharasi V, Srikanth J & Santhalakshmi G, Molasses-based medium requires no nitrogen supplement for culturing three entomopathogenic fungi. *J Biol Cont*, 19 (2005) 135.

32 Singh AK, Gauri, Bhatt RP & Pant S, Optimization and comparative study of the sugar waste for the growth of *Rhizobium* cells along with traditional laboratory media. *Res J Microbiol*, 6 (2011) 715.

33 Bekele H, Dechassa N & Argaw A, Effects of different carbon and nitrogen sources in broth culture on the growth of *Rhizobium leguminosarum* bv phaseoli and symbiotic effectiveness of haricot bean (*Phaseoulus vulgaris* L.) in Eastern Harorghe soils of Ethiopia. *Afr J Microbiol Res*, 7 (2013) 3754.

34 Singh AK, Singh G, Bhatta RP, Pant S, Naglot A & Singh L, Sugar waste, an alternative growth and complete medium for fast growing *Rhizobium* cells. *Afr J Microbial Res*, 5 (2011) 3289.

35 Zhao G, Ji S, Sun T, Ma F & Chen Z, Production of bio-flocculants prepared from waste water supernatant of anaerobic co-digestion of corn straw and molasses waste water treatment. *Biores*, 12 (2017) 1991.