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Full Length Research Paper

Sugars waste, an alternative growth and complete medium for fast growing *Rhizobium* cells

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The sugar waste (molasses) was tested for various physiochemical parameters. After examination of various physiochemical parameters, growth and population count of *Rhizobium meliloti* MTCC-100 was monitored at different concentrations of sugar waste (10, 20, 30 and up to 100%) in terms of optical density (OD) and colony forming unit (C.F.U.). Growth and cell count of *Rhizobium* were highest in 10% sugar waste concentration. Growth pattern of the bacteria was observed at 10% sugar waste along with different synthetic media (tryptone yeast extract medium, rhizobium minimal medium and yeast extract medium). Growth of bacteria in 10% sugar waste was found to be superior to standard media (TY, RMM and YEM) used for *Rhizobium.* The important environmental parameters like pH and temperature were optimized for 10% sugar waste as growth medium. A pH of 7.0 and temperature of 28°C were found to be the most suitable for the fast growing *R. meliloti* MTCC-100.

Key words: *Rhizobium meliloti,* sugar waste, synthetic media, colony forming unit.

INTRODUCTION

The staggered increasing world population demands sustainable crop production, which has become a threat by affecting total agricultural land area and extra burden on agriculture. The imprudent use of chemical fertilizers, pesticides and fungicides has resulted in the deterioration of soil health and also harmful effects on soil biota. Amongst soil biota, microorganisms play a significant role in regulating the dynamics of organic matter decom-position and the availability of plant nutrients such as nitrogen, phosphorous and sulphur. Thus, it has led an emphasis on the integrated management strategy to improve the interaction of roots of the plants with soil microorganisms (Mishra et al., 2006) . To obtain good quality nitrogen inoculants, it is desired to obtain effective rhizobia with good nodulation and nitrogen fixing capacity to the host plant. Therefore, there is need for the present scenario to develop an economical as well as productive nutrient medium for the growth of such rhizobial cells.

Most researchers prefer YEM broth for rhizobial culture, which contains mannitol as a carbon source and yeast extract as a source of nitrogen and growth factors (Vincent 1970; Ferreria and Castro 2005; Kucuk and Kivinac, 2008; Alemayehu, 2009). Both mannitol and yeast extract are the expensive ingredients of that medium making it not suitable for commercial production of inoculants. In view of the growing demand of rhizobial inoculants, the use of cheap carbon sources and nutritional supplements as substrate is required to reduce the production cost of the biofertilizers and to support the growth of rhizobia, equal or better than the known growth in the available media.

In the present study, this goal was achieved for fast growing Rhizobia, namely *Rhizobium meliloti*, by using the product of sugar cane industries, that is, sugar waste (Molasses), as a suitable growth medium. Dark brown thick syrup remained as a residue of inverted sugar crystallization, known as molasses, and was the basic

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raw material used for a lot of microbiological processes. 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; Mahmoud et al., 1978; El – Enshasy et al., 2008).

MATERIALS AND METHODS

Organism

R. meliloti MTCC101 strain was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India, and routinely maintained on YEM agar slants.

Substrate

The sugar waste (molasses) was collected from Uttarakhand State Sugar Corporation Ltd. Unit: Doiwala, Dehradun, Uttrakhand (India).

Chemical analysis

The pH, dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD) and reducing sugar content of the collected sugar waste were determined (APHA-AWWA-WEF 1992).

Starter culture

50 ml of culture was prepared by inoculating *Rhizobium* strain into TY broth and RMM, respectively and incubated at 28 ± 2°C for 16 to 24 h.

Media optimization

Sugar waste

1% of the starter culture was inoculated into different concentrations of sugar waste that, is 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% and growth was monitored by recording optical density (OD) and C.F.U. / ml at regular time interval (Cuppers and Smelt 1993). The viable count of bacterial population was also observed (Cappuccino and Sherman 2002).

Comparison of sugar waste with synthetic media

Effect of different media that is 10% sugar waste, RMM and YEM on *Rhizobium* culture was monitored in terms of absorbance at 600 nm after every 12 h up to 60 h.

Effect of culture parameters on growth

pH

At constant temperature of 28 ± 2°C and 10% sugar waste medium, *Rhizobium* strain was inoculated and maintained at different pH that is, 6.0, 6.5, 7.0, 7.5 and 8.0 and growth was monitored in terms of absorbance at 600 nm after every 12 h up to 60 h. The viable count of the bacterial population was also observed.

Temperature

Rhizobial culture was inoculated into 10% sugar waste media having constant pH and maintained at different temperatures that is, 26, 27, 28, 29 and 30°C. The growth was observed by recording optical density (OD) at 600 nm after every 12 h up to 60 h. The viable count of the bacterial population was also determined.

RESULTS AND DISCUSSION

Physiochemical characteristics of sugar waste

Three representative samples of sugar waste were tested for physiochemical characteristics. The sugar waste was brown in colour with a pH of 6.8 ± 0.2 and dissolved oxygen 40 mg/l. The biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were found 232 mg/l and 450 mg/l, respectively. The reducing sugar content of sugar waste was 2.75 ± 0.17 g/l. The above results are supported by the findings of Sharma and Singh (2000).

Effect of concentration of sugar waste on growth and population count of *R. meliloti* MTCC 100

Keeping the two parameters constant that is pH and temperature, 7.0 and 28 ± 2°C, respectively. Growth and population count of *R. meliloti* at different concentrations of sugar waste (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%) was monitored by recording OD at 600 nm and C.F.U. /ml after 48 h incubation. There was a considerable decline in OD and C.F.U. /ml values of the test organism above 10% sugar waste concentration. At 10% concentration OD and C.F.U. /ml were 0.744 ± 0.01 and 8.47 ± 0.1, respectively. 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. The effect of sugar waste concentration above 10% was clearly visible on the growth and population count of bacteria (Figures 1 and 2). Minimum growth was observed at 100% sugar waste concentration that is 0.003 ± 0.001 in terms of OD and at 60% sugar waste concentration that is 1.98 ± 0.12 in terms of C.F.U. /ml. Tuzimura and Meguro (1968) reported that many types of sugars could be used to substitute mannitol as carbon and energy source for *Rhizobium japonicum*. Among the sugars used, cane sugar was the best because maximum growth was observed in cane sugar and it was cheapest and constantly available in the country. Furthermore, Somsak et al. (1975) evaluated rhizobial growth in the presence of varied levels (8 to 10 g/l) of cane sugar. The author reported the maximum absorbance at the

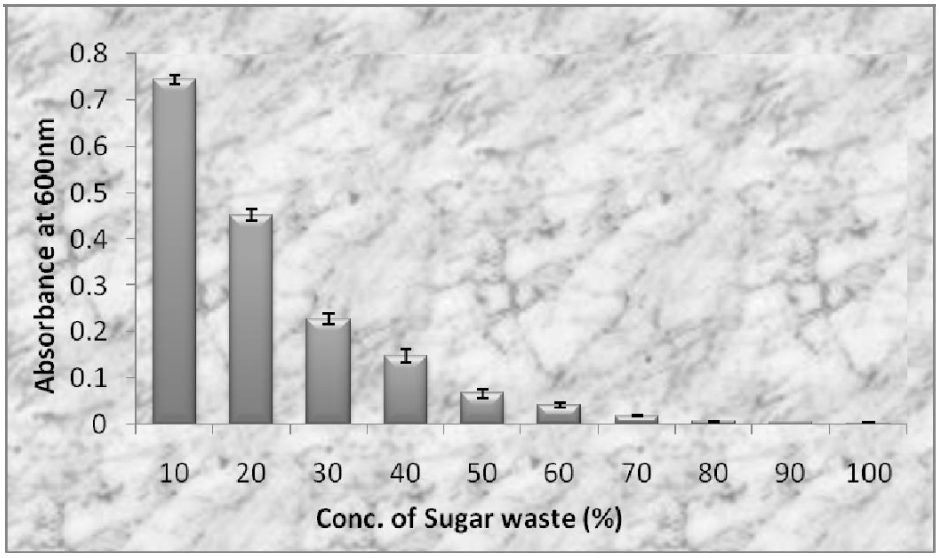

Figure I. Effect of concen tration of sugar waste on growth of *R. meliloti* (48 h Incubation).

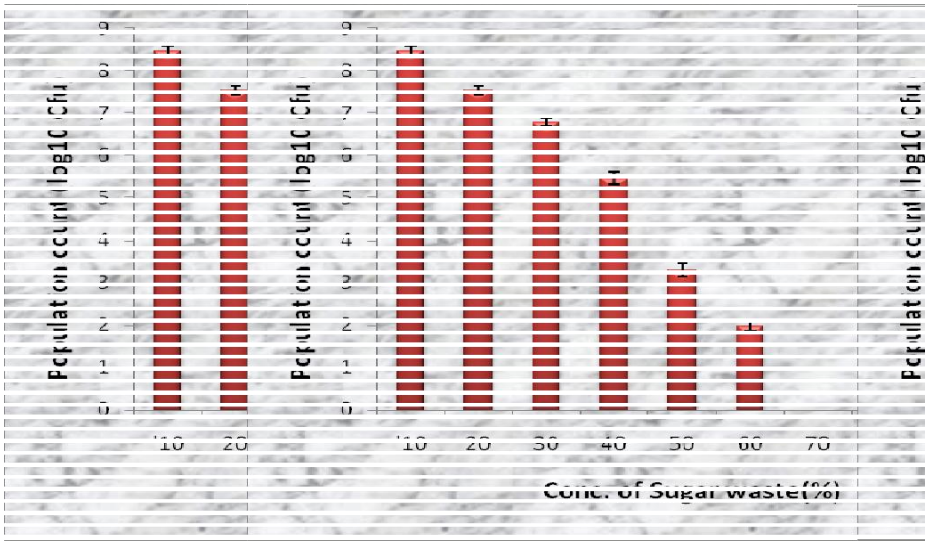

Figure 2. Effect of conc entration of sugar waste on population of *R. meliloti.*

concentration of 8 g/l. These results suggested that cane sugar at the concentration of 8 g/l would be sufficient for optimal growth of *R. japonicum*.

Growth profile of *R. meliloti* (MTCC 101) on sugar waste concentration and synthetic med ia

At pH 7 and temperature 28°C the growth of *R. meliloti* was observed at 10% sugar waste conc entration along with other synthetic media like RMM an YEM. In each case, the numbers of bacteria reached t o the maximum at 48 h and then attain a stationary stage (Figure 3). The growth of *R. meliloti* was found maximum in 10% sugar waste concentration at 48 h that is 0.744 ± 0.012 and minimum in RMM, medium tha is 0.164 ± 0.012.

Annapurna et al. (1988) used 1% aqueous solutions of commercial quality of molasses, malt extract, jaggery, peptone and yeast extract as the sole source of nutrients for the two species of *Rhizobiu m* that is *Rhizobium trifolii* (RC 1 to 4), a fast grower and *R. Japonicum* (SB - 16), a slow grower. YEM broth was used as standard medium for comparison, among different media used jaggery solution supported maximum growth (OD: 10.17) for *R. trifolii* and 9.98 (OD) for *R . Japonicum* followed by molasses 9.65 OD (for *R. trif olii* and 9.22 (OD) for *R.*

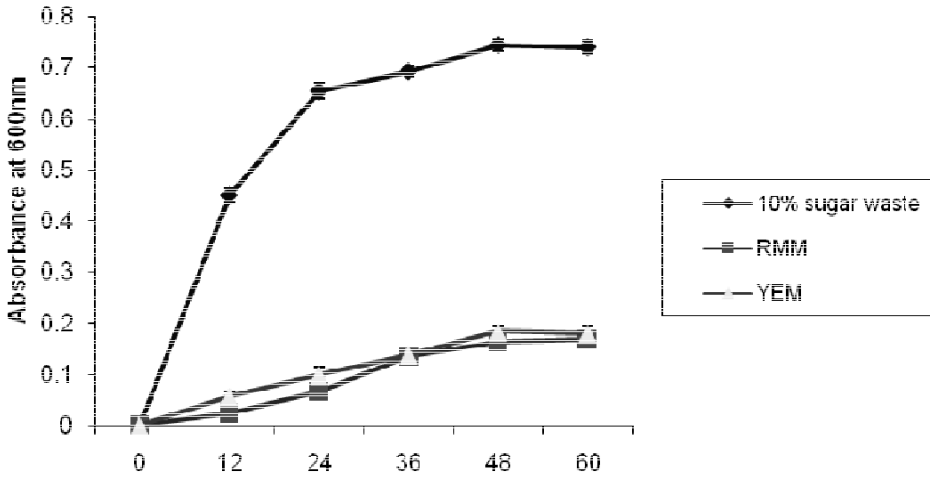

Figure 3. Growth Profile of *R. meliloti* on sugar waste and synthetic media (RMM and YEM).

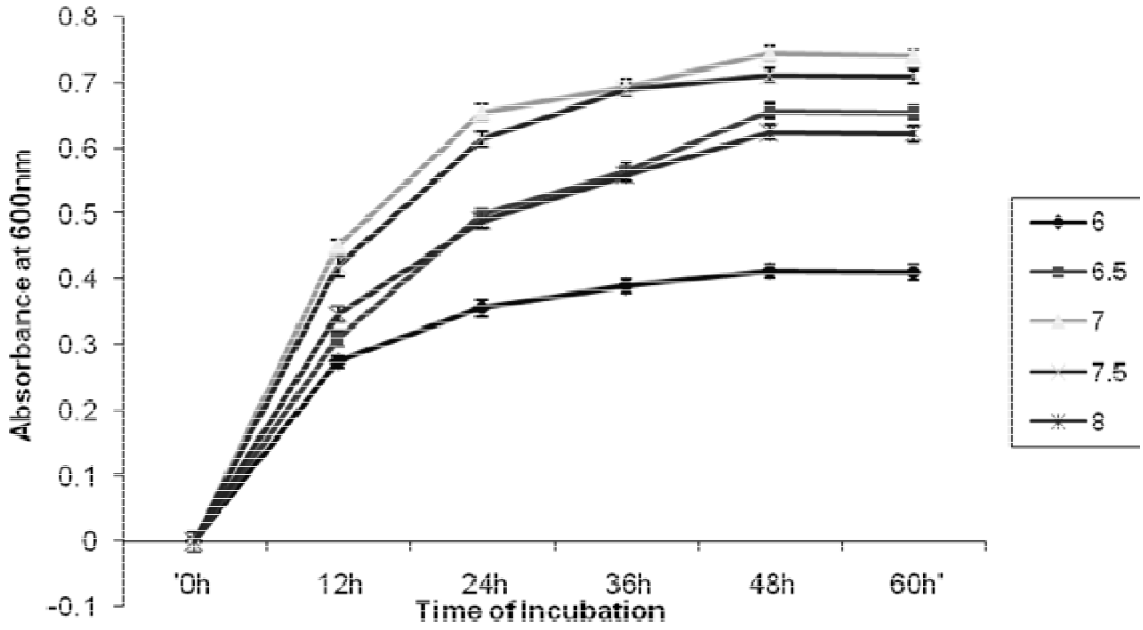

Figure 4. pH dependent growth profile of *Rhizobium meliloti* (Temperature -28°C, 10% sugar waste).

Japonicum) though it was not comparable to that of YEM broth which supported 10.31 (OD) for *R. trifolii* and 10.13 (OD) for *R. Japonicum,* therefore according to this finding jaggery solution and molasses can be used as an alternate of YEM broth in commercial preparation of inoculants. This will considerably reduce the production cost. Similar observation has been reported by Daniel et al. (2009) in the composition of the alternative culture media, which included glycerol, molasses, glutamate, yeast extract and salts that were optimised for *Rhizobium*. Consequently, no significant difference was observed in the alternate culture media, which is the traditional one (Yeast extract mannitol agar medium).

Growth of *R. meliloti* (MTCC 101) at different pH

At constant, temperature and concentration of sugar waste that is 28°C and 10%, respectively, the growth of *R. meliloti* at different pH (that is 6.0, 6.5, 7.0, 7.5 and 8.0) was recorded in terms of optical density (600 nm) after every 12 h up to 60 h (Figure 4). There was a

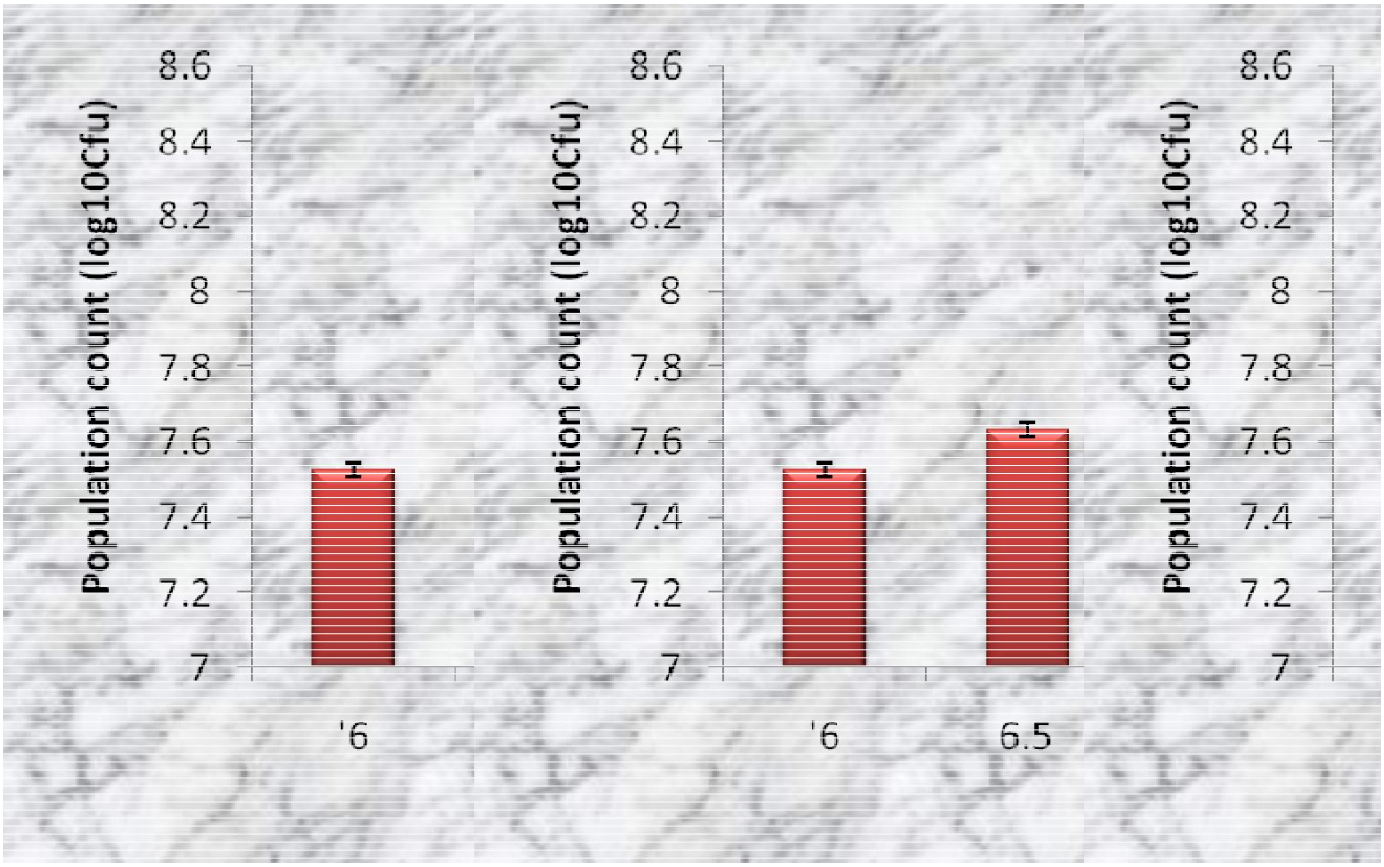

Figure 5. Effect of pH on population of *R. meliloti* (Temperature - 28°C, sugar waste-10%).

considerable increase in OD values reach ing a maximum of 0.744 ± 0.011 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 alka line pH (above 7.0) was clearly visible on the growth. Min imum growth of *Rhizobium meliloti* was observed at pH 6.0 that is, 0.412
± 0.011. Similarly Ali et al. (2009) obse rved that there was considerable increase in OD values with increasing pH up to 7.0. Rodrigues et al. (2006) als o reported that pH 7.0, is the most optimum pH for the growth of root nodulating bacteria.

Effect of pH on Population of *R. meliloti* (MTCC 101)

The growth of *R. meliloti* at different pH (6 .0, 6.5, 7.0, 7.5 and 8.0) was monitored in terms of C. F.U. after 48 h incubation. There was a considerable inc rease in C.F.U. values reaching a maximum of 8.481 ± 0.003 with increasing pH up to 7.0 after 48 h (Figu re 5). This is in accordance with the research of Mensah et al. (2006), in which they recorded maximum absorbance for broth experiment, as well as exhibited he avy growth as measured by population count at pH 7.0 for *Rhizobium* species.

Growth and population count of *R. meliloti* (MTCC 100) at different temperature

Keeping the two parameters constant that is, pH and concentration of sugar waste, 7.0 and 10%, respectively, growth of *R. meliloti at* differe nt temperature (that is 26, 27, 28, 29, and 30°C) was rec orded as mentioned above (Figure 6). There was a significant increase in the OD values reaching a maximum of 0.744 ± 0.01 with the increase in temperature that is up to 28°C in 48 h. Minimum growth of *R. meliloti* was observed at 26°C that is, 0.175 ± 0.011. Populatio n count of *R. meliloti* at different temperature was monitored in terms of C.F.U. after 48 h of incubation. Con siderable increase in the C.F.U. values was recorded, reaching a maximum of 8.47
± 0.001 with increasing temp erature that is up to 28°C after 48 h. In our study the maximum growth of *R. meliloti* was observed at 28°C ; therefore, it is assumed to be the most suitable te mperature for *Rhizobium.* This is in agreement with the earlier findings of Appunu and Dhar (2006) and Ogutco t al. (2008). However, the effect of temperature (below an d above 28°C) was clearly visible on the growth (Figure 7). But both growth and population count of *R. meliloti* was found constant after 48 h of incubation. It has been reported previously in the

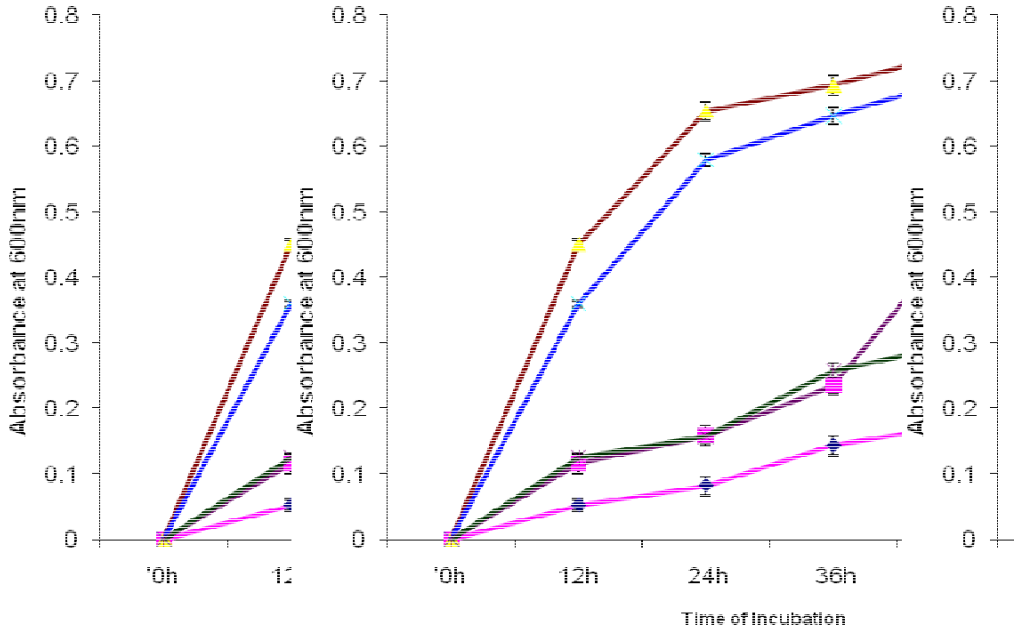

Figure 6. Temperature depe ndent growth profile of *R. meliloti* (pH-7.0 and 10% sugar was te).

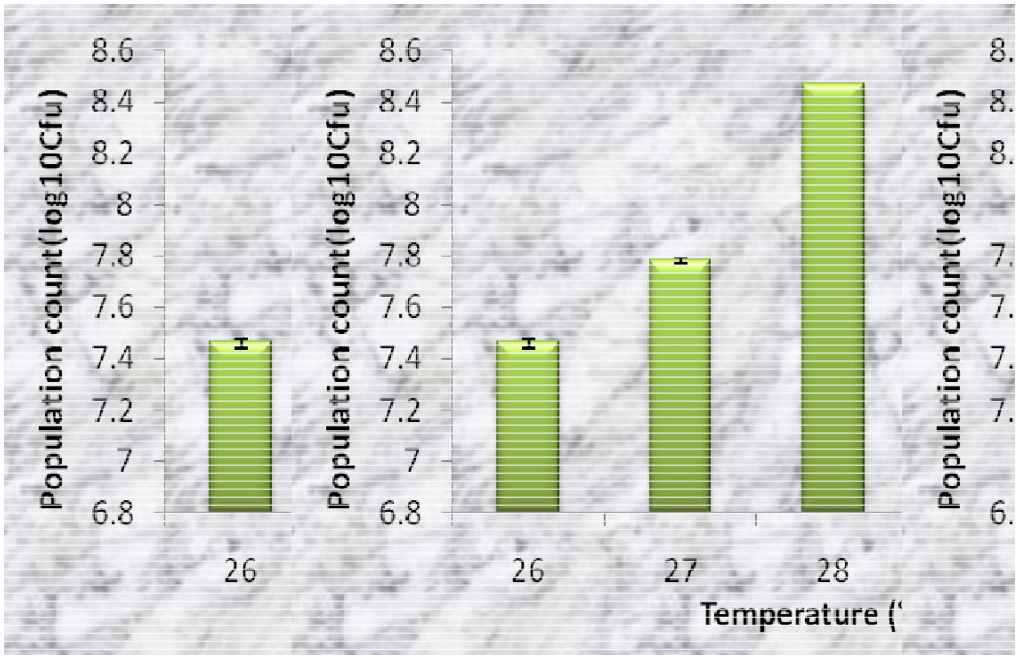

Figure 7. Effect of temperature on population of *R. meliloti* (pH-7.0 and sugar waste-10 %).

literature that the temperature for g rowth of root nodulating bacteria ranged from 25 to 30°C (Gaur 1993).

The present study concludes that 10% of sugar waste (molasses) is a suitable growth medium for the rhizobial inoculants in terms of productivity, cheapness, availability and inertness, eco friendly and su perior to other available standard synthetic media.

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