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RESEARCH ARTICLE

Spent Wash as an Alternative Medium for Growth of Rhizobium

Kumar Gaurav, Ashok Kumar Singh and Gauri Singh*
Dept. of Microbiology, Dolphin (PG) Institute of Biomedical and Natural Sciences, Manduwala, Dehradun (Uttarakhand), India
gaurisingh2007@gmail.com*; +91 9568011274

Abstract

The present study was aimed to use spent wash as a substitute for expensive commercial media for Rhizobial cultures. Growth of Rhizobium strains at different concentrations of spent wash was observed and found maximum at 50% spent wash. Bacteria were grown on 50% spent wash which produced remarkable results and competed with standard media like tryptone yeast extract, Rhizobium minimal and yeast extract mannitol media. Media was optimized using 50% spent wash along with two main ingredients used for Rhizobium namely yeast extract (1-7 g/L) and mannitol (1-7 g/L) in terms of OD at different intervals of time namely 24, 48 and 72 h. The maximum growth observed in 5 g/L in yeast extract and 2 g/L in mannitol at 24 h incubation period in all the strains. This study provides the information of nutritive, inexpensive and suitable medium for Rhizobial cultures.

Keywords: Rhizobium, spent wash, standard media, yeast extract, incubation period.

Introduction

Rhzobium is a genus of gram negative soil bacteria that fix atmospheric nitrogen. Rhizobium forms an endosymbiotic nitrogen fixing association with roots of legumes. Beijernick in the Netherland was first to isolate and cultivate a microorganisms from nodules of legumes in 1888. They colonize the rhizosphere or the interior of the plant growth by increasing the supply or availability of primary nutrients to host plant (Zaharn, 1999). Most important feature in the inoculation of legume seeds or soils with commercial inoculants containing pre-selected strains of root-nodule bacteria is a common agriculture practice, which helps to ensure an effective symbiosis, particularly when natural soil populations of these bacteria are deficient, ineffective or only partially effective (Stephens and Rask, 2000). The starting phase in the commercial production of legume inoculants is mass culturing of a selected rhizobial strain in liquid medium (Thompson, 1991). As in case of industrial fermentations, the economy of such a process is largely governed by the price of media utilized. The suitability of culture media for large-scale production of Rhizobium depends upon the utilization of carbon and nitrogen source and multiplication rate of bacteria. For industrial production of rhizobial inoculants, it is important to identify inexpensive and easily available sources of nutrients for culture medium. Nutrient media such as yeast extract mannitol, tryptone yeast extract, and rhizobial minimal media are found to be very suitable for the growth of rhizobia. The standard medium includes mannitol, sucrose or glycerol as the carbon source, yeast extract as a source of nitrogen, growth factors, and mineral salts. Rebah *et al*. (2007) showed that fast-growing bacteria can grow on a large variety of carbon substrate. Although, in such media preparations, a single source of carbon cannot be used for all strains, because rhizobial strains of different genera often differ in carbon utilization (Tittabutr *et al.*, 2005). The YMB medium (Yeast Mannitol Broth) has been used mostly for a laboratory-scale production (Verma *et al.*, 2010; Singh *et al.,* 2015). However, its industrial use is limited due to high cost (Tyagi *et al.,* 2002). Most of the researchers have looked for alternative cheap media for producing biofertilizers. A large number of agricultural and industrial byproducts such as proteolyzed pea husks, molasses and water hyacinth (Gulati, 1979), malt sprouts (Bioardi and Ertola, 1985), deproteinized leaf extracts (Chanda *et al*., 1987), cheese whey (Estrella *et al*., 2004), waste water sludge (Rebah *et al*., 2007), Jaggery solution (Jain *et al*., 2000), Potato extract broth (Martyniuk and Oron, 2011), sugar waste (Singh *et al*., 2011) and Dairy sludge (Singh *et al*., 2013). These products support the growth of rhizobia equal to or better than the known growth in the available media. Spent wash contain valuable nitrogen and phosphate and organic matter. Spent wash has lower levels of heavy metals or other harmful components than sewage sludge. There are about 579 sugar mills and 285 distilleries in India. The alcohol industry produces a huge amount of wastes every day, which is rich in organic material and characteristically less toxic and easily amenable for microorganisms. Alcohol is produced in India by the fermentation of molasses. The mother liquor left after the sugar production is spent wash. It is dark brown in colour with high temperature, low pH and high ash content. So considering the above facts in view, the present study was aimed to use spent wash as a substitute for expensive commercial media for rhizobial cultures.

Materials and methods

Substrate and isolates: The spent wash was used as a substrate and collected from well known distillery situated in Dehradun. Rhizobium RT1 and RT2 strains were used. Bacteria were isolated from the root nodules of *Trifolium alexandrinum* according to Vincent (1970), collected from different agroclimatic regions of Dehradun. About 50 mL of starter culture was prepared by inoculating *Rhizobium* strains into Yeast extract mannitol broth (YEM) and incubated at 28±2°C for 24 h.

Optimization of spent wash and comparison with lab media: About 1% of the starter culture was inoculated into different concentrations of spent wash (10-100%) and growth was monitored by recording optical density at regular time intervals. Effect of different media i.e. 50% spent wash, RMM, TY and YEM on *Rhizobium* was monitored in terms of absorbance at 600 nm after every 24-72 h by inoculating bacteria with these media components.

Optimization of spent wash with components of standard media: Yeast extract and mannitol are the two main components of the standard media which are very helpful for the growth of *Rhizobium*. So, the growth of bacteria were also observed in 50% spent wash along with different concentrations of yeast extract (1-7 g/L), similarly 50% spent wash along with different concentrations of mannitol (1-7 g/L).

Statistical analysis: Experiments were performed in triplicate and the result was expressed as a mean ± standard deviation of three replications.

Results

Rhizobium from root nodules of *T. alexandrinum:* A total of 9 root nodule bacteria were isolated from *Trifolium alexandrinum* growing in field. The isolates were designated from RT1 to RT9. The isolates were recognized on the basis of their colony morphology on YEMA plates. The general microscopic characteristics of the selected isolates showed rod shaped and gram negative in nature and also similar to those reported earlier (Gauri *et al*., 2011). Only two (RT1 and RT2) isolates showed maximum nodulation with *T. alexandrinum* and selected further.

Effects of concentration of spent wash on growth of Rhizobium strains: Growth of *Rhizobium* at different concentrations of spent wash (10-100%) was monitored by recording OD at 600 nm after 48 h incubation. At 50%, OD values were 2.488 for RT1 and 2.779 for RT2. Minimum growth for RT1 and RT2 was observed at 10% spent wash i.e. 0.034 and 0.279 respectively (Fig. 1).

Growth of RT1 and RT2 on spent wash and synthetic medium: Growth of RT1 and RT2 were observed at 50% spent wash concentration along with YEM, RMM and TY medium separately within same conditions by recording OD at 600 nm after 24 h (Fig. 2). Growth of RT1 and RT2 were found maximum in 50% spent wash i.e. 2.488 for RT1 and 2.779 for RT2 and minimum in RMM medium i.e. 1.116 and 1.139 respectively.

Fig. 1. Growth of RT1 and RT2 on different concentration of spent wash.

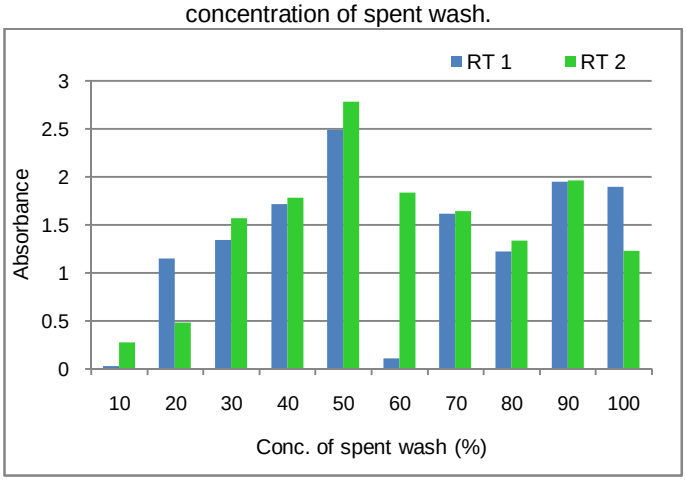


Fig. 2. Absorbance of RT1 and RT2 on standard medium.

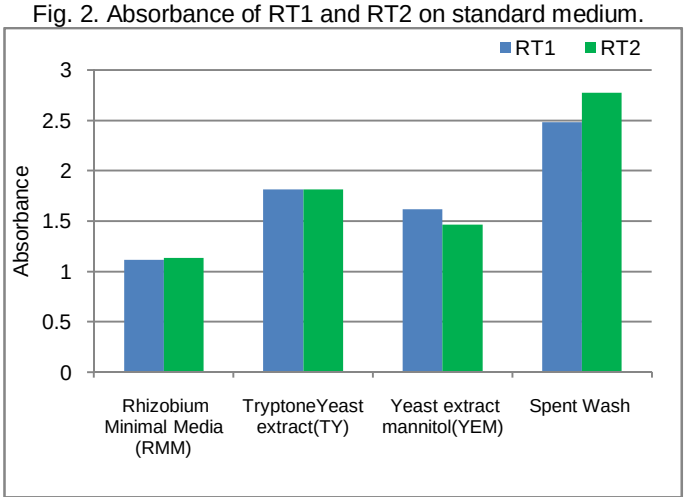
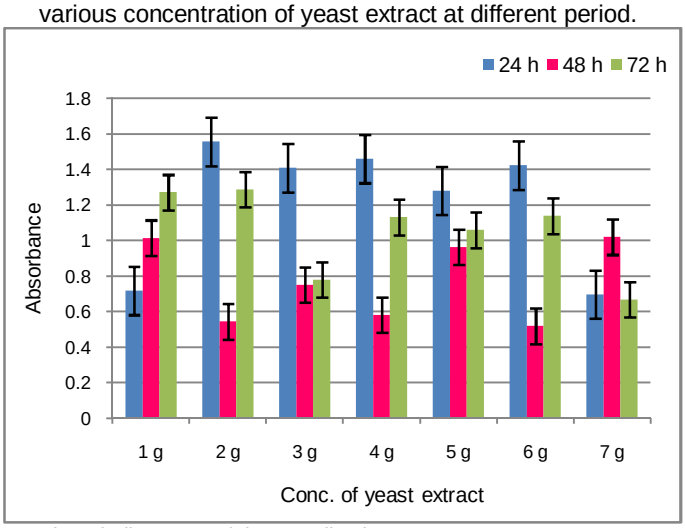


Fig. 3. Growth of *Rhizobium* (RT1) on 50% spent wash at various concentration of yeast extract at different period.



Error bars indicate SD of three replications.

Media optimization using 50% spent wash along with different concentrations of yeast extract: Growth of *Rhizobium* strains were monitored at 50% spent wash along with different concentration of yeast extract. *Rhizobium* strains were exposed to yeast extract (1-7 g/L) in terms of optical density at different time intervals i.e. 24, 48 and 72 h. Rhizobial cell viability was maintained throughout the 72 h growth period. Maximum growth was observed in 7 g/L of yeast extract in RT1 and 5 g/L of yeast extract in RT2 at 24 h incubation period (Fig. 3 and 4).

Media optimization using 50% spent wash along with different concentration of mannitol: Growth of *Rhizobium* strains were monitored at 50% spent wash concentration along with various concentrations of mannitol (1-7 g/L) in terms of optical density at different time intervals i.e. 24, 48 and 72 h. Rhizobial cell viability was maintained throughout the 72 h growth period. Maximum growth was observed in RT1 and RT2 at 2 g/L i.e. 1.230±0.134 and 1.558±0.008 respectively of mannitol at 24 h incubation period (Fig. 5 and 6).

Discussion

In this study, spent wash obtained from Doiwala Sugar factory which is situated in Dehradun is used as a growth media for fast growing rhizobia. Two isolates (RT1 and RT2) isolated from root nodules of barseem were selected in this study. Growth of isolates was observed at different concentrations of spent wash (10-100%) by recording OD at 600 nm. Growth of RT1 and RT2 were maximum at 50% concentration. Similar work has been done using other substrate for rhizobial growth. Singh *et al*. (2011) optimized different concentrations of sugar waste and found maximum growth at 10% sugar waste. Singh *et al*. (2013) optimized different concentration of dairy sludge for fast growing rhizobia and observed maximum growth at 60% dairy sludge. 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 our study, growth of both bacteria was observed at 50% spent wash along with traditional media such as YEM, RMM and TY. Growth of RT1 and RT2 was found maximum in 50% spent wash and minimum in RMM medium. The results of Ormeno and Zuniga (1998) showed that when mannitol is replaced with 12.5 g/L of glycerol in a LNB growth medium, a Bradyrhizobium strain showed a better growth (79%) compared to YEM medium. Sellami *et al*. (2015) studied the growth of fast-growing *Rhizobium legumunosarium* in industrial wastewater. They found that all wastewater samples sustained the bacterial growth. Almost a similar study was done by Mimb *et al*. (2014) using two selected LNB strains (VUID1 and AHYP21), 16 local media (C and N sources) and groundnuts.

Fig. 4. Growth *Rhizobium* (RT2) on 50% spent wash at various concentration of yeast extract at incubation different period.

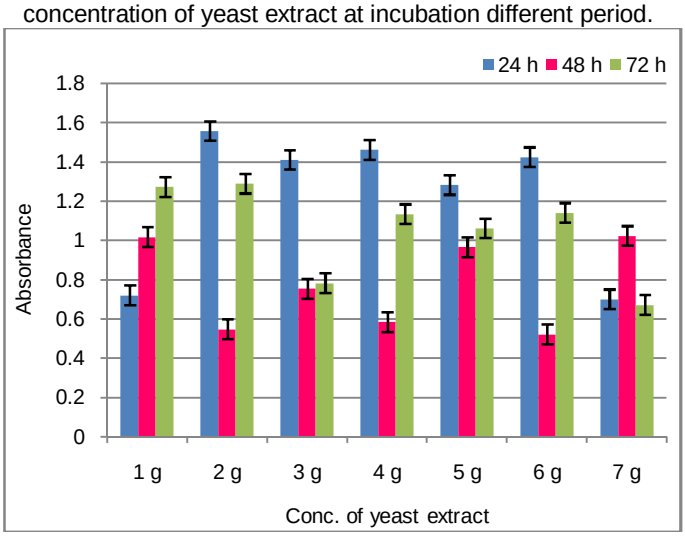


Fig. 5. Growth of *Rhizobium* (RT1) on 50% spent wash at various concentration of mannitol at different period.

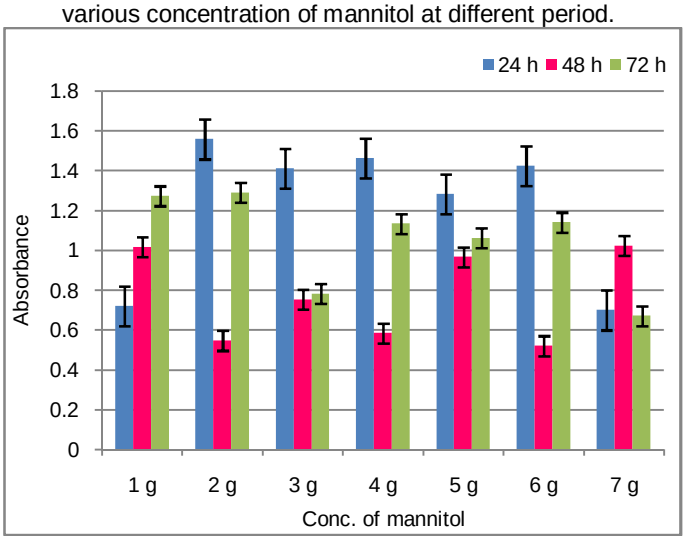
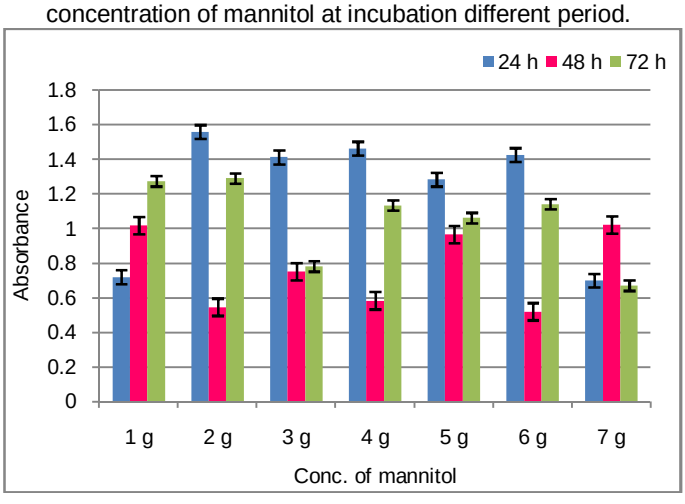


Fig. 6. Growth *Rhizobium* (RT2) on 50% spent wash at various concentration of mannitol at incubation different period.



The LNB biomass obtained from the local media was greater than the one obtained from the standard YEM media. In an another work, Molasses and hydrolysates of water-hyacinth and pea husk at proportions of 1:2:2 as substitute for mannitol (10 g/L) gave a better yield of *R. trifolii* and *R. japonicum* than the standard yeast extract mannitol medium (Gulati, 1987). The composition of a culture medium is one of the most important parameters to be analyzed in biotechnological processes with industrial purposes, because around 30-40% of the production costs were estimated to be accounted for the cost of the growth medium. The concept of 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 (Kivanc *et al.,* 2006). Yeast extract and mannitol are two main components of the standard media for the growth of *Rhizobium* therefore 50% spent wash along with different concentrations of yeast extract and mannitol were further optimized. Growth in both conditions was studied at different time intervals. According to our findings maximum growth were observed in 7 g/L of yeast extract in RT1 and 5 g/L of yeast extract in RT2 and 2 g/L of mannitol at 24 h incubation period in all strains.

Conclusion

This study can provide alternative substrates for biofertilizers production by growing rhizobia in spent wash and may help to reduce pollution problems related to wastewater treatment. Therefore our study recommends that the use of 50% spent wash as a suitable growth media as it is very nutritive, easily available and very good to the standard media used for growth of *Rhizobium*.

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